The investigation of Tau cleaved by Caspase-6 in the cerebrospinal fluid as a biomarker for early Alzheimer disease

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ABSTRACT

Mounting biochemical and immunohistochemical evidence implicates Caspase-6 (Casp6) activation as an early event in familial and sporadic Alzheimer disease (AD). Given the need for early AD diagnosis and the ample evidence implicating the activation of Casp6 as an initiating event in AD, we propose to investigate if Casp6 activity in the cerebrospinal fluid (CSF), measured by Tau cleaved by Casp6 (TauΔCasp6) protein, may serve as a biomarker for early AD. To address our hypothesis, we designed a sandwich enzyme-linked immunosorbent assay (ELISA), which specifically detects tau protein that has been cleaved by Casp6. This sandwich ELISA was used to detect TauΔCasp6 in the post-mortem CSF of non-cognitively impaired, mild-cognitively impaired and mild, moderate and severe AD groups. We assessed levels of TauΔCasp6 in brains by immunohistochemistry and compared levels found in situ to those found in CSF. Finally, we compared the levels of TauΔCasp6 detected in CSF to cognitive scores obtained pre-mortem to determine the clinical validity of our sandwich ELISA test. Here, we show that levels of TauΔCasp6 in post-mortem CSF are significantly increased in AD and correlate with the severity of the disease. Furthermore, we find that TauΔCasp6 levels in the CSF positively correlate with levels of TauΔCasp6 in affected brain regions of AD and negatively correlate with memory test scores. The results from this study implicate TauΔCasp6 as a novel AD biomarker.
RÉSUMÉ

Plusieurs recherches biochimiques et immunohistochimiques ont impliqué l’activation de la Caspase-6 (Casp6) comme un événement précoce dans les formes familiaux et sporadiques de la maladie Alzheimer (MA). Le besoin de diagnostiquer la MA de façon hâtive nous a incité à investiguer si l’activité de la Casp6 dans le liquide céphalo-rachidien (LCR), mesurée par la présence de la protéine Tau clivée par la Casp6 (TauΔCasp6), pourrait servir comme un marqueur biologique pour la MA. Pour répondre à notre hypothèse, nous avons établi un dosage immuno-enzymatique de forme sandwich sur support solide (ELISA) qui détecte la protéine TauΔCasp6. Ce sandwich ELISA a été utilisé pour déterminer le niveau de TauΔCasp6 dans le LCR post-mortem d’individus sans déficience cognitif, avec une déficience intermédiaire, ou dans les groupes d’individus Alzheimer de niveau faible, modéré et sévère. De plus, nous avons évalué le niveau de TauΔCasp6 dans les cerveaux par immunohistochimie et comparé ces niveaux à ceux trouvés dans le LCR. Finalement, nous avons comparé les niveaux de TauΔCasp6 mesurées dans le LCR aux scores cognitifs de ces individus pour déterminer la validité clinique de notre méthode. Nos résultats démontrent que le niveau de TauΔCasp6 dans le LCR dans les cas Alzheimer est augmenté de façon significative et corrèle avec la sévérité de la maladie. Par ailleurs, nous avons trouvé que le niveau de TauΔCasp6 dans le LCR corrèle positivement avec le niveau de TauΔCasp6 dans les régions affectées du cerveau Alzheimer et corrèle négativement avec les scores des tests de mémoire. Ces résultats promettent l’utilisation de TauΔCasp6 comme un marqueur biologique pour diagnostiquer hâtivement la MA.
ACKNOWLEDGMENTS

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It is an honor for me to acknowledge all of the LeBlanc laboratory members past and present. Andrea Lee, Prateep Pakavathkumar and Sarah Peters: this thesis is as much yours as it is mine.

Last but not least, thank you to my parents Mary and Lenny and my brothers Aaron and Jerome for believing in me when I didn’t.

This thesis is dedicated to Giancarlo Da Soghe. There are no words.
**ABBREVIATIONS**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid beta peptide</td>
</tr>
<tr>
<td>Aβ_{40}</td>
<td>Amyloid beta peptide 40 amino acids in length</td>
</tr>
<tr>
<td>Aβ_{42}</td>
<td>Amyloid beta peptide 42 amino acids in length</td>
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<td>AD</td>
<td>Alzheimer disease</td>
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<td>ADAS-Cog</td>
<td>Alzheimer's disease Assessment Scale-Cognitive</td>
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<td>ADNI</td>
<td>Alzheimer disease Neuroimaging Initiative</td>
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<td>AEBSF</td>
<td>4-(2-Aminoethyl)-benzenesulfonyl fluoride</td>
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<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>Asp_{13}</td>
<td>Aspartic amino acid 13</td>
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<tr>
<td>Asp_{401}</td>
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<tr>
<td>Asp_{421}</td>
<td>Aspartic amino acid 421</td>
</tr>
<tr>
<td>BACE1</td>
<td>β-site amyloid precursor protein-converting enzyme 1</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
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<td>CA1-4</td>
<td>cornu ammonis 1-4</td>
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<td>CARD</td>
<td>Caspase activation and recruitment domain</td>
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<td>Caspase</td>
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<td>Caspase-1</td>
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<td>Caspase-14</td>
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<td>Caspase</td>
<td>Cysteine-dependent aspartate specific proteases</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CERAD</td>
<td>Consortium to Establish a Registry for Alzheimer's Disease</td>
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<td>CJD</td>
<td>Creutzfeldt-Jakob disease</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>DED</td>
<td>Death effector domain</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ERC</td>
<td>Entorhinal cortex</td>
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<td>18FDG</td>
<td>18(^{\text{F}})Fluorodeoxyglucose</td>
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<tr>
<td>FTD</td>
<td>Frontotemporal dementia</td>
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<tr>
<td>GCS</td>
<td>Global cognitive score</td>
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<tr>
<td>HC</td>
<td>Healthy control</td>
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<tr>
<td>Kan+</td>
<td>Kanomycin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LBD</td>
<td>Lewy body dementia</td>
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<td>MAP</td>
<td>Microtubule associated protein</td>
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<tr>
<td>MCI</td>
<td>Mild-cognitive impairment</td>
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<tr>
<td>MMSE</td>
<td>Mini-mental state examination</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MSA</td>
<td>Multiple system atrophy</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
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<tr>
<td>nAD</td>
<td>Non-Alzheimer disease dementia</td>
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<tr>
<td>NCI</td>
<td>Non-cognitive impairment</td>
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<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
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<tr>
<td>NINCDS-ADRDA</td>
<td>National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association</td>
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<tr>
<td>NP</td>
<td>Neuritic plaque</td>
</tr>
<tr>
<td>NPDPK</td>
<td>Non-proline directed protein kinase</td>
</tr>
<tr>
<td>NPT</td>
<td>Neuropil thread</td>
</tr>
<tr>
<td>OND</td>
<td>Other neurodegenerative disease</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDPK</td>
<td>Proline-directed protein kinase</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>Phospho-tau(_{181})</td>
<td>Tau protein phosphorylated at threonine 181</td>
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<tr>
<td>Phospho-tau(_{199})</td>
<td>Tau protein phosphorylated at serine 199</td>
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<td>Tau protein phosphorylated at threonine 231</td>
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<td>Tau protein phosphorylated at serine 396</td>
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<tr>
<td><strong>Phospho-tau</strong>$_{404}$</td>
<td>Tau protein phosphorylated at serine 404</td>
</tr>
<tr>
<td><strong>PIB</strong></td>
<td>Pittsburgh Compound B</td>
</tr>
<tr>
<td><strong>PNS</strong></td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td><strong>PSP</strong></td>
<td>Progressive supranuclear palsy</td>
</tr>
<tr>
<td><strong>ROS</strong></td>
<td>Religious Orders Study</td>
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<tr>
<td><strong>SDS</strong></td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td><strong>SDS-PAGE</strong></td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td><strong>Tau</strong>$\Delta$<strong>Casp6</strong></td>
<td>Tau cleaved by Caspase-6 at the C-terminal site KSPVVSGD</td>
</tr>
<tr>
<td><strong>Tau FL</strong></td>
<td>Full-length tau protein (isoform 3)</td>
</tr>
<tr>
<td><strong>TBS</strong></td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td><strong>TBST</strong></td>
<td>Tris Buffered Saline with Tween 20</td>
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<tr>
<td><strong>TLCK</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Trans-ERC</strong></td>
<td>Trans-entorhinal cortex</td>
</tr>
<tr>
<td><strong>VaD</strong></td>
<td>Vascular dementia</td>
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INTRODUCTION

Caspases are proteases playing essential roles in the regulation of tissue homeostasis including apoptosis and inflammation (Cerretti et al., 1992; Thornberry et al., 1992; Yuan et al., 1993). The essential role of caspases in cell death and the vast neuronal loss observed in neurodegenerative diseases has caused a spike in the investigation of caspases in Alzheimer disease (AD).

Mounting evidence implicates Caspase-6 (Casp6) activity as an early insult in AD. Active Casp6 is abundant in neurofibrillary tangles (NFTs), neuropil threads (NPTs) and neuritic plaques (NPs) in the hippocampus and temporal cortex of familial AD brains and at all stages of sporadic AD (Guo et al., 2004; Albrecht et al., 2007; Albrecht et al., 2009). Accumulation of active Casp6 in AD neuropathological hallmarks indicates that Casp6 is intimately linked to the pathophysiology of both sporadic and familial AD.

Furthermore, active Casp6 is localized to NFTs, NPTs and NPs in the entorhinal cortex of some aged non-cognitively impaired (NCI) brains (Albrecht et al., 2007). In fact, aged NCI individuals with active Casp6 in the entorhinal cortex had lower global cognitive scores (GCS) than those that did not have active Casp6 in their brains (Albrecht et al., 2007). The abundance of active Casp6 in NCI brains with lowered GCS implicates Casp6 as an early instigator of AD pathology and memory impairment. These results suggest that Casp6 activity may be used as a biomarker for early AD.
Therefore, the main objective of this study is to evaluate if Casp6 activity in the cerebrospinal fluid (CSF), measured as Tau cleaved by Casp6 (TauΔCasp6) protein, may serve as a biomarker for early AD.

1. LITERATURE REVIEW

1.1 Caspases

Caspases are a superfamily of cysteine-dependent aspartate specific proteases playing essential roles in the regulation of tissue homeostasis including apoptosis and inflammation (Cerretti et al., 1992; Thornberry et al., 1992; Yuan et al., 1993). Caspases are named for the cysteine side chain required for catalytic activity and their specificity for cleaving substrates after aspartic acid residues (Alnemri et al., 1996). The first caspase, Caspase-1 (Casp1), originally named interleukin 1-β cleaving enzyme, was discovered in the early 90’s (Cerretti et al., 1992; Kronheim et al., 1992; Thornberry et al., 1992). Following this discovery, the study of caspases swelled and presently twelve human caspases have been identified including Caspase-1 to -10, Caspase-12 and Caspase-14 (Alnemri et al., 1996; Van de Craen et al., 1997; Eckhart et al., 2000).

1.1.1 Classification of Caspases

Caspases can be characterized based on function, N-terminal (prodomain) length or substrate specificity. Based on function, caspases can be separated into apoptotic initiator (Casp2, 8, 9 & 10) or effector (Casp3, 6 & 7) caspases and inflammatory caspases (Casp1, 4, 5 and 12) (Pop et al., 2009). Casp14 is
distinct because it is neither apoptotic nor inflammatory but rather is involved in the terminal differentiation of epidermal keratinocytes (Eckhart et al., 2000). Caspases contain either a short (Casp3, 6, 7, and 14) or long (Casp1, 2, 4, 5, 8, 9, 10, and 12) prodomain (Eckhart et al., 2008). Casp2, 9 and 12 contain caspase activation and recruitment domains (CARD) and Casp8 and 10 contain death effector domains (DED) that are absent in the short prodomain caspases (Eckhart et al., 2008). Caspases cleave their substrates following preferred tetrapeptide motifs (Thornberry et al., 1997).

Based on substrate specificity, three groups emerge: Group I (Casp1, 4 & 5) which prefer the sequence WEHD, Group II (Casp2, 3, & 7) which prefer DEXD (where X is any amino acid) and Group III (Casp6, 8, 9 & 10) which prefer (L/V)EXD (Thornberry et al., 1997; Garcia-Calvo et al., 1999). Within groups, each caspase displays optimal substrate specificity. For example, Casp6 recognizes and optimally cleaves protein substrates following VEID sequences (Earnshaw et al., 1999).

1.1.2 Caspase Activation

Caspases are synthesized as proteolytically inactive proenzymes consisting of an N-terminal prodomain of varying length followed by a large 20 kilodalton (kDa; p20) subunit, usually a linker region, and a small 10 kDa (p10) subunit (Pop et al., 2009). Initiator caspases exist as inactive monomers and effector caspases as inactive dimers (Renatus et al., 2001; Boatright et al., 2003). Caspases remain in this inactive form until a signal (i.e. apoptotic insult) initiates activation (Pop et al., 2009).
The mode of caspase activation differs between inflammatory, initiator and effector caspases. Following an apoptotic stimulus, oligomeric activation platforms assemble (Shi, 2004). Adaptor proteins within the activation platform recruit initiator caspases via CARD and DED domain interactions (Shi, 2004). Initiator caspase recruitment facilitates clustering and oligomerisation resulting in auto-processing and subsequent activation (Shi, 2004). Inflammatory caspases are activated similarly to initiator caspases. Upon an inflammatory stimulus, pro-inflammatory caspases are recruited to activation complexes referred to as inflammasomes wherein they become activated (Martinon et al., 2007). Once activated, initiator and inflammatory caspases act as signaling molecules transmitting downstream signals through specific substrate cleavage. Classically, it was thought that effector caspases were solely cleaved by exo-caspases, for example an active initiator caspase (Pop et al., 2009). Recent evidence, however, demonstrates that Casp6 is capable of activation via self-processing (Klaiman et al., 2009). Once activated, effector caspases cleave a host of cellular protein targets in order to achieve a desired biological outcome (Pop et al., 2009).

1.2. Alzheimer Disease

It is estimated that 25 to 30 million people worldwide currently suffer from dementia and this number is expected to triple by 2040 (Ferri et al., 2005). Dementia is a clinical state characterized by a loss of function in multiple cognitive domains and caused by a range of illnesses (Association, 2000). AD is the most common form of dementia. Symptoms of AD-type dementia
include memory impairment (the ability to learn new and recall previously learned information) as well as disturbances in language function (aphasia), motor abilities (apraxia), ability to recognize or identify objects (agnosia) and executive function such as planning (Association, 2000). Such symptoms can occur years before a clinical diagnosis of probable AD in a transitional stage between normal aging and dementia known as mild cognitive impairment (MCI) (Petersen et al., 1999). MCI is characterized by subjective and objective memory impairment, preservation of activities of daily living and cognitive functioning in the absence of dementia (Petersen et al., 2001). Not all MCI patients will convert to AD, in fact, some will remit while others convert to other forms of dementia (Petersen et al., 1999). MCI can present as either amnestic, where memory impairment is the most prominent symptom or non-amnestic where a cognitive domain other than memory is impaired and both can involve single or multiple cognitive domains (Petersen et al., 2005). It is believed that amnestic MCI represents preclinical or prodromal AD and convert to probable AD at a rate of 10-15% per year while NCI aged matched controls convert at a rate of 1-2% per year (Tierney et al., 1996; Bowen et al., 1997; Devanand et al., 1997; Petersen et al., 1999; Grundman et al., 2004). Most studies identify episodic memory impairment as the earliest sign and symptom of MCI (Salmon, 2011). Episodic memory, also referred to as autobiographical memory, is the memory of specific events, situations and experiences that happened in one’s own life (Salmon, 2011). Impairments in episodic memory in MCI manifest as the inability to learn and retain new
information (Salmon, 2011). Recent studies show that other than episodic memory, there are also early deficits in language, attention, executive functions and visuospatial abilities (Backman et al., 2004, 2005; Twamley et al., 2006). In fact, a recent study demonstrated that episodic memory, semantic memory and executive function all decline in MCI, however episodic and semantic memory are far more impaired than executive function (Mickes et al., 2007). Semantic memory is the memory of meanings, understandings and concepts unrelated to specific experiences (Salmon, 2011). It is the general knowledge one has about the world (Salmon, 2011). Examples of semantic memory deficits exhibited in MCI include impairments in the ability to recall over-learned facts such as the number of days in the year and produce proper nouns such as the name of a famous person (Ahmed et al., 2008; Seidenberg et al., 2009). Executive function, on the other hand, is an umbrella term for many types of cognitive processes including working memory, attention, problem solving, verbal reasoning, inhibition, and mental flexibility (Salmon, 2011). Working memory, for example, is used to temporarily and actively store items with which you will be doing higher order cognitive processes (Salmon, 2011). Impairments in working memory tasks that require attention, such as mental math, are frequently observed in MCI (Salmon, 2011). As patients progress from MCI to AD, these symptoms progress and become more apparent affecting activities of daily living, making a clinical AD diagnosis possible.
1.2.1 Forms of AD

AD exists in both familial and sporadic forms. Familial AD accounts for approximately 5-10% of all AD cases with sporadic AD comprising the remaining cases (Hutton et al., 1998b). AD can be further divided into early-onset AD, diagnosis under the age of 65 and late-onset AD, diagnosed after 65 years of age (Hutton et al., 1998b). Most sporadic cases are late-onset while the majority of familial AD cases are early-onset (Hutton et al., 1998b).

Familial AD (FAD) is caused by autosomal dominant inherited single gene mutations (Hutton et al., 1998b). The first mutation causing FAD was identified in the amyloid precursor protein (APP) gene located on chromosome 21 (Goate et al., 1991). Later on, through familial studies, mutations were discovered in the presenilin I and II genes located on chromosome 14 and 1, respectively (Levy-Lahad et al., 1995; Sherrington et al., 1995). While mutations in the APP gene were discovered earliest, mutations in presenilin I genes account for most familial AD cases (Levy-Lahad et al., 1995; Sherrington et al., 1995). In fact, over 152 pathogenic mutations have been identified thus far (Serretti et al., 2007).

While genetic risk does not play a major role in sporadic AD several genes have been identified that increase disease susceptibility in carriers. Of these genes, apolipoprotein E (APOE) accounts for most of the known genetic risk associated with sporadic AD (Raber et al., 2004). The APOE gene has three
alleles: ε4, ε3, and ε2 (Corder et al., 1993). APOE ε4 allele heterozygotes are seven times more likely to develop the disease than APOE ε3 carriers (Corder et al., 1993). In contrast, it has been suggested that the APOE ε2 allele plays a protective role (Corder et al., 1994). Many other candidate genes have been identified but evidence thus far suggests that they pose only a minor risk (Serretti et al., 2007). In fact, age remains the biggest risk factor for sporadic AD. However, AD is a heterogeneous disease wherein unhealthy lifestyle choices, cardiovascular diseases and low levels of education are also associated with an increased risk (Mayeux, 2003). Still, the official cause of sporadic AD remains unknown.

1.2.3 Pathogenesis

In 1907, Dr. Alois Alzheimer first described bundles and miliary foci in an autopsied patient’s brain, now referred to as NFTs, NPTs and NPs, respectively (Fig 1-1) (Alzheimer et al., 1995). The accumulations of NFTs and NPTs, along with neuronal and synaptic loss are first detected in the medial temporal lobe limbic structures (the entorhinal cortex (ERC) and hippocampus) and then progress to higher associative areas located in the frontal and parietal lobes (Salmon, 2011).
Figure 1-1. The pathological hallmarks of AD. Micrograph of an AD hippocampal brain tissue section stained with a PHF-1 antibody that specifically recognizes phosphorylated tau protein. In AD, phosphorylated tau accumulates in NFTs (red arrow), NPs (black arrow) and NPTs (yellow arrow).

1.2.3.1 The Amyloid Cascade Hypothesis

NPs are extracellular brain deposits composed of an amyloid beta (Aβ) peptide core surrounded by a ring of dystrophic neurites (Glenner et al., 1984; Khachaturian, 1985; Masters et al., 1985). Many proteins other than Aβ peptides are localized to the core including immunoglobulins, proteoglycans, inflammatory molecules, metal ions, proteases, cholinesterases, complement factors and even Apo ε (Armstrong, 1998; Atwood et al., 2002). Furthermore, glial cells including astrocytic processes and activated microglia are associated with NPs (Itagaki et al., 1989).

Aβ peptides are generated during normal cell metabolism from proteolytic processing of APP via the amyloidogenic pathway (Kang et al., 1987; Haass et
APP is a type I transmembrane protein comprised of a large extracellular and short cytoplasmic domain (Kang et al., 1987). Sequential cleavage of the extracellular domain by BACE1 (β-site APP-converting enzyme 1), followed by differential γ-secretase cleavage at several adjacent sites in the transmembrane region, liberate Aβ peptide species of 38-43 amino acids in length extracellularly (Anderson et al., 1992; Vassar et al., 1999). The majority of these secreted soluble Aβ peptide species are 40 (Aβ40; ~90%) and 42 (Aβ42; 10%) amino acids in length (Seubert et al., 1992). Normally, Aβ peptides are degraded by insulin-degrading enzyme, neprilysin, endothelin-converting enzyme and microglia (Carson et al., 2002).

Since 1991, Aβ protein has been at the center of AD research with the advent of the amyloid cascade hypothesis. According to this hypothesis, the mismetabolism of APP causes an imbalance between Aβ42 protein production and clearance which leads to insoluble protein deposition in the brain causing synaptic dysfunction, neuronal cell death, tangle formation and ultimately dementia (Hardy et al., 1991). NPs first appear in the neocortex followed by the ERC and finally the hippocampus (Thal et al., 2002). Support for the amyloid cascade hypothesis come from the fact that 1) there is extensive accumulation of Aβ in AD brains 2) FAD genes are involved in APP processing with some causing an increase in Aβ42 production 3) people with Down’s syndrome contain an extra APP gene and develop Aβ plaques and advanced AD within the fourth decade of life 4) transgenic mouse models
expressing pathogenic forms of APP or presenilin I have increased levels of Aβ and amyloid plaques (Tanzi et al., 2005) and 5) duplication of the APP locus causes accumulation of Aβ deposits in the brain and results in autosomal dominant early-onset AD (Rovelet-Lecrux et al., 2006; Sleegers et al., 2006; Rovelet-Lecrux et al., 2007; Guyant-Marechal et al., 2008; Kasuga et al., 2009; Thonberg et al., 2011; McNaughton et al., 2012). The amyloid cascade hypothesis has come under great scrutiny in recent years with evidence that 1) in early phases of AD, NFTs can be found in great quantities where Aβ plaque deposition is low (Braak et al., 1991) 2) there is a weak to no correlation between the density of Aβ plaques and dementia severity (Hyman et al., 1993; Berg et al., 1998) 3) single transgenic mouse models accumulate massive quantities of Aβ deposition but do not display NFTs or neuronal degeneration (Eriksen et al., 2007) and 4) some AD individuals show no Aβ deposits in positron emission tomography scans (Braak et al., 1991; Hyman et al., 1993; Berg et al., 1998; Eriksen et al., 2007; Li et al., 2008). In addition, an Aβ42 immunization clinical trial resulting in decreased Aβ plaque load without improvements in survival rate or severity of dementia caused further detriment to the faltering hypothesis (Holmes et al., 2008). These are but a few lines of evidence demonstrating that while Aβ is an important contributing factor to the pathogenesis of AD, Aβ alone cannot and does not account for this multifactorial disorder.
1.2.3.2 Tau Protein in AD

NFTs and neuropil threads (NPTs) are intracellular neuronal deposits of abnormally phosphorylated tau protein (Grundke-Iqbal et al., 1986a).

1.2.3.2.1 Tau Gene and Protein

Tau protein, originally discovered in porcine in 1975, is encoded by a 100 kb single copy gene, *MAPT*, located on chromosome 17q21.1 (Weingarten et al., 1975; Neve et al., 1986). Alternative splicing of this 16-exon gene (-1, 1-4, 4A, 5-14) produces three messenger ribonucleic acid (mRNA) transcripts of 2, 6 and 9 kilobases (kb) in length (Neve et al., 1986; Goedert et al., 1992; Wang et al., 1993). In the central nervous system (CNS), exons 1-5, 7, 9 and 11-13 are constitutive while exons 2, 3 and 10 are alternatively spliced (Andreadis, 2005). There is little to no expression of exons 6 or 8 in the human and exon 4A is constitutively included in the 9kb transcript, which is exclusively expressed in the peripheral nervous system (PNS) (Andreadis et al., 1992; Couchie et al., 1992; Goedert et al., 1992; Andreadis, 2005). Exon -1 is part of the promoter region and exon 14 is part of the 3’ untranslated region, therefore both exons are transcribed but not translated (Andreadis, 2005).

Alternative mRNA splicing of exons 2, 3, and 10 results in six major protein isoforms ranging in size from 352-441 amino acids (37-46 kDa) (Cleveland et al., 1977; Goedert et al., 1988; Goedert et al., 1989a; Goedert et al., 1989b; Andreadis et al., 1992). Splicing of exons 2 and 3 where exon 2 can appear alone but exon 3 never appears independently of exon 2 results in isoforms
containing 0 (0N), 1 (1N) or 2 (2N) 29 amino-terminal inserts (Lee et al., 1988; Goedert et al., 1989b; Andreadis et al., 1992; Andreadis et al., 1995). Splicing of exon 10 generates isoforms with either three or four 31-32 amino acid carboxy-terminal repeats (Goedert et al., 1989b; Andreadis et al., 1992). Isoforms including exon 10 contain 4 tandem repeats (4R-tau) while those excluding exon 10 only contain 3 (3R-tau) (Goedert et al., 1989b; Andreadis et al., 1992). Exon 10 alternative splicing has been particularly well characterized since the discovery that a mutation within this exon is one of the major causes of frontotemporal dementia and parkinsonism linked to chromosome 17 (Hutton et al., 1998a; Poorkaj et al., 1998; Spillantini et al., 1998).

1.2.3.2.2 Tau localization

Tau protein is found throughout the CNS and PNS as well as skeletal muscle (Trojanowski et al., 1989; Nunez et al., 1997; Wei et al., 1998). On a cellular level, tau is primarily neuronal but is also found in oligodendrocytes and astrocytes (Binder et al., 1985; LoPresti et al., 1995). Subcellularly, tau is mainly localized to growing and mature axons but is also expressed in the cell soma, dendrites and nucleus (Binder et al., 1985; Kosik et al., 1989; Wang et al., 1993; Kempf et al., 1996; Thurston et al., 1997).

Exons 2, 3, and 10 are adult specific and therefore all six-protein isoforms are expressed in the adult whereas only the smallest protein isoform 0N/3R (37 kDa/352 aa) is expressed in the fetus and neonatally (Goedert et al., 1989a;
Kosik et al., 1989; Goedert et al., 1990). Inclusion of exon 4A generates a 110 kDa protein known as “Big Tau” which is specific to the adult PNS (Andreadis et al., 1992; Couchie et al., 1992; Goedert et al., 1992).

1.2.3.2.3 Tau biological functions

Tau contains two functional domains: a projection domain and a microtubule (MT)-binding domain. The acidic N-terminal region is termed the projection domain because it protrudes from the surface of MTs (Hirokawa et al., 1988). This domain can be further divided into an N-terminal acidic rich region and a C-terminal proline rich region (Wang et al., 2008; Andreadis, 2012). The exact function of the projection domain remains unclear, however several potential functions have been proposed. It has been suggested that the acidic portion, modulated by exons 2 and 3, is involved in (1) microtubule bundling and (2) signal transduction through interaction with the axonal plasma membrane (Kanai et al., 1992; Brandt et al., 1995). Inclusion of exon 4A, which occurs exclusively in the PNS, denotes a role for the projection domain in MT spacing in neuronal axons and dendrites (Chen et al., 1992; Couchie et al., 1992; Goedert et al., 1992). A potential role in signal transduction is furthered by evidence that PPxxP (where P is proline and x is any amino acid) or PxxP motifs in the proline rich region, modulated by exon 6, can interact with plasma membrane associated proteins comprising Src homology 3 domains such as non-receptor tyrosine kinase families fyn and src (Arrasate et al., 2000; Lee et al., 2004; Lee et al., 2005). Interactions with fyn and src may result in tyrosine phosphorylation of tau, targeting tau
protein to the plasma membrane and enabling tau to participate in cell signaling (Lee et al., 2005; Wang et al., 2008; Gendron et al., 2009). Recent work also indicates that the proline rich region influences neurite extension, a well-established function of tau protein (Caceres et al., 1990; Liu et al., 1999; Ramirez et al., 1999; Luo et al., 2004). Knowing that tyrosine phosphorylation via tyrosine kinases is essential for growth cone function suggests that the projection domain's involvement in neurite extension is influenced by tyrosine phosphorylation of tau by fyn and src (Ignelzi et al., 1994; Goldberg et al., 1996; Wang et al., 2008). Lastly, interaction of the proline rich region with the C-terminal MT-binding domain suggests a role for the projection domain in MT binding and stabilization (Goode et al., 1997).

The basic C-terminal region of tau is referred to as the MT-binding domain because as the name implies, it enables tau protein to bind MTs (Lee et al., 1989). The MT-binding domain is composed of three or four MT-binding repeats, encoded by exons 9-12 and modulated by exon 10, where the 4R-tau isoform has a higher affinity for MTs than the 3R-tau (see 1.1.4.2.2 Protein isoforms) (Goedert et al., 1989b; Andreadis et al., 1992; Mandelkow et al., 1995). The MT-binding repeats enable tau to bind tubulin and promote the assembly and stability of MTs in neuronal axons, the major biological function of tau protein (Witman et al., 1976; Lee et al., 1989). Tau is a member of the microtubule associated protein (MAP) family, which interacts
with microtubules to carry out a variety of functions, some of which are shared and may explain why tau knock-out mice are viable (Mandelkow et al., 1998; Wang et al., 2008). Tau modulates MTs by either binding to the outer surface of already formed MTs promoting stability or tubulin dimers promoting nucleation required for mature MT formation (Al-Bassam et al., 2002; Kar et al., 2003).

1.2.3.2.4 Tau phosphorylation

During normal development, tau protein undergoes various forms of post-translational modifications including phosphorylation, truncation, nitration, glycation, glycosylation, ubiquitination, and polyamination (Wang et al., 2008). Phosphorylation of tau has been most widely investigated and will be the main focus here. In reference to the longest tau protein isoform, there are 80 potential serine and threonine phosphorylation sites (Sergeant et al., 2008). The majority of tau phosphorylation sites are located in the proline rich region and flanking the MT-binding repeats (Sergeant et al., 2008). In normal adult brains, 1-3 moles of phosphate are present per mole of tau with phosphorylation on up to forty serine/threonine residues and five tyrosine residues being reported (Sergeant et al., 2008; Wang et al., 2008). Similar to alternative splicing, tau phosphorylation state is developmentally regulated, with a higher amount of phosphorylation present embryonically (Goedert et al., 1993). In 1984, it was discovered that tau in a dephosphorylated state promotes MT assembly more efficiently than phosphorylated tau protein (Lindwall et al., 1984). Tau phosphorylation is carried out by protein kinases
and subsequent dephosphorylation by protein phosphatases. Kinases are divided into two major groups based on substrate specificity: proline-directed protein kinases (PDPKs) and non-proline directed protein kinases (NPDPKs) (Gendron et al., 2009). Phosphorylation of serine/threonine residues by PDPKs inhibits tau from promoting MT assembly (Gendron et al., 2009). Phosphorylation of serine/threonine residues by NPDPKs, specifically in the MT-binding repeats, promotes the dissociation of tau from MTs (Gendron et al., 2009). Protein kinases and phosphatases work to carefully regulate the phosphorylation state of tau (Drewes et al., 1995; Sengupta et al., 1998; Wang et al., 1998; Zheng-Fischhofer et al., 1998; Jicha et al., 1999).

1.2.3.2.5 Tau hypothesis

Like the Aβ cascade hypothesis, there exists a “tau hypothesis” believed to be a major pathway in the progression from normal aging to AD. According to the tau hypothesis, an imbalance between the rate of tau phosphorylation and dephosphorylation results in tau hyperphosphorylation (Wang et al., 2008). Hyperphosphorylated tau has decreased MT-promoting ability and sequesters other MAPs causing inhibition of MT assembly and promoting disassembly and hence impaired MT-based axonal transport (Grundke-Iqbal et al., 1986b; Iqbal et al., 1986; Alonso et al., 1994; Alonso et al., 1996; Praprotnik et al., 1996; Alonso et al., 1997; Stokin et al., 2005). Simultaneously, hyperphosphorylated tau is prone to aggregation forming twisted or straight filaments known as paired helical filaments (PHFs), which deposit in the cell body as NFTs, in neurites as NPTs and within dystrophic
neurites associated with NPs (Grundke-Iqbal et al., 1986b; Iqbal et al., 1986; Kosik et al., 1986; Iqbal et al., 1989; Alonso et al., 2001; Wang et al., 2007). It is theorized that hyperphosphorylated tau deposits together with disturbed axonal transport result in the synaptic dysfunction, neuronal cell death and dementia observed in AD (Iqbal et al., 2005).

It is important to note that the ability of hyperphosphorylated tau to promote tau aggregation into PHFs remains an area of debate (Schneider et al., 1999). While it is clear that hyperphosphorylation of tau does occur before NFT formation (Braak et al., 1995), it is still of great debate as to whether tau hyperphosphorylation and aggregation are true causes of AD or simply consequences. In support of the tau hypothesis is evidence demonstrating the correlation between NFT pathology and memory scores. The trans-ERC region is the first brain region displaying NFT pathology in AD (Braak et al., 1991). As the disease progresses, NFTs spread from the trans-ERC region to the hippocampus and then neocortex (Braak et al., 1991). The spread of NFT pathology is staged into Braak stages (Braak et al., 1991). Stages I and II are characterized by NFTs and NPTs in the trans-ERC and ERC (Braak et al., 1991). Stages III and IV are assigned when tau is located in the hippocampus (Braak et al., 1991). Finally, when neurofibrillary tau presents in the isocortex, one has reached stage V and VI (Braak et al., 1991). The temporal progression of AD symptoms reflects this spread of NFT pathology throughout the brain (Braak et al., 1991, 1997; Berg et al., 1998). In fact,
several groups have shown that NFTs correlate with dementia severity better than NPs (Berg et al., 1993; Raghavan et al., 1994; Gomez-Isla et al., 1996; Berg et al., 1998; Guillotet et al., 2003).

In contrast to the amyloid cascade hypothesis where increased Aβ directly causes tangle formation, the tau hypothesis states that NFT and Aβ plaque formation occur in parallel rather than one being upstream of the other (Maccioni et al., 2010). Indeed, post-mortem AD brain tissue sections have shown that large quantities of NFTs can be found in absence of Aβ plaques (Braak et al., 1991) and single transgenic mouse models accumulate massive quantities of Aβ deposition but do not display NFTs or neuronal degeneration (Eriksen et al., 2007). These pieces of evidence indicate that tau is not necessarily upstream of increased Aβ production or the cause of plaque formation. Rather, Aβ plaque formation occurs along with NFT accumulation as a non-specific event which is not responsible for the cognitive deficits occuring in AD (Maccioni et al., 2010).

1.2.4 Neurodegeneration

Neurodegeneration is an umbrella term referring to the progressive loss of structure or function of neurons, including cell death (Przedborski et al., 2003). Along with NPs, NFTs and NPTs, neurodegeneration, specifically neuronal and synaptic loss, is a key feature of AD pathogenesis (Terry et al., 1981; Davies et al., 1987; Terry et al., 1991). Neurodegeneration occurs
predominantly in the temporal lobe ERC and hippocampus but also extends to higher order associative areas in the frontal and parietal cortices (Davies et al., 1987; Hamos et al., 1989; West et al., 1994; Gomez-Isla et al., 1996). Neurodegeneration appears early on in the disease process, as extensive neuronal loss is observed in the ERC in individuals with MCI and mild AD (Gomez-Isla et al., 1996; Kordower et al., 2001). Moreover, neurodegeneration likely precedes the accumulation of NFTs, NPTs and NPs, which does not provide supporting evidence to the Aβ cascade or tau hypotheses (Nixon et al., 2005). In fact, several groups have shown that synaptic loss is a better correlate of cognitive impairment compared to NFTs and/or NPs (Scheff et al., 1990; Terry et al., 1991; Scheff et al., 1993; Scheff et al., 1996; Scheff et al., 1998). Albeit interesting, the finding that synaptic loss correlates well with cognitive impairment is not surprising. As previously mentioned, a large amount of synaptic loss occurs in the ERC, especially at early disease stages. The ERC serves as a hub receiving a multitude of cortical inputs and projecting this information to the hippocampal formation via the perforant pathway (Andersen, 2007). The hippocampal formation encompasses the hippocampus proper (cornu ammonis 1-4 (CA1-4)), dentate gyrus and subiculum (Martin, 2003; Andersen, 2007). The ERC can be divided into six (I-VI) layers of neurons, which send and receive projections to and from the hippocampal formation (No, 1933). In humans, the perforant pathway is made up of projections from layer II to the CA3 and dentate gyrus and layer III to the CA1 and subiculum (Andersen, 2007). The hippocampus is
primarily involved in consolidation of new memories (episodic memory) as well as navigation, spatial orientation and emotional responses (Andersen, 2007). The perforant pathway is essential in order for the hippocampus to carry out its functions (Andersen, 2007). Hence, it is expected that neurodegeneration in the ERC and hippocampus, which are so deeply involved in memory would correlate well with cognitive decline. Whether these neuronal and synaptic changes are directly, indirectly or not at all due to the accumulation of NFTs and/or NPTs remains unclear.

1.3 Casp6 and Neurodegeneration

The essential role of caspases in cell death and the vast neuronal loss observed in neurodegenerative diseases has caused a surge in the investigation of caspases in AD. In particular, mounting evidence implicates Casp6 activation as an early event and causative factor in familial and sporadic AD.

1.3.1 Casp6 and Cell Death

Discovered in 1995, the CASP6 gene can be alternatively spliced into CASP6α and CASP6β mRNA transcripts, which are translated into proCasp6a and proCasp6b, respectively (Fernandes-Alnemri et al., 1995). ProCasp6a possesses caspase activity whereas proCasp6b is a catalytically inactive protein capable of inhibiting proCasp6a activation (Fernandes-Alnemri et al., 1995; Lee et al., 2010). In 1996, it was discovered that Casp6 is involved in apoptosis via cleavage of lamin A, a neurofilament protein essential for maintaining nuclear structure (Orth et al., 1996). Cleavage of lamin A by
active Casp6 results in nuclear breakdown, contributing to eventual apoptosis (Orth et al., 1996; Ruchaud et al., 2002). However, a recent study using the mammalian cancer cell line HEK293 demonstrated that unlike effector Casp3 and -7, which induce rapid apoptosis, activation of Casp6 does not lead to cell death (Klaiman et al., 2009; Gray et al., 2010). While informative and important, studying Casp6 in cancer cell lines does not provide a biologically relevant setting for investigating neurodegenerative brain diseases. Studies using cultured neurons have provided a better understanding of Casp6 in AD. Serum deprivation of primary human neurons causes activation of effector Casp6 in the absence of effector Casp3 and 7, resulting in a protracted type of cell death (LeBlanc et al., 1999; Zhang et al., 2000). Similarly, Casp6 is required for axonal degeneration of adult axons in the rodent brain as well as cultured mouse motor and sensory neurons where it is activated in a punctate pattern indicative of axonal fragmentation (Nikolaev et al., 2009; Park et al., 2010). Likewise, primary human neurons transfected with familial APP mutants display neuritic beading in a Casp6 dependent but Aβ independent manner (Sivananthan et al., 2010). Together, these findings indicate that activation of Casp6 in neurons results in progressive neuritic degeneration reminiscent of the neurodegeneration observed in AD.

1.3.2 Neuronal Substrates of Casp6

Casp6 cleaves a number of protein substrates altered in AD including APP, tau, valosin containing protein (p97) and several synaptic and cytoskeleton
proteins including spinophilin, tubulin, drebrin, and actinin (LeBlanc et al., 1999; Gamblin et al., 2003; Horowitz et al., 2004; Klaiman et al., 2008; Halawani et al., 2010). Cleavage of APP by Casp6 results in an increase of Aβ protein production (LeBlanc, 1995; LeBlanc et al., 1999). However, caspase dependent increases in Aβ can occur independently of APP cleavage (Tesco et al., 2003). As previously discussed, the amyloid cascade hypothesis poses that an increase in Aβ protein production results in the deposition of Aβ in plaques in AD. Increased production of Aβ protein due to Casp6 could account for the imbalance in Aβ production and clearance. Other than hyperphosphorylation, tau protein localized to NFTs, NPTs and NPs can also be cleaved (Gamblin et al., 2003; Guo et al., 2004; Horowitz et al., 2004). Casp3 can cleave tau protein after aspartic amino acid 421 (Asp421) while Casp6 is capable of cleaving tau following Asp13 and Asp401 (Gamblin et al., 2003; Guo et al., 2004; Horowitz et al., 2004). Casp3 cleaved tau protein has been shown to be more fibrillogenic than full-length tau in vitro (Gamblin et al., 2003). Casp6 cleavage of tau protein could account for decreased solubility and increased aggregation of this truncated tau protein in AD. In addition to cleavage of tau protein, Casp6 cleaves p97, a ubiquitin-dependent ATPase that plays a central role in ubiquitin proteasome system-mediated protein degradation (Halawani et al., 2010). Cleavage of p97 by Casp6 could explain the impairment of the ubiquitin proteasome system observed in AD (Halawani et al., 2010). Synaptic and cytoskeletal proteins are essential for the maintenance of neuronal function and architecture. Cleavage of synaptic
and cytoskeleton proteins by Casp6 could result in the synaptic impairment and microtubule dysfunction observed in AD (Klaiman et al., 2008). Collectively, these data strongly suggest that Casp6 is an upstream event in AD.

1.3.3 Casp6 in AD Brains

Immunoprecipitation and immunohistochemical studies using active Casp6 specific antiserum have shown that Casp6 is active, present and abundant in AD brains. Active Casp6 is increased by two to three-fold in the frontal and temporal cortex of AD compared to NCI brains (Guo et al., 2004). Furthermore, this increase was not found in cerebellar tissue, which is a brain area unaffected by AD pathology (Guo et al., 2004). This result indicates that Casp6 is specifically activated in AD afflicted brain areas. Within the frontal and temporal cortex of AD brains, active Casp6 is not localized to the nucleus, a requirement for Casp6 induced apoptosis, but rather is observed in the cell soma and neurites (Ruchaud et al., 2002; Guo et al., 2004). This finding supports the theory that Casp6 contributes to neuritic degeneration in AD. More specifically, active Casp6 is abundant in NFTs, NPTs and NPs in the hippocampus and temporal cortex of familial AD brains and at all stages (mild, moderate, severe & very severe) of sporadic AD (Guo et al., 2004; Albrecht et al., 2007; Albrecht et al., 2009). Accumulation of active Casp6 in AD hallmarks indicates that Casp6 is intimately linked to the pathophysiology of both sporadic and familial AD. Furthermore, active Casp6 is localized to NFTs, NPTs and NPs in the hippocampus and ERC of MCI and some NCI
brains (Albrecht et al., 2007). In fact, within the NCI group, a negative correlation \((r^2 = 0.6976)\) was found between the level of Casp6 activity and GC score (Albrecht et al., 2007). The abundance of active Casp6 in MCI brains and in NCI individuals with lowered GC scores implicates Casp6 as an early instigator of AD pathology and memory impairment.

1.4 AD Diagnosis

At present, AD can only be definitely diagnosed post-mortem based on histopathological examination; regarded as the gold standard. A possible (patient may or may not have AD) or probable (patient likely has AD) diagnosis, however, can be assigned with extensive clinical and neuropsychological assessment. The diagnostic criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) is most commonly used in clinical diagnosis (McKhann et al., 1984). The NINCDS-ADRDA set forth criteria for possible, probable and definite AD (Table 1-1). A “possible AD” diagnosis can be assigned when cognitive decline is atypical in onset or progression and there are focal neurological findings or co-morbidities which could explain the dementia (McKhann et al., 1984). A “probable AD” diagnosis can be given when onset of cognitive decline occurs insidiously with a progressive decline in episodic memory (for at least 6 months) and at least one other cognitive domain (McKhann et al., 1984). Episodic memory and other cognitive impairments must be established via clinical and confirmed by neuropsychological examinations.
(McKhann et al., 1984). Furthermore, co-morbidities must not be present which could account for the dementia (McKhann et al., 1984). The mini-
mental state examination (MMSE), Alzheimer’s Disease Assessment Scale-
Cognitive (ADAS-Cog) and the Consortium to Establish a Registry for
Alzheimer’s Disease (CERAD) battery of neuropsychological tests are
commonly used to assess cognitive status (Folstein et al., 1975; Rosen et al.,
1984; Morris et al., 1989). A definite AD diagnosis can only be given to
patients presenting with “probable AD” which is confirmed at autopsy via
neuropathological assessment (McKhann et al., 1984). Confirmation of AD
neuropathologically requires that NPs are present with a CERAD frequency
score of 3 (1-3 scale) and NFTs with a Braak stage of V or VI in the neocortex
(Hyman et al., 1997). Criteria set forth by the NINCDS-ADRDA shows
diagnostic accuracy upwards of 80% in distinguishing healthy controls (HC)
from those with AD but a variable and disappointing ability to distinguish AD
from non-AD dementias (nAD) and other neurodegenerative diseases (OND)
(23-88%) (Knopman et al., 2001; Ballard et al., 2011). Early and accurate
diagnosis is becoming increasingly important with the advent of
neuroprotective agents designed to impede disease progression. It is
important to note that current AD therapies are symptomatic in that they
lessen AD associated symptoms, however, they do not prevent AD onset nor
have they proven to slow the progression of the disease (Ballard et al., 2011).
Hence, research in the field of early diagnosis has been rampant with studies
focused on biomarkers to help accurately diagnose AD and predict incipient
AD in MCI. In 2007, an expert consensus group put forth suggestions to improve upon the original NINCDS-ADRDA criteria established in 1984. These ameliorations focus on implementing neuroimaging and chemical biomarkers to improve upon early diagnosis (Dubois et al., 2007).

**Table 1-1. Criteria for diagnosing possible, probable and definite Alzheimer disease**

<table>
<thead>
<tr>
<th></th>
<th>Possible</th>
<th>Probable</th>
<th>Definite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Onset of cognitive decline</strong></td>
<td>atypical</td>
<td>insidious</td>
<td>insidious</td>
</tr>
<tr>
<td><strong>Progression of cognitive decline</strong></td>
<td>atypical</td>
<td>insidious + decline of additional cognitive domain</td>
<td>insidious + decline of additional cognitive domain</td>
</tr>
<tr>
<td>Evidence of co-morbidities</td>
<td>present</td>
<td>not present</td>
<td>not present</td>
</tr>
<tr>
<td>Clinical examination &amp; neuropsychological assessment</td>
<td>not required</td>
<td>required</td>
<td>required</td>
</tr>
<tr>
<td>Neuropathological assessment</td>
<td>not required</td>
<td>not required</td>
<td>required</td>
</tr>
</tbody>
</table>

(McKhann et al., 1984)

**1.4.1 AD Biomarkers**

A biomarker is an objective measure of a biological or pathogenic process that can be used to evaluate the presence, severity and risk of disease in order to guide clinical diagnosis (Blennow et al., 2010). An ideal core AD biomarker should:

a) reflect a fundamental feature of AD neuropathology and be confirmed neuropathologically at autopsy,
b) have sensitivity and specificity values above 80% for detecting AD and distinguishing from other dementias,
c) be efficacious in early or preclinical AD (MCI) stages,
d) monitor disease severity or progression,
e) indicate efficacy of therapeutic intervention and
f) be readily-available, reliable, reproducible, non-invasive, simple to perform and inexpensive (Reagan, 1998).

1.4.1.1 Diagnostic Accuracy

The diagnostic accuracy of an AD biomarker is evaluated based on its sensitivity and specificity. Sensitivity measures the proportion of individuals with AD who are correctly identified as such (Reagan, 1998). Hence, a sensitivity of 100% indicates that a biomarker test is able to identify 100% of patients with AD (Reagan, 1998). Specificity measures the proportion of individuals without AD (i.e. HC, nAD & OND) who are correctly identified as such (Cerretti et al., 1992). Hence, a specificity of 100% indicates that a biomarker test is able to identify 100% of patients without AD (Reagan, 1998). As previously discussed, clinical criteria for AD show accuracy rates ~80% when compared to autopsy-confirmed cases and therefore an ideal biomarker should have sensitivity and specificity levels exceeding this value (Reagan, 1998).

1.4.1.2 Neuroimaging Techniques

Magnetic resonance imaging (MRI) and positron emission tomography (PET) are recommended as neuroimaging biomarkers to detect early structural and
functional changes, respectively (Dubois et al., 2007). MRI is a non-invasive medical test that uses magnetic and radio waves without the use of radiation to produce detailed three-dimensional images of internal body structures (Dubois et al., 2007). As previously discussed, AD pathology first occurs in the temporal lobe resulting in atrophy. Atrophy of the medial temporal lobe including the hippocampus, ERC or amygdala can be determined qualitatively, by visual scoring, or quantitatively, through volumetry, referenced against well characterized age matched controls (Dubois et al., 2007). Significant hippocampal atrophy can be detected by MRI in preclinical stages of AD and predicts the conversion of MCI to AD with diagnostic accuracies of 80% (Hampel et al., 2008). Currently, hippocampal volumetry is the most sensitive structural biomarker for AD (Hampel et al., 2008). However, volumetry is extremely time-consuming because it is measured manually and therefore is not practical to be implemented in clinical diagnosis.

PET is a non-invasive imaging technique that uses radioactive tracers to produce three-dimensional images of functional processes in the body (Hampel et al., 2008). ¹⁸Fluorodeoxyglucose (¹⁸FDG), a glucose analog, is most commonly used as a radioactive tracer and allows the visualization of glucose uptake and hence cortical metabolism (Hampel et al., 2008). ¹⁸FDG uptake is reduced in the temporal and parietal cortices of mild to moderate AD patients (Hampel et al., 2008). A reduction in cortical metabolism in MCI
via FDG-PET predicts conversion to AD with accuracy rates higher than 80% (Hampel et al., 2008). Interest in PET scanning as an AD diagnostic tool has grown in recent years with the development of a marker known as Pittsburgh Compound B (PIB) (Hampel et al., 2008). PIB can be used to visualize amyloid-β plaques and shows increased uptake in AD patients compared to healthy controls and is proving to be a conceivable biomarker (Hampel et al., 2008). At present, however, PET is expensive and not widely available and therefore not ideal for everyday clinical use.

1.4.1.3 Biological Markers

Biological markers are found in the blood, urine, saliva and CSF. The CSF is a clear, colorless fluid that fills the ventricles and the subarachnoid spaces surrounding the brain and spinal cord (Nolte, 2002). Unconstrained by the blood brain barrier, the CSF comes in direct contact with the brain interstitial fluid draining the brain of metabolic byproducts and therefore containing a plethora of potential biomarkers (Nolte, 2002). Biochemical studies have yet to yield a viable AD biomarker however three neurochemical proteins, $\text{A}\beta_{42}$, total tau and phospho-tau, have been established as core feasible biomarkers by international consensus and independent multi-center research groups (Table 1-2).
### Table 1-2. Sensitivity and specificity of Aβ42, total tau and phospho-tau$_{181}$ detection in CSF

<table>
<thead>
<tr>
<th></th>
<th>sensitivity</th>
<th>specificity</th>
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</thead>
<tbody>
<tr>
<td>Aβ$_{42}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD vs HC</td>
<td>86%</td>
<td>89%</td>
</tr>
<tr>
<td>MCI→ AD</td>
<td>79%</td>
<td>65%</td>
</tr>
<tr>
<td>total-tau</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD vs HC</td>
<td>81%</td>
<td>89%</td>
</tr>
<tr>
<td>MCI→ AD</td>
<td>86%</td>
<td>56%</td>
</tr>
<tr>
<td>phospho-tau$_{181}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD vs HC</td>
<td>76%</td>
<td>91%</td>
</tr>
<tr>
<td>MCI→ AD</td>
<td>84%</td>
<td>47%</td>
</tr>
<tr>
<td>Aβ$<em>{42}$ + total-tau + phospho-tau$</em>{181}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCI→ AD</td>
<td>83%</td>
<td>88%</td>
</tr>
</tbody>
</table>

Aβ$_{42}$ = amyloid beta peptide 42 amino acids in length; phospho-tau$_{181}$ = tau protein phosphorylated at serine 181; AD = Alzheimer disease; HC = healthy control; MCI = mild cognitive impairment (Blennow, 2004; Mattsson et al., 2009).

### 1.4.1.3.1 CSF Aβ$_{42}$

Aβ$_{42}$ has plausibility as a core biomarker because it accumulates in amyloid plaques reflecting a fundamental feature of AD neuropathology. In the last two decades, numerous studies have reported a significant decrease in CSF Aβ$_{42}$ levels compared to HC. It was originally hypothesized that this decrease was due to the accumulation of soluble Aβ$_{42}$ into plaques with lower levels diffusing into CSF. Although, this theory has been brought into question with evidence that decreased levels of CSF Aβ$_{42}$ are found in other diseases exempt from amyloid pathology such as Creutzfeldt-Jakob disease (CJD),
amyotrophic lateral sclerosis (ALS) and multiple system atrophy (MSA) (Blennow et al., 2003). Although, several studies have reported that high PIB binding correlates negatively with low CSF Aβ42 levels, indicating that decreased levels of Aβ42 in the CSF may, at least in part, be due to increased aggregation in the brain (Fagan et al., 2006; Forsberg et al., 2008).

A comprehensive biomarker study was conducted in 2004 in order to determine the diagnostic accuracy of CSF Aβ42 for AD (Blennow, 2004). Averaged over 16 CSF Aβ42 studies and totaling over 650 AD and 500 HC subjects, this study revealed that CSF Aβ42 has a sensitivity and specificity of 86% and 89%, respectively in distinguishing AD from HC (Table 1-2) (Blennow, 2004). The ability of CSF Aβ42 to distinguish AD from OND and nAD is unclear. CSF Aβ42 may aid in the differential diagnosis of depression, Parkinson’s disease (PD) and progressive supranuclear palsy (PSP) since normal levels of CSF Aβ42 have been reported in these disorders (Blennow, 2004). However, CSF Aβ42 levels may be decreased in frontotemporal dementia (FTD), Lewy body dementia (LBD), vascular dementia (VaD), ALS, CJD, and MSA, limiting its use in differential diagnosis (Minati et al., 2009; Schipper, 2010).

In 2009, a cross-sectional and longitudinal multi-centre study including 529 AD, 750 MCI and 304 HC patients characterized the predictive value of AD CSF biomarkers (Table 1-2) (Mattsson et al., 2009). Patients were
cognitively assessed at baseline and follow-up (2 years later) and categorized as AD, MCI or HC (Mattsson et al., 2009). Baseline CSF Aβ42 levels showed sensitivity and specificity levels of 79% and 65%, respectively in being able to predict the conversion of MCI to AD, within two years (Table 1-2) (Mattsson et al., 2009). Hence, CSF Aβ42 levels are simply not sensitive or specific enough to be implemented clinically in predicting incipient AD. However, in 2010, the Alzheimer Disease Neuroimaging Initiative conducted a cross-sectional and longitudinal multi-institutional study characterizing the utility of CSF biomarkers in the clinical diagnosis of AD (Petersen et al., 2010). This study included 229 HC, 398 MCI and 192 AD subjects (Petersen et al., 2010). Results from this study showed that CSF Aβ42 levels distinguish between HC, MCI and AD groups with a statistical significance of p<0.001 (Petersen et al., 2010).

1.4.1.3.2 CSF total tau

Total tau protein has plausibility as a core AD biomarker because it is a major protein component of neuronal axons and axonal degeneration is a principal hallmark of AD pathology. Over the last twenty years, studies have consistently reported a significant increase in the levels of CSF total tau protein in AD compared to HC groups. Evidence demonstrating an increase in CSF total tau following acute brain trauma (i.e. stroke) supports the hypothesis that total tau is increased in the CSF due to release from degenerating axons (Hesse et al., 2001; Ost et al., 2006; Zetterberg et al., 2006). Furthermore, the highest increases in CSF total tau levels are found in
disorders with the most rapid neuronal degeneration such as CJD (Otto et al., 1997). Conversely, a recent study demonstrated a statistically significant and positive correlation between the level of CSF total tau and NFT pathology in AD brains at autopsy (Tapiola et al., 2009). This result suggests that the level of CSF total tau protein is a reflection of NFT load as opposed to or in addition to axonal degeneration. However, neurodegeneration in AD is progressive so levels of CSF total tau protein should increase slowly in comparison to acute disorders such as stroke. In 2012, Saman and colleagues proposed a novel theory to explain the increase in CSF tau proteins in AD (Saman et al., 2012). Results from this study showed that a neuroblastoma cell line (M1C) secretes tau protein primarily through a process known as exosomal secretion (Saman et al., 2012). Exosomal secretion is known to mediate the release of other aggregation-prone proteins in neurodegenerative diseases such as Aβ protein (Saman et al., 2012). The authors conclude that the levels of CSF tau protein are increased in AD patients due to exosomal secretion rather than neurodegeneration.

An analysis of 20 CSF total tau studies including 2000 AD subjects and 1000 HC shows that levels of CSF total tau protein have a sensitivity and specificity of 81% and 91%, respectively for distinguishing AD from HC groups (Table 1-2) (Blennow, 2004). Similar to CSF Aβ42, CSF total tau protein is not efficacious in discriminating AD from nAD and OND. Levels of CSF total tau protein are known to be elevated in stroke, MS, VaD, FTD and LBD resulting
in lowered specificity levels (~70%) (Clark et al., 2003; Blennow, 2004; Minati et al., 2009). However, normal levels of CSF total tau protein have been reported in depression, alcoholic dementia, PD and PSP (Blennow et al., 2003).

The aforementioned 2009 multi-centre study reported that CSF total tau protein has a sensitivity and specificity of 86% and 56%, respectively in being able to predict the conversion of MCI to AD (Table 1-2) (Mattsson et al., 2009). While the sensitivity of CSF total tau meets the requirement for an ideal biomarker, CSF total tau is not specific enough to distinguish AD from several nAD/OND and predict incipient AD. However, the 2010 ADNI studied confirmed that CSF total tau can distinguish between HC, MCI and AD groups with a statistical significance of p<0.001 on par with Aβ42.

1.4.1.3.3 CSF phospho-tau

Phospho-tau is a plausible AD biomarker because it accumulates in NFTs and NPTs reflecting a fundamental feature of AD neuropathology. Several phospho-tau epitopes are significantly elevated in AD compared to HC. An increase in phospho-tau in the CSF is believed to reflect both the phosphorylation state of tau protein and the accumulation of NFTs in the brain. This hypothesis is supported by several studies reporting that CSF phospho-tau epitopes, specifically those phosphorylated at threonine 181 (phospho-tau_{181}) or 231 (phospho-tau_{231}), significantly and positively correlate with NFT load in autopsied brains (Hampel et al., 2005; Buerger et
An increase in the CSF levels of several phospho-tau epitopes has been identified and includes: phospho-tau$_{181}$, phospho-tau$_{231}$, phospho-tau at serine 199 (phospho-tau$_{199}$) as well as combinations of phospho-tau$_{181}$ & phospho-tau$_{231}$, phospho-tau$_{231}$ & phospho-tau at serine 235, (phospho-tau$_{235}$), and phospho tau at serines 396 & 404 (phospho-tau$_{396}$, phospho-tau$_{404}$) (Blennow et al., 2010). Of these, phospho-tau$_{181}$, 199 & 231 show the most promise with phospho-tau$_{181}$ being the best characterized of the three.

An average of 11 CSF phospho-tau studies including 800 AD and 370 HC subjects revealed that CSF phospho-tau epitopes have a sensitivity and specificity of 80% and 92%, respectively in distinguishing AD from HC (Table 1-2) (Blennow, 2004). Seven of these studies (359 AD and 184 HC) focused on CSF phospho-tau$_{181}$ and revealed a sensitivity and specificity of 76% and 91%, respectively in distinguishing AD from HC groups (Blennow, 2004). Compared to CSF A$\beta_{42}$ and total tau, phospho-tau epitopes are most efficacious at distinguishing AD from nAD and OND. A comparative CSF phospho-tau study revealed that phospho-tau$_{181, 199}$ and 231 can distinguish AD from nAD with a sensitivity of 85% and specificities of 85%, 81% and 72% respectively (Hampel et al., 2004). Furthermore, the three biomarkers distinguished AD from OND with extremely high specificities of 95%, 86% and 91%, respectively (Hampel et al., 2004). More specifically, phospho-tau$_{231}$ displayed the highest specificity (92%) in being able to distinguish AD
from FTD and phospho-tau$_{181}$ demonstrated the highest specificity (68%) in being able to distinguish AD from LBD (Hampel et al., 2004). These results reveal that phospho-tau epitopes demonstrate promise in being able to differentiate between various neurodegenerative diseases, an area where both CSF Aβ$_{42}$ and total tau are lacking.

In the 2009 multi-centre study, it was reported that phospho-tau$_{181}$ had a sensitivity and specificity of 84% and 47% respectively for predicting the conversion of MCI to AD (Table 1-2) (Mattsson et al., 2009). So, while phospho-tau epitopes show greater promise in differential diagnosis compared to Aβ$_{42}$ and total tau, phospho-tau epitopes show low diagnostic accuracy for predicting incipient AD (Mattsson et al., 2009). That being said, the 2010 ADNI studied confirmed that CSF phospho-tau can distinguish between HC, MCI and AD groups with a statistical significance of p<0.001 on par with Aβ$_{42}$ and total tau.

1.4.1.3.4 Biomarker combinations: Aβ$_{42}$, total tau and phospho-tau$_{181}$

A thorough AD biomarker discussion is not complete without addressing studies combining multiple biomarkers. The combination of decreased Aβ$_{42}$ and increased total tau and phospho-tau$_{181}$ is currently the most accurate chemical biomarker for early AD. In line with previous reports, the 2009 multicentre study showed that biomarker combinations have a higher predictive value than that of any single biomarker. More specifically, the study reported that a combination of baseline biomarker levels could predict
incipient AD in MCI individuals with a sensitivity and specificity of 83% and 88%, respectively (Table 1-2) (Mattsson et al., 2009). Taking the analysis a step further, the study also showed that a combination of baseline biomarker levels could distinguish between MCI with incipient AD versus MCI with incipient nAD/OND with specificity values between 57% and 86% (Mattsson et al., 2009). In summary, this multi-centre study demonstrated that a combination of CSF A\(\beta_{42}\), total tau and phospho-tau\(\text{181} \) identify incipient AD in MCI with a high accuracy. However, the results were much lower than those previously reported by single-centre studies highlighting a need for a standardization of technical and clinical procedures. In fact, one of the major goals of ADNI is to improve upon current standards in order to establish uniform standards for neuroimaging and chemical biomarker testing. The true diagnostic ability of current core feasible biomarkers can only truly be established with such long-term initiatives. Therefore, despite tremendous advances in the biomarker field, the necessity for standardization limits the use of neuroimaging and chemical biomarkers for clinical diagnosis. Hence, there remains a gap in the AD field for early clinical diagnosis.

1.5 Hypothesis and Thesis Objectives

Given the need for early AD diagnosis and the ample evidence implicating active Casp6 as an early event and causative factor in AD, we propose to investigate if Casp6 activity in the brain may serve as a biomarker for early AD. Since a cytoskeleton protein such as tau is more stable than an enzyme
such as Casp6, we decided to use TauΔCasp6 protein in the CSF as a measure of Casp6 activity.

To address our hypothesis, we designed a sandwich enzyme-linked immunosorbent assay (ELISA), which specifically detects tau protein that has been cleaved by Casp6 (TauΔCasp6) at the Casp6 C-terminal site KSPVVSGLD. This sandwich ELISA was used to detect TauΔCasp6 in the post-mortem CSF of NCI, MCI and mild, moderate and severe AD groups. Then we assessed levels of TauΔCasp6 in AD brains by immunohistochemistry and compared these levels found in situ to those found in CSF. Finally, we compared the levels of TauΔCasp6 detected in CSF to cognitive scores obtained pre-mortem to determine the clinical validity of our sandwich ELISA test.

2. MATERIALS AND METHODS

2.1 Cloning of TauΔCasp6 and Tau FL proteins

TauΔCasp6 and Tau full-length (Tau FL) complementary deoxyribonucleic acid (cDNA) were amplified from a red fluorescent protein-tau cDNA construct (kind gift from Dr. Yasuo Ihara, University of Tokyo, Japan) via Polymerase Chain Reaction (PCR). TauΔCasp6 cDNA (1032 bp) was amplified using the following forward and reverse primers, respectively: 5’ TTCAGGATCCGCTGAGCCCCGCCAGGAG 3’ and 5’ ACCGCTCGAGTTAGTCCCCAGACACCCTGG 3’. Tau FL cDNA (1149 bp) was
amplified using the following forward and reverse primers, respectively: 5’ TTCAGGATCCGCTGAGCCCCGCCAGGAG 3’ (identical to that used to amplify TauΔCasp6) and 5’ ACACCGCTCGAGTTACAAACCCTGCTTGGCCAG 3’. It is important to note that an ATG start codon was not included in the forward primer. Instead, the start codon of the cloning vector, pET28a+ (Novagen, USA), was used so that the vector’s N-terminal His tag would be incorporated into both Tau FL and TauΔCasp6 proteins upon protein translation. The PCR reactions were performed using 500 ng of RFP-tau cDNA, 20 µM of each primer, 10 mM Deoxyribonucleotide Triphosphate or dNTP, 0.025 units/µl of FideliTaq deoxyribonucleic acid (DNA) polymerase, FideliTaq 10x buffer and H2O (USB, Ohio). A mixture of H2O and 20 µM of each primer was used as a negative control. The reactions were denatured for 10 minutes (min) and 30 seconds (s) at 94°C, annealed for 1 min at 52.8°C for 35 cycles, and extended for 2 and 10 min at 68°C. The PCR DNA products were analyzed on a 1% agarose gel followed by gel extraction purification with the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, USA). Purified PCR DNA was digested using BamHI and XhoI restriction enzymes (Roche, ON). Digestion reactions were performed using 20 units of each restriction enzyme, 1 µg of purified PCR DNA, 10x Buffer H and H2O (Roche, ON). The reactions were incubated for 2 hours at 37°C followed by purification with the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, USA). Purified Tau FL and TauΔCasp6 PCR DNA were each cloned via ligation into the pET28a+ prokaryotic expression vector between BamHI and XhoI
restriction sites (Novagen, USA). Ligation reactions were performed using 1 unit of T4 DNA ligase, 10x T4 DNA ligase buffer, a 1:6 ratio of purified PCR products (25 ng) to pET28a+ vector (150 ng) and H2O (Roche, ON). A mixture of 150 ng of pET28a+ vector, 1 unit of T4 DNA ligase, 10x T4 DNA ligase buffer and H2O was used as a negative control for vector re-ligation. The ligation reactions were incubated overnight at 4°C. Tau FL-pET28a+, TauΔCasp6-pET28a+ and pET28a+ constructs were separately transformed into Rosetta™ competent cells (Novagen, USA). Transformation reactions were performed using 50 ng of each construct and Rosetta™ competent cells. Fifty ng of pET28a+ empty vector and Rosetta™ competent cells were used as a positive control for transformation. The transformation reactions were incubated on ice for 20 min, heat-shocked at 42°C for 1.5 min, incubated on ice for 1 min, added to 800 µl of LB media (5 g of bacto yeast, 10 g of bacto-tryptone and 10 g of NaCl in 1L of H2O), shaken at 37°C for 45 min and centrifuged at 8000 rpm for 10 min to pellet the cells. Pellets were resuspended, plated onto LB + Kanomyacin (Kan+) bacterial plates, and incubated overnight at 37°C. Starter cultures were prepared by shaking colonies in 3 mL of LB media supplemented with 30 µg/ml of Kan+ overnight at 37°C. Glycerol stocks were prepared by mixing starter culture with 30% sterile glycerol. DNA constructs were isolated by an alkaline lysis miniprep method (Birnboim et al., 1979). Each starter culture was centrifuged at 130,000 rpm (max speed) for 20 s to pellet the cells and the supernatant was discarded. The pellet was resuspended in Buffer 1 (50 mM Tris-HCl, 10 mM
ethylenediaminetetraacetic acid or EDTA, 100 µg/mL RNase A, pH 8.0) by vortexing. Cells were lysed by rapid inversion with Buffer 2 (1% sodium dodecyl sulfate (SDS), 0.2M NaOH) followed by 5 min incubation on ice. Ice-cold Buffer 3 (3.0M Potassium Acetate, pH 5.5) was added to each tube and mixed rapidly by inversion several times followed by 5 min incubation on ice in order to isolate plasmid DNA from other macromolecules. Tubes were centrifuged at max speed for 5 min and the supernatant was removed and placed into clean tubes (repeat 3x). Nucleic acids were precipitated by addition of isopropanol, incubation at room temperature for 2 min followed by centrifugation at max speed for 5 min. The supernatants were discarded and the pellets were washed by inversion with absolute ethanol and centrifuged at max speed for 2 min. The supernatants were discarded and the alcohol was allowed to evaporate by leaving the DNA pellets under the chemical hood for 15 min followed by resuspension in H2O. Restriction enzyme digestion with *Bam*HI and *Xho*I restriction enzymes was used to test for positive clones. Clones containing TauΔCasp6 or Tau FL DNA should yield DNA bands of 1032 bp and 1149 bp, respectively upon digestion. Positive clones were confirmed by sequencing at Genome Quebec.

### 2.2 Recombinant protein expression and purification of TauΔCasp6 and Tau FL

Tau FL and TauΔCasp6 Rosetta™ cell glycerol stocks were streaked on LB + Kan⁺ plates and incubated overnight at 37°C. Starter cultures were prepared by mixing 1 colony from each plate with LB media supplemented with 30
µg/ml of Kan⁺, shaking overnight at 37°C. Starter cultures were diluted in 2 L of 2xYT media (16 g of bacto-tryptone, 5 g of bacto-yeast, 2.5 g of NaCl) supplemented with 30µg/mL of Kan⁺ and shaken at 37°C until the OD₆₀₀ reached ~0.55-0.65. Protein expression was induced with 0.2 mM IPTG for 4 hrs shaking at 37°C. Cells were collected by centrifugation at 6000 g for 15 min and lysed by addition of 25 mM Phosphate Buffer (0.2 M sodium phosphate, mono-sodium salt, 0.2 M sodium phosphate, di-sodium salt), 300 mM NaCl, 1 mg/mL lysozyme (SIGMA, ON) and fresh protease inhibitors (38 mg/mL 4-(2-Aminoethyl)-benzenesulfonyl fluoride or AEBSF, 0.1 µg/mL Nα-tosyl-L-lysine chloromethyl ketone or TLCK, 0.1 µg/mL pepstatin A and 0.5 µg/mL) left on ice for 30 min, and sonicated 10 s on/off for 4 min on ice. It is essential to add protease inhibitors to prevent proteolysis because tau proteins are prone to degradation. Lysates were cleared by centrifugation at 45,000 g for 45 min at 4°C and filtered through a 0.22 µm pore (Millipore, ON). The lysate was loaded at a speed of 1 ml/min onto a 3 mL column packed with Nickel Sepharose™ 6 Fast Flow Beads (GE Healthcare, USA) pre-equilibrated with 5x column volume equilibration buffer (25 mM Phosphate Buffer, 300 mM NaCl). Bound proteins were washed with 5x column volume wash buffer 1 (25 mM Phosphate Buffer, 300 mM NaCl, 2mM imidazole). The proteins were eluted with 5x column volume elution buffer (25 mM Phosphate Buffer, 300 mM NaCl, 200 mM imidazole) over 15 x 1 mL fractions. Each protein fraction was separated on 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels and detected by immunoblotting with
anti-human tau HT7 monoclonal antibody (200 μg/mL) generated to the N-terminal peptide PPGQK of tau at 1:3,000 (Pierce Endogen, ON) followed by an anti-mouse HRP secondary antibody (0.8 mg/mL) at 1:10,000 (Jackson Immuno Research, USA) or staining with 2.5 g/L Coomassie Brilliant Blue R250 in 45% methanol and 10% acetic acid (Bioshop, ON). TauΔCasp6 identity was confirmed by running recombinant TauΔCasp6 protein on a non-denaturing gel and immunoblotting with TauΔCasp6 no. 10635 antiserum at 1:5000 developed against the C-terminal KSPVVSMD epitope of tau generated by cleavage with Casp6 (Guo et al., 2004). The 10635 antiserum can only recognize TauΔCasp6 protein in a native state. Expected sizes for Tau FL and TauΔCasp6 recombinant proteins are 53 and 50 kDa, respectively (Goedert et al., 1990). The purist protein fractions were pooled and dialyzed in dialysis buffer (25mM Phosphate Buffer, 25 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol or DTT at pH 6.8) with 20 kDa molecular weight cut off cassettes (Thermo Scientific, IL) for 4 hrs at 4°C. EDTA and are included in the dialysis buffer to lengthen protein shelf life because tau proteins are prone to degradation. Furthermore, it is imperative to avoid freeze-thaw cycles, which decrease protein stability. For long-term storage (months to years), recombinant tau proteins should be stored at -80°C. For short-term storage (days to weeks), prepare small working volumes (10-20 μL) and store at -20°C.
2.3 Collection of Brain Tissues, Fixation, and Preparation of Slides

Brain tissue was obtained from subjects who participated in the Religious Orders Study (ROS), established by Dr. David Bennett in 1993. The ROS includes 1100 older nuns, priests and brothers who have agreed to yearly clinical evaluations and brain donation at time of death. The clinical evaluations include a medical history, and neurologic and cognitive assessments performed on a yearly basis. The Braak staging and clinical evaluations were done by the ROS and scores were unveiled once the study was complete. The ROS provides baseline and last valid neuropsychological assessment scores for mini-mental state examination, visuospatial ability, and perceptual speed as well as episodic, semantic, and working memory. A global cognitive score was generated by converting the values from 19 different cognitive tests into Z scores (using the mean and standard deviation at baseline) and averaged. Participants were diagnostically classified by a clinician following the NINCDS-ADRDA criteria. An AD diagnosis was assigned to persons with a history of cognitive decline and evidence of impairment in memory and other cognitive domains. MCI referred to individuals who displayed cognitive impairment upon neuropsychological evaluations but were not clinically diagnosed with dementia. NCI referred to persons with no cognitive impairment.
2.4 Immunostaining of Tissue Sections with Anti-active Casp6 and TauΔCasp6

Formalin-fixed, paraffin-embedded 4-µm thick hippocampal tissue sections were deparaffinized and rehydrated in xylene (2 x 5 min), 100% ethanol (2 x 5 min), 95% ethanol (1 x 3 min) and H₂O (1 x 5 min), sequentially. Sections were treated with antigen retrieval buffer (10mM Tris Base, 1mM EDTA, 0.05% Tween 20, pH 9) for 20 min at 97°C. Sections were immunostained using the Dako Autostainer Plus, an automated slide processor (Dako, ON). The Dako Autostainer Plus was programmed to perform a specified protocol at room temperature using antibodies and reagents prepared by the user. The Dako Autostainer Plus treated sections with 450 µL of EnVision™ Flex Peroxidase Blocking Reagent (Dako, ON) for 5 min before washing with 450 µL of 1x EnVision™ Flex Wash Buffer (diluted from 20x concentrate; Dako, ON). Sections were blocked with 450 µL of Serum-Free Protein Block (Dako, ON) for 30 min followed by a washing cycle. Sections were treated with 450 µL of primary antibody prepared in EnVision™ Flex Antibody Diluent for 30 min. The antibody to active Casp6 (p20Csp6) no. 10630 antiserum was developed against the p20 subunit C-terminal PLDVVD sequence of Casp6 (Guo et al., 2004). The TauΔCasp6 no. 10635 antiserum was developed against the C-terminal KSPVVSGD epitope of tau generated by cleavage with Casp6 (Guo et al., 2004). Dilutions for 10635 and 10630 antisera were 1:25000 and 1:1000, respectively. EnVision™ + System mouse or rabbit-HRP ready-to-use secondary antibodies (Dako, ON) were applied for 30 min.
Staining was visualized with 450 μL of EnVision™ + System diaminobenzidine (DAB; Dako, ON) incubated for 10 min and counterstained with Hematoxylin for 5 min (Dako, ON). Sections were manually dehydrated in 95% ethanol (1 x 3 min) and 100% ethanol (1 x 5 min, 1 x 3 min) and cleared in xylene (2 x 3 min). Sections were mounted in Permount mounting medium (Fisher Scientific, ON) and coverslipped with Premium Cover Glass (Fisherfinest, ON).

### 2.5 Assessment of Immunohistochemical Staining

The MIRAX SCAN was used to laser scan tissue sections and generate high-resolution digital images, which were analyzed using the MIRAX Viewer Program (Zeiss, DE). The Atlas of the Human Brain was used as a reference to identify the hippocampus proper (CA1-CA4 and subiculum) and the ERC, trans-ERC and temporal cortices in each tissue section (Jurgen, 2008). In some sections, not all of these brain areas were present. Scoring was done in collaboration with neuropathologist, Dr. Steffen Albrecht, in a blinded manner. The densities of NFTs, NPs and NPTs were scored semi-quantitatively as absent (0), absent to low (0-1), low/mild (1), mild to moderate (1-2), moderate (2), moderate to severe (2-3) or high/severe (3). Scoring diagrams developed by CERAD for assessing AD pathology in autopsy brains were used as guides. Alzheimer type tau pathology (NFTs, NPTs, and NPs) was assessed using conventional well-established neuropathological diagnostic criteria (Mirra et al., 1993).
2.6 Assessment of TauΔCasp6 protein levels in the CSF using a sandwich ELISA

The TauΔCasp6 no. 10635 antiserum was developed against the C-terminal KSPVVSGD epitope of tau generated by cleavage with Casp6 (Guo et al., 2004). The TauΔCasp6 antiserum was prepared at a 1:125 dilution in 1x Phosphate-Buffered Saline (PBS diluted from 10x concentrate: 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄, 2 g KH₂PO₄, pH 7.4). One hundred microliters of TauΔCasp6 antiserum solution was added to each well of a 96-well clear, flat bottom plate and incubated overnight at 4°C (Nunc, USA). Each well was washed with 300 μL of Tris Buffered Saline with tween (TBST; 50 mM Tris, 150 mM NaCl, 0.05% tween 20, pH 7.4) 4 x 2 min, shaking at a speed setting of 3 (Barnstead/Lab-line Rotator Model 1314). Plates were blocked with 300 μL of bovine serum albumin in TBST (BSA-TBST; 2.5% solution of bovine serum albumin fraction V crystalline powder dissolved in TBST, pH 7.4) for 2 hours at room temperature, shaking at a speed setting of 3. Each well was washed with 300 μL of TBST 4 x 2 min, shaking at a speed setting of 3. Control samples were prepared in Tris Buffered Saline (TBS; 50mM Tris, 150mM NaCl, pH 7.4). CSF samples were not diluted. One hundred μL of control/CSF samples were loaded into each well and allowed to incubate for 3 hours at room temperature, shaking at a speed setting of 3. Each well was washed with 300 μL of TBST 6 x 1 min, shaking at a speed setting of 3. Anti-human tau HT7 monoclonal antibody generated to the N-terminal peptide PPGQK of tau (200 μg/mL) was used as the detection antibody (Pierce
Endogen, ON). The HT7 antibody was prepared at a 1:1000 dilution in 1% BSA-TBS. One hundred µL of the HT7 antibody solution was added to each well and incubated overnight at 4°C, shaking at a speed setting of 3. Each well was washed with 300 µL of TBST 6 x 1 min, shaking at a speed setting of 3. HRP anti-mouse IgG Fc antibody (0.8 mg/mL) was used as the developing antibody (Jackson Immuno Research, ON). The HRP anti-mouse antibody was prepared at a 1:100 000 dilution in 1% BSA-TBS. One hundred µL of the HRP anti-mouse antibody solution was added to each well and incubated for 1 hour at room temperature, shaking at a speed setting of 3. Each well was washed with 300 µL of TBST 6 x 1 min, shaking at a speed setting of 3. The ELAST ELISA Amplification System was used to increase assay sensitivity (PerkinElmer, USA). Biotinyl tyramide solution was prepared according to the producer’s manual. One hundred µL of the biotinyl tyramide solution was added to each well and incubated for 15 minutes at room temperature, shaking at a speed setting of 3 and protected from light. Each well was washed with 300 µL of TBST 6 x 1 min, shaking at a speed setting of 3. HRP avidin reaction was prepared according to the producer’s manual. One hundred µL of the biotinyl tyramide solution was added to each well and incubated for 15 minutes at room temperature, shaking at a speed setting of 3 and protected from light. Ready-to-use Enhancer K-Blue TMB was used as the HRP substrate (Neogen, USA). One hundred microliters of the substrate was added. Fifty µL of 0.5 M sulfuric acid was added to each well to stop color development. Plates were read at a wavelength of 450 nm. Recombinant
TauΔCasp6 protein standard curves comparing protein concentration in pg/mL (x-axis) to absorbance readings at 450 nm (y-axis) were used quantitatively assess the amount of TauΔCasp6 in each CSF sample.

2.7 Statistical Evaluations

InStat 3 statistical software was used for all statistical evaluations. One-way Analysis of Variance was used to test if the data are sampled from populations that follow Gaussian distributions. Post-hoc Bonferroni multiple comparisons test was used to evaluate group differences. Spearman Rank correlation and linear regression were used for correlative analyses.

3. RESULTS

3.1 Recombinant TauΔCasp6 protein is specifically detected using the TauΔCasp6 sandwich ELISA

N-terminally His-tagged bacterially expressed TauΔCasp6 and Tau full-length (Tau FL) proteins were purified as positive and negative controls, respectively for use in the TauΔCasp6 sandwich ELISA. With the addition of the His tag, Tau FL and TauΔCasp6, had expected molecular weights of 41 kDa and 37 kDa, respectively. Coomassie Brilliant Blue staining and immunoblotting with an anti-human tau HT7 monoclonal antibody generated to the N-terminal peptide PPGQK revealed that Tau FL and TauΔCasp6 did not run at the predicted masses (Fig 3-1A). Instead, Tau FL and TauΔCasp6 migrated with apparent molecular weights of 53 kDa and 50 kDa,
respectively (Fig 3-1A). This is expected since tau proteins migrate anomalously on SDS-PAGE gels, appearing at molecular weights higher than those predicted (Goedert et al., 1988; Lee et al., 1988; Goedert et al., 1990). Although the Tau FL isoform, purified herein, has an actual molecular weight of 41 kDa, the protein has an apparent mass of 53 kDa when separated on an SDS-PAGE gel (Goedert et al., 1990).

Coomassie Blue stain and immunoblotting also revealed that Tau FL and TauΔCasp6 are prone to proteolysis (Fig 3-1A). Despite significant proteolysis, the TauΔCasp6 antiserum developed against the C-terminal KSPVVSVD epitope of TauΔCasp6 did not show immunoreactivity with the degradation products (Fig 3-1B) (Guo et al., 2004). TauΔCasp6 and Tau FL proteins were resolved under non-denaturant conditions and immunoblotted with the TauΔCasp6 antiserum, which detects TauΔCasp6 protein under native conditions only (Fig 3-1B) (Guo et al., 2004). The TauΔCasp6 antiserum showed specificity for cleaved but not full-length tau or the degradation products (Fig 3-1B). It was vital that the TauΔCasp6 antiserum showed specificity for TauΔCasp6 because the antiserum was used to “capture” TauΔCasp6 in the TauΔCasp6 sandwich ELISA (Fig 3-1C).

To assess levels of TauΔCasp6 in the CSF, a TauΔCasp6 sandwich ELISA was developed (Fig 3-1C). Coating wells with the TauΔCasp6 antiserum was the first of six steps (Fig 3-1C). The TauΔCasp6 antiserum had to specifically
capture TauΔCasp6 from recombinant protein or post-mortem CSF samples (Fig 3-1C). The captured samples were “sandwiched” by the addition of the anti-human tau HT7 monoclonal antibody generated to the N-terminal peptide PPGQK (Fig 3-1C). An HRP anti-mouse IgG Fc antibody was used to specifically detect bound HT7 antibody (Fig 3-1C). An amplification system was used to increase the number of HRP enzymes available to cleave TMB substrate therefore enhancing the colorimetric signal (Fig 3-1C). The ELISA detected recombinant TauΔCasp6 protein within a linear ($r^2 = 0.999$) range of 15.6-1000 pg/mL and a detection limit of 3.9 pg/mL (Fig 3-1D). The TauΔCasp6 sandwich ELISA showed little detection of the recombinant Tau FL protein even at 1000 pg/mL protein concentrations (Fig 3-1D). The TauΔCasp6 sandwich ELISA is a suitable method for specifically and quantitatively measuring TauΔCasp6 in biological samples.
Figure 3-1

A. Coomassie

B. Western Blot

C. (7) TMB substrate
   (6) Streptavidin-HRP
   (5) Biotinyl tyramide
   (2) Recombinant controls or CSF samples
   (4) Anti-mouse HRP antibody
   (3) Anti-human tau HT7 monoclonal antibody
   (1) TauΔCasp6 antiserum

D. 

\[ \text{Absorbance (450 nm)} \]

\[ \begin{align*}
\text{TauΔCasp6} & : r^2 = 0.999 \\
\text{Tau FL} & : r^2 = 0.428
\end{align*} \]

\[ \text{Protein concentration (pg/mL)} \]
Figure 3-1. Validation of TauΔCasp6 sandwich ELISA. A. Coomassie blue stain (1μg) and western blot (100ng) analysis with anti-human tau HT7 monoclonal antibody on recombinant Tau FL and TauΔCasp6 proteins. B. Western blot (100ng) analysis using TauΔCasp6 antiserum on recombinant Tau FL and TauΔCasp6 proteins separated under non-denaturant conditions. C. Schematic diagram of in-house developed sandwich TauΔCasp6 ELISA. D. Representative plot of TauΔCasp6 (square) and Tau FL (diamond) standard curves.
3.2 Demographics of the NCI, MCI and AD cases used in this study.

A total of 24 cases were used in this study: 7 NCI, 5 MCI and 12 AD (Table 3-1). There are no significant differences in age or education (in years) between the three groups. Although the number of females and males is not equivalent within groups, overall there are 11 males and 13 females. The average MMSE score of the AD group is significantly lower (p<0.01) when compared to the NCI and MCI groups. This is expected, as individuals with dementia tend to score lower on the MMSE than those without dementia.
Table 3-1. Demographics of the NCI, MCI and AD cases used in this study.

<table>
<thead>
<tr>
<th></th>
<th>NCI</th>
<th>MCI</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Age at death (years)</td>
<td>83.35 ± 8.36</td>
<td>84.09 ± 9.77</td>
<td>89.94 ± 4.04</td>
</tr>
<tr>
<td>Education (years)</td>
<td>19.43 ± 3.60</td>
<td>17.00 ± 4.00</td>
<td>18.92 ± 2.23</td>
</tr>
<tr>
<td>Gender</td>
<td>5 males, 2 females</td>
<td>3 males, 2 females</td>
<td>3 males, 9 females</td>
</tr>
<tr>
<td>MMSE</td>
<td>27.71 ± 1.80*</td>
<td>28.18 ± 1.29*</td>
<td>13.35 ± 9.13</td>
</tr>
</tbody>
</table>

Data represent mean ± SD. NCI = non-cognitive impairment; MCI = mild-cognitive impairment; AD = Alzheimer disease; MMSE = mini-mental state examination. *p<0.01 vs AD via Bonferroni Multiple Comparisons Test.
3.3 The level of TauΔCasp6 detected in the CSF is significantly increased in AD compared to NCI individuals

In order to determine if there were group differences between the levels of TauΔCasp6 detected in the CSF, the aforementioned TauΔCasp6 sandwich ELISA was used to measure TauΔCasp6 in the 24 NCI, MCI and AD cases (Fig 3-2A).

The level of TauΔCasp6 in the CSF was significantly higher (p<0.05) in the AD group compared to the NCI group (Fig 3-2B). Even though the MCI group had a higher level of TauΔCasp6 in the CSF compared to the NCI group and a lower level versus the AD group, the difference did not reach statistical significance (Fig 3-2B).

In order to assess if there is a correlation between the level of TauΔCasp6 in the CSF and disease severity, the AD group was divided into mild (N = 5), moderate (N = 3) and severe (N = 4) AD cases. A comparison between all individuals in the five cognitive groups with the level of CSF TauΔCasp6 revealed a positive and statistically significant correlation (Fig 3-2C). Linear regression analysis yielded an extremely significant (p<0.0001) $r^2$ value of 0.498. This result indicates that the level of TauΔCasp6 in the CSF increases as AD worsens.
Figure 3-2

A. CSF Tau\DeltaCasp6 levels (pg/mL) in NCI, MCI and AD

<table>
<thead>
<tr>
<th></th>
<th>NCI</th>
<th>MCI</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>73.5</td>
<td>108</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>111</td>
<td>119</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>113</td>
<td>157</td>
<td>151.5</td>
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<td>154.5</td>
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<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>Average</th>
<th>SD</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
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<tbody>
<tr>
<td></td>
<td>210.5</td>
<td>44</td>
<td>164</td>
<td>225</td>
<td>277</td>
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<tr>
<td></td>
<td>136</td>
<td>32</td>
<td>23</td>
<td>78</td>
<td>83</td>
</tr>
</tbody>
</table>

B. 

![Graph showing CSF Tau\DeltaCasp6 levels (pg/mL) in NCI, MCI, and AD.](image)

C. 

![Graph showing correlation between CSF Tau\DeltaCasp6 levels (pg/mL) and AD severity.](image)
Figure 3-2. Levels of TauΔCasp6 in the CSF of NCI, MCI and AD groups. A. Table of the mean levels and standard deviations of TauΔCasp6 in pg/mL measured in the CSF of NCI, MCI and AD (mild, moderate, severe) groups. B. Plot of mean levels of TauΔCasp6 in pg/mL detected in the CSF of NCI, MCI and AD groups. Mean level measured in the AD group (217 pg/mL) is significantly higher than the NCI group (136pg/mL) but not the MCI group (147pg/mL). *p<0.05 using Bonferroni Multiple Comparisons Test. C. Plot of individual levels of TauΔCasp6 in pg/mL detected in the CSF of NCI, MCI and mild, moderate and severe AD groups. Dementia level positively associates with the level of TauΔCasp6 detected in the CSF. * p<0.0001 by linear regression analysis.
3.4 *The level of TauΔCasp6 in the CSF positively correlates with the level of active Casp6 and TauΔCasp6 detected in brains*

An increase in the level of TauΔCasp6 in the CSF of the AD group may be a reflection of an increased accumulation of active Casp6 or TauΔCasp6 in NFTs, NPTs and NPs in brains. This potential was assessed by immunohistochemically staining hippocampal tissue sections of all 24 NCI, MCI and AD cases using the active Casp6 or TauΔCasp6 antiserum. The antibody to active Casp6 (p20Csp6) no. 10630 antiserum was developed against the p20 subunit C-terminal PLDVVD sequence of Casp6 (Guo et al., 2004). The TauΔCasp6 no. 10635 antiserum was developed against the C-terminal KSPVVSVD epitope of tau generated by cleavage with Casp6 (Guo et al., 2004). The specificity of the active Casp6 and TauΔCasp6 antisera were previously defined via immunohistochemistry and western blotting (Guo et al., 2004). The levels of active Casp6 or TauΔCasp6 in the ERC, subiculum and hippocampus (CA1-CA4) were assessed by assigning a score of 0-3 based on the abundance of NFTs, NPTs and NPs (**Fig 3-3A**). Areas without immunoreactivity were given a score 0, those with sparse staining received a score of 1, moderate staining a score of 2 and frequent staining a score of 3 (**Fig 3-3A**).

A comparison between the levels of TauΔCasp6 detected in the CSF and the levels of TauΔCasp6 (**Fig 3-3B**) or active Casp6 (**Fig 3-3C**) measured in the brain revealed positive and significant correlations. Spearman Rank
correlation analysis yielded significant $r$ values for TauΔCasp6 in the ERC, subiculum, CA1, CA3 and CA4 areas of the hippocampus as well as the temporal cortex. Significant $r$-values for active Casp6 were found in the trans-ERC, ERC, subiculum, CA2, CA3 and CA4 areas of the hippocampus as well as the temporal cortex. These results indicate that the level of TauΔCasp6 detected in the CSF is mirroring Casp6 activity or accumulation of TauΔCasp6 in some brain areas highly affected in AD. These findings also suggest that the increase in TauΔCasp6 in the CSF could be a direct result of the accumulation of TauΔCasp6 in brains.

In the brain areas first affected by AD pathology (Fig 3-3B a-d), most cases display a high TauΔCasp6 staining score whereas in the areas affected later on in the disease process (Fig 3-3B e-h), the majority of cases show a weaker TauΔCasp6 staining score. This result indicates that the spread of TauΔCasp6 in the brain reflects NFT progression defined by Braak staging.
Figure 3-3

A.

B. TauCasp6

C. Active Casp6
Figure 3-3. Comparison between levels of active Casp6 or TauΔCasp6 quantified in brains and levels of TauΔCasp6 detected in the CSF in all individuals. A. Semi-quantitative scoring method for TauΔCasp6 and active Casp6 antisera. NFTs, NPs and NPTs were scored as absent (0), low/mild (1), moderate (2), or high/severe (3). B. Plot of individual levels of TauΔCasp6 measured in the (a) trans-ERC, (b) ERC, (c) subiculum, (d-g) CA1-CA4 and (h) temporal cortex compared to levels of TauΔCasp6 in pg/mL measured in CSF. * p<0.05, **p<0.01 by Spearman Rank correlation analysis. C. Plot of individual levels of active Casp6 measured in the (a) trans-ERC, (b) ERC, (c) subiculum, (d-g) CA1-CA4 and (h) temporal cortex compared to levels of TauΔCasp6 in pg/mL measured in CSF. * p<0.05, **p<0.01 by Spearman Rank correlation analysis.
3.5 The level of TauΔCasp6 in the CSF negatively correlates with memory scores in all individuals

Given the above finding, the association between the level of TauΔCasp6 detected in the CSF and cognitive assessment scores was investigated. Various cognitive test scores were compared to the level of TauΔCasp6 detected in the CSF of all 24 NCI, MCI and AD cases.

A comparison between GCS or MMSE score and the level of TauΔCasp6 detected in the CSF revealed significant negative correlations (Fig 3-4 a, b). Spearman Rank correlation analyses yielded statistically significant (p<0.01) r-values of -0.547 and -0.555 for GCS and MMSE score, respectively (Fig 3-4 a, b). A comparison between episodic, working or semantic memory scores with the level of TauΔCasp6 measured in the CSF also revealed statistically significant negative correlations (Fig 3-4 c, d, e). Spearman Rank correlation analyses yielded r-values of -0.472 (p<0.01), -0.509 (p<0.05) and -0.549 (p<0.01) for episodic, working and semantic memory scores, respectively (Fig 3-4 c, d, e). Visuospatial ability (VSA) and perceptual speed (PS) scores were also compared to the level of TauΔCasp6 detected in the CSF (Fig 3-4 f, g). These comparisons revealed negative but not statistically significant trends (Fig 3-4 f, g).
Figure 3-4

A. CSF Tau/ΔCasp6 (pg/mL) vs. GC score, $r = -0.547^{**}$

B. CSF Tau/ΔCasp6 (pg/mL) vs. MMSE score, $r = -0.555^{**}$

C. CSF Tau/ΔCasp6 (pg/mL) vs. Episodic memory score, $r = -0.472^{*}$

D. CSF Tau/ΔCasp6 (pg/mL) vs. Semantic memory score, $r = -0.549^{**}$

E. CSF Tau/ΔCasp6 (pg/mL) vs. Working memory score, $r = -0.509^{*}$

F. CSF Tau/ΔCasp6 (pg/mL) vs. Visuospatial ability score, $r = -0.416$

G. CSF Tau/ΔCasp6 (pg/mL) vs. Perceptual speed score, $r = -0.417$
Figure 3-4. Comparison between memory scores and the levels of TauΔCasp6 measured in the CSF across all individuals. Plot of individual GC (a), episodic memory (b), working memory (c), semantic memory (d), visuospatial ability (e), perceptual speed (f) and MMSE (g) scores compared to the level of TauΔCasp6 in pg/mL measured in the CSF. *p<0.05, **p<0.01 by Spearman Rank correlation analysis.
4. DISCUSSION

Given the need for early AD diagnosis and the ample evidence implicating active Casp6 as an early event in AD, we investigated if Casp6 activity in the brain may serve as a biomarker for early AD. Since cytoskeleton proteins like tau are more stable than enzymes such as Casp6, we used TauΔCasp6 protein in the CSF as a measure of Casp6 activity. We find that levels of TauΔCasp6 in post-mortem CSF are significantly increased in AD compared to NCI and in fact increase with disease severity. Furthermore, we show that TauΔCasp6 levels in the CSF positively correlate with levels of TauΔCasp6 in some affected regions of AD and negatively correlate with memory test scores.

4.1 *TauΔCasp6 levels in the CSF increase with the severity of AD*

The results described in this thesis demonstrate a novel finding that TauΔCasp6 accumulates in the CSF of AD. Specifically, we found 1) a significant difference between the CSF TauΔCasp6 levels in AD versus NCI and 2) an extremely significant positive correlation between the severity of AD and the amount of TauΔCasp6 in the CSF. Ideally, an AD biomarker should be efficacious at detecting the disease at all stages, especially early on, i.e. MCI (Reagan, 1998). In our study, CSF TauΔCasp6 levels detected in the MCI group are not significantly different from the NCI or AD group. The inability of TauΔCasp6 to distinguish between MCI and NCI or AD can be explained by the heterogeneity of the MCI group. The MCI population is composed of
individuals who may or may not convert to AD. In order to accurately determine if \( \text{Tau}^\Delta \text{Casp6} \) in the CSF can detect AD at early stages, a longitudinal study, like those completed for \( \text{A}\beta_{42} \), total tau and phospho-
\( \text{tau}_{181} \) is required. With a longitudinal study, levels of \( \text{Tau}^\Delta \text{Casp6} \) in the CSF of NCI, MCI and AD individuals can be measured at baseline and at follow-up. At follow-up the MCI group can be separated into MCI who convert to AD and those who do not. If baseline levels of \( \text{Tau}^\Delta \text{Casp6} \) in the CSF can distinguish “MCI-converters” from NCI and “MCI non-converters” then \( \text{Tau}^\Delta \text{Casp6} \) is a true “early” biomarker.

Despite vast evidence demonstrating the accumulation of truncated tau in AD brains, this is the first study to report an increase of cleaved tau in the CSF of AD versus healthy controls (Gamblin et al., 2003; Guo et al., 2004; Horowitz et al., 2004). While several enzymes can cleave tau protein, we show here that tau specifically cleaved by Casp6 accumulates in the CSF of AD. It remains a matter of debate in the biomarker field as to how tau proteins are released from the brain and accumulate in the CSF. However, results from a recent study show that neuroblastoma cells secrete phospho-tau through a process known as exosomal secretion (Saman et al., 2012). The authors conclude that the levels of CSF phospho-tau are increased in AD patients due to exosomal secretion (Saman et al., 2012). \( \text{Tau}^\Delta \text{Casp6} \) could be released into the CSF via a similar or the same mechanism. Indeed, many studies show that tau protein is cleaved prior to being hyperphosphorylated in AD brains.
(Gamblin et al., 2003; Horowitz et al., 2004; Rissman et al., 2004; Guillozet-Bongaarts et al., 2005; de Calignon et al., 2010). If tau cleavage precedes hyperphosphorylation then exosome released tau would be phosphorylated and cleaved, potentially explaining the increase in CSF TauΔCasp6 in AD.

4.2 TauΔCasp6 levels in the CSF reflect the levels of TauΔCasp6 in affected brain regions of AD

Our results solidify that the levels of TauΔCasp6 in the CSF are an accurate reflection of those levels accumulating in the brain. Specifically, the levels of TauΔCasp6 in the CSF positively and significantly correlate with levels detected in the ERC, CA1, CA3, CA4 and temporal cortex. Ideally, a core feasible biomarker should reflect a fundamental feature of AD neuropathology (Reagan, 1998). It is peculiar that a significant correlation is not reached in the trans-ERC considering that this is a highly affected, not to mention the first affected, area in AD. It is possible that even though there is a great deal of TauΔCasp6 deposited in the trans-ERC, TauΔCasp6 is incapable of “escaping” from this particular brain region into the CSF and therefore does not correlate. Alternatively, since AD neuropathology begins in the trans-ERC, this brain region may be so saturated with TauΔCasp6 deposits and therefore levels do not correlate with those in CSF. A lack of correlation in the CA2 might be due to the volume encompassed by the CA2 region. A small volume indicates that the CA2 does not contribute tremendously to the amount of circulating TauΔCasp6 in the CSF and could account for the lack of
correlation. Furthermore, the CA2 region of the hippocampus is not highly involved in AD in comparison to the CA1, for example, and may not accumulate enough pathology to correlate with TauΔCasp6 levels found in CSF.

Over the last 15 years, only a hand-full of studies has investigated the relationship between CSF biomarker levels and AD neuropathological changes in the brain. Of these, none have looked specifically at the trans-ERC, ERC, CA2, CA3 or CA4. However, two of these studies have assessed neuropathological load in the CA1 region of the hippocampus and the temporal cortex (Tapiola et al., 1997; Strozyk et al., 2003; Buerger et al., 2006). In these studies, total tau and phospho-tau_{231} CSF levels significantly correlate with NFT load in the temporal cortex in accordance with our study (Tapiola et al., 1997; Buerger et al., 2006). However, neither total tau nor phospho-tau_{231} CSF levels correlate with NFT load in the CA1 while our study shows a significant correlation in the CA1 (Tapiola et al., 1997; Buerger et al., 2006). One explanation for this inconsistency could be due to differing scoring methods. Tapiola and colleagues scored brain sections based on the accumulation of total tau in NFTs only (Tapiola et al., 1997). However, in AD tau protein accumulates in NFTs, NPTs and NPs. Therefore, a true assessment of total tau load in the brain must include NFTs, NPTs and NPs. Buerger and colleagues addressed this issue by scoring phospho-tau_{231} NFT and NP load (Buerger et al., 2006). Even still, this study did not find a correlation between
phospho-tau$_{231}$ load in the CA1 and levels in the CSF (Buerger et al., 2006). Alternatively, inconsistencies between our study and those aforementioned could be due to dissimilarities between the types of tau under investigation. Tapiola and colleagues were investigating total tau, Buerger and associates were measuring phospho-tau$_{231}$ while our study involved TauΔCasp6 (Tapiola et al., 1997; Buerger et al., 2006). Indeed, many studies show that tau protein is cleaved prior to being hyperphosphorylated in AD brains (Gamblin et al., 2003; Horowitz et al., 2004; Rissman et al., 2004; Guillotet-Bongaarts et al., 2005; de Calignon et al., 2010). If tau cleavage does indeed precede hyperphosphorylation in AD brains, then cleaved tau protein could be secreted into the CSF first and therefore correlate more accurately.

### 4.3 TauΔCasp6 levels in the CSF reflect memory test scores

The results from this study reveal that the levels of TauΔCasp6 in the CSF are an accurate reflection of memory test scores. Specifically, the levels of TauΔCasp6 in the CSF correlate inversely and significantly with GCS, MMSE score and episodic, working and semantic memory scores but not with VSA or PS scores. A lack of correlation between the level of TauΔCasp6 in the CSF and VSA or PS scores is not surprising. Unlike episodic, semantic and working memory, VSA and PS deficits occur in patients with late-stage AD (Salmon, 2011). Hence, when VSA and PS begin to decline in the end stages of AD, high levels of TauΔCasp6 are already circulating in the CSF and could account for the lack of correlation.
Comparisons between levels of total tau, phospho-tau$_{181}$ and Aβ$_{42}$ in the CSF with memory scores yield similar correlations. Aβ$_{42}$, total tau and phospho-tau$_{181}$ significantly correlate with MMSE scores in MCI and episodic memory scores in APOE ε4 carries with severe memory impairment (Andersson et al., 2007; Mattsson et al., 2009). However, a recent study from 2010 showed no clear correlation between any of the three core feasible biomarkers and cognitive functioning in AD (Spies et al., 2010). Thus, the relationship between the core feasible biomarkers and cognitive functioning remains unclear. To our knowledge, no biomarker study has compared the levels of CSF Aβ$_{42}$, total tau and phospho-tau$_{181}$ to VSA or PS scores.

4.4 Theoretical implications and practical applications

Taken together, these results implicate TauΔCasp6 as a biomarker of specific pathological and cognitive changes associated with AD. At present, clinical and neuropsychological assessments are laborious, time-consuming and expensive and lead to a “probable” or “possible” AD diagnosis at best. However, early and accurate clinical diagnosis is becoming increasingly important with the advent of therapeutic agents designed to impede disease progression. If TauΔCasp6 in the CSF proves to be an early AD biomarker, it could be used clinically to identify individuals who are in the earliest stages of the disease but who do not yet show clinical symptoms. Though it is possible that a treatment to prevent or slow the progression of AD may never come to realization, identifying preclinical AD by measuring CSF TauΔCasp6...
remains beneficial because it prolongs the patient’s autonomy and allows them as well as their families to prepare for the future. It also provides time for patients to enroll themselves in clinical trials to determine which current treatments work best. Enrolling patients in clinical trials also aids future patients because it allows researchers to determine the best clinical outcomes. That being said, TauΔCasp6 could also be implemented in clinical drug trials to monitor therapeutic efficacy. Since TauΔCasp6 is an excellent marker of the neuropathological and cognitive changes occurring in AD, TauΔCasp6 could be implemented as a clinical outcome measure to determine if a particular therapy results in clearance of AD hallmarks or improvement in memory test scores.

4.5 Limitations and future experiments

Further studies are required to confirm how effective TauΔCasp6 is at detecting early stages of AD since 1) our results do not show a significant difference in TauΔCasp6 between MCI and NCI or AD and 2) the positive correlation between severity of AD and levels of TauΔCasp6 is driven by the advanced AD (moderate and severe) rather than the earlier groups (MCI and mild). Furthermore, our study focused on a population of NCI, MCI and AD individuals however an ideal biomarker must be able to distinguish AD from nAD and OND. Hence, studies investigating differences between AD and other diseases must be conducted in order to confirm biomarker sensitivity and specificity. Furthermore, even though each CSF sample was tested in
triplicate, the TauΔCasp6 ELISA was only performed once and therefore must be repeated to confirm reliability and reproducibility. Lastly, fresh pre-mortem samples must be tested in future studies to confirm results from this pilot study using post-mortem CSF.

5. CONCLUSION

Globally, the results from this thesis introduce TauΔCasp6 protein in the CSF as a novel AD biomarker. This work is extremely important due to the desperate need for early clinical diagnosis of AD and the lack of standardization for current core feasible AD biomarkers. Primarily, this work shows that TauΔCasp6 is increased in the CSF of AD in comparison to NCI. Evidence that levels of TauΔCasp6 in the CSF accurately reflect levels in brain as well as memory scores substantiates the potential of TauΔCasp6 in the CSF as a future clinical diagnostic tool.

This work also contributes to the growing body of evidence implicating the activation of Casp6 as a crucial event in AD. Presently, the official cause of sporadic AD remains unknown. Since AD is a multifactorial disorder solidifying Casp6 activation as a key player in neurodegeneration is crucial to understanding the complex disease process. Collectively, this work provides a much-needed novel AD biomarker and advances our understanding of Casp6 activation as a key event in AD.
REFERENCES


