THE INHERITANCE OF LOSS: CASPASE 6 ACTIVITY AND EFFECT IN HUMAN NEURONS CAUSED BY FAMILIAL ALZHEIMER'S DISEASE ASSOCIATED MUTANTS

By

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ABBREVATIONS

Amyloid Beta	Aβ
Actylcholinesterase	AChE
A Disintegrin And Metalloprotease	ADAM
Alzheimer's Disease	AD
7-amino-4-trifluoromethylcoumarin	AFC
Analysis of Variance	ANOVA
Anterior Pharynx Defective 1	APH1
Apolipoprotein E	APOE
Amyloid Precursor Like Protein	APLP 1/2
Amyloid Precursor Protein	APP
β-site APP cleaving enzyme	BACE
CAspase Recruitment Domain	CARD
Caspase 6	Casp6
Cyclin Dependent Kinase 5	CDK5
Caenorhabditis Elegans	C. Elegans
Caenorhabditis Elegans Death gene	CED
Choline acetyltransferase	ChAT
Cytomegalovirus	CMV
C-terminal fragment	CTF
Cytotoxic T Lymphocyte	CTL
Death Effector Domain	DED
Dominant Negative	DN
Down's Syndrome	DS
Elongation Factor 1 alpha	EF-1α
Enhanced Green Florescent Protein	EGFP

Electron Microscopy	EM
Endoplasmic Reticulum	ER
Familial Alzheimer Disease	FAD
Golgi-localized-γ-ear-containing-ARF binding protein	GGA3
Glycogen Synthase Kinase 3β	GSK 3β
Huntington's Disease	HD
Immunoreactive	IR
JNK Interacting Protein 1	JIP-1
c-Jun N term Kinase	JNK
Kinesin Light Chain	KLC
London mutation	Lond
Mammalian Ced-3 Homologue 2	Mch2
Mild Cognitive Impairment	MCI
Minimum Essential Media	MEM
Mini Mental Status Examination	MMSE
Magnetic Resonance Imaging	MRI
Nerve Growth Factor	NGF
Phosphate Buffered Saline	PBS
Presenilin Enhancer 2	PEN2
Presenilin 1 gene/protein	PSEN1/PS1
Presenilin 2 gene/protein	PSEN2/PS2
Polyvinylidene Fluoride	PVDF
Sporadic Alzheimer's Disease	SAD
Single Nucleotide Polymorphism	SNP
Sortilin-related Receptor gene	SORL1
Swedish mutation	Swd

Red Fluorescent Protein Tau	RFP-Tau
Red Fluorescent Protein Ubiquitin	RFP-Ubq
Tau cleaved by Caspase 6	TauΔCasp6
Tyrosine Kinase Receptor B	TrkB
Tubulin cleaved by Caspase 6	TubulinΔCasp6
Terminal transferase dUTP nick end labelling	TUNEL

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ABSTRACT

Active Caspase 6 associates with the neuropathological hallmarks of sporadic Alzheimer disease (SAD), indirectly increases amyloid beta (Aβ) and cleaves Tau and other cytoskeleton proteins. Given the similar neuropathology of SAD and familial AD (FAD), my objective is to assess if FAD-associated amyloid precursor protein (APP) mutations activate Caspase 6 in human neurons. Human neurons were transfected with a bigenic vector expressing enhanced green florescent protein (EGFP) and either wild type APP, the Swedish or London mutations. Normally diffuse EGFP beads in neurons over-expressing APP or APP mutants in an Aβ independent manner and appears to co-localize with beaded Tau and Ubiquitin. Additionally, the number of EGFP-positive APP or APP mutant-transfected neurons decreases with time in culture and undergoes Aβ dependent cell death. Treatment with a caspase 6 inhibitor or dominant negative Caspase 6 attenuates EGFP beading, EGFP-positive neuronal dropout and cell death. Therefore, FAD-mutants and APP over-expression activate Caspase 6 in human neurons resulting in morphological changes and Aβ dependent cell death that may contribute to the neuropathological features of FAD.

RÉSUMÉ

La forme active de la Caspase 6 est impliquée dans les effets neuropathologiques typiques de la forme sporadique de la maladie d'Alzheimer (SAD) en augmentant indirectement la production du peptide amyloïde-bêta (Aβ) et en clivant Tau ainsi que d'autres protéines du cyotoskelette. Compte tenu des neuropathologies similaires de SAD et de la forme familiale de la maladie d'Alzheimer (FAD), mon objectif était de déterminer si les mutations de la protéine précurseur de l'amyloïde (APP) associées à FAD, activent la Caspase 6 dans les neurones humains. Les neurones humains ont été transfectés avec un vecteur bigénique exprimant la protéine fluorescente verte (EGFP; Enhanced Green Fluorescent Protein) et soit l'APP sauvage ou sa forme mutée associée a FAD soit la mutation Suédoise ou la mutation de Londre. Normalement présente sous forme diffuse dan le neurone, l'EGFP forme des agrégats dan les neurites del cellules qui sur experiment en présence de l'APP sauvage ou les mutants. La protein Tau et d'Ubiquitine semble se co-localiser avec les agrégations EGFP. Notons que cette accumulation ne dépend pas de la présence de Aβ. De plus, le nombre de neurones exprimant une forme ou l'autre d'APP où EGFP est visible diminue et il y a augmentation de la mort cellulaire chez ces cellules liée à la présence de Aβ. Le traitement avec l'inhibiteur de la Caspase 6 ou un mutant dominant négatif de la Caspase 6 atténue la formation d'agrégats d'EGFP, la diminution du nombre de neurones positifs à l'EGFP et la mort cellulaire. Par conséquent, les mutants FAD activent la caspase 6 dans les neurones humains et mènent à des changements de morphologie et à la mort cellulaire liée à la présence Aβ, pouvant contribuer au profil neuropathologique de la FAD.

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This is dedicated to ***Travis Ratnam***, you inspire me, thank you for believing in me.

The title 'The Inheritance of Loss' was motivated by Kiran Desai's 2006 Booker prize award winning fictional novel.

INTRODUCTION: RATIONALE & OBJECTIVE

My objective was to determine whether Caspase 6 (Casp6) is activated by familial Alzheimer's disease (FAD) associated mutations. Alzheimer's disease (AD) is the most prevalent form of dementia in the elderly (Ferri et al, 2005). Occurring either sporadically (late onset) or genetically (early onset), the neuropathological features of both forms are similar consisting of plaques containing the amyloid beta (Aβ) peptide, hyperphosphorylated Tau in neurofibrillary tangles, synaptic loss and widespread neurodegeneration (Selkoe, 2001). More importantly, the etiology of the disease is still unclear and a definite diagnosis of AD can only be made post-mortem via the identification of the aforementioned pathological hallmarks.

Caspases, the executioners of apoptosis have been investigated in the pathogenesis of AD. Casp6 has been shown to be activated and localized to the neuropathological hallmarks at all stages of sporadic AD including Mild Cognitive Impairment (MCI), a transitional zone between normal aging and AD (Albrecht et al, 2007; Guo et al, 2004). Even in aged brains without cognitive impairment, active Casp6 immunoreactivity is increased with decreased global cognitive scores implying an early role for Casp6 in neurodegeneration.

Since Casp6 is active in sporadic AD, we rationalized that the same may be true of FAD. Missense mutations in three genes have been linked to FAD: the amyloid precursor protein (APP), Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2) genes. A partial hypothesis of the disease cascade has been garnered from these genes. The Aβ peptide which accumulates in senile plaques is excised from APP (Kang et al, 1987) and missense mutations in APP such as the Swedish and London mutations alter amounts and isoforms of the Aβ peptide (Citron et al, 1992; De Jonghe et al, 2001). As well, these mutations can cause direct and indirect death by multiple mechanisms making cells more vulnerable to age related stresses and triggering caspases (Eckert et al, 2003; Hashimoto et al, 2000; Luo et al, 1999; Marques et al, 2003; McPhie et al, 2003; McPhie et al, 2001; Niikura et al, 2004; Zhao et al, 1997).

Casp6 cleaves APP, Presenilin (PS), Tau as well as a number of other cytoskeleton-associated proteins and interestingly, does not translocate to the nucleus but remains neuritic in neurons of severe AD (Gamblin et al, 2003; Horowitz et al, 2004; Klaiman et

al, 2008; LeBlanc et al, 1999; Pellegrini et al, 1999; van de Craen et al, 1999; Weidemann et al, 1999). Cytoskeleton defects have been reported in AD (Lampert, 1971; Price et al, 1986; Rose et al, 2000; Stokin et al, 2005; Terry, 1963). Compellingly, it was recently demonstrated that expression of APP-associated missense mutations can cause axonal dysfunction measured by vesicular stalling and abnormal organelle and vesicle filled swellings in *Drosophila* and numerous transgenic murine AD models (Gunawardena & Goldstein, 2001; Salehi et al, 2006; Stokin et al, 2008; Stokin & Goldstein, 2006; Stokin et al, 2005; Wirths et al, 2007). Since Casp6 can cleave cytoskeleton associated proteins, it could be contributing to the FAD mutant induced axonal dysfunction.

Down's syndrome (DS) patients who have a duplication of chromosome 21, which includes the APP gene, develop clinical and neuropathological features of AD (Burger & Vogel, 1973). Additionally, families with duplications of regions of chromosome 21 that include APP have early onset AD without the clinical features of DS (Cabrejo et al, 2006; Rovelet-Lecrux et al, 2006; Sleegers et al, 2006). This has been further supported in murine and *Drosophila* models where over-expression of APP disrupts axonal trafficking of vesicles, synaptic proteins and nerve growth factor (NGF) (Gunawardena & Goldstein, 2001; Salehi et al, 2006; Torroja et al, 1999) implying that over-expression of APP, like APP mutations can cause AD neuropathology.

We hypothesized that if Casp6 is important to the neuropathology of AD, it would also be activated in FAD. Additionally, since FAD-associated missense mutations and APP over-expression can cause axonal dysfunction and make cells vulnerable to stresses by triggering caspases, these same mutations may activate Casp6 initiating a cascade of events that lead to neurodegeneration. I investigate this objective in primary human fetal neurons. These represent one of the closest *in vitro* model systems to study AD since the disease is restricted to humans. Unlike most cultured cells, human fetal neurons are terminally differentiated cells that have not been immortalized, therefore data obtained from their gene and protein expression is more relevant. As well, due to the neurons' specific cellular architecture and specialized protein trafficking, neuritic dysfunction and degeneration can be more ideally studied. Finally, results from this system have already been validated in AD brains.

I. LITERATURE REVIEW

1.1 ALZHEIMER DISEASE: A SHORT HISTORY

In 1907 Dr. Alois Alzheimer presented the clinical and neuropathological features of 'An unusual illness of the cerebral cortex' seen in a 55-year old woman he had observed for five years at the Frankfurt asylum (Alzheimer et al, 1995). The disease that now bears his name, bestowed by Dr. Emil Kraepelin, has emerged as the most common cause of dementia with an exponential increase in incidence over the age of 65 (Blennow et al, 2006). AD occurs sporadically or genetically with the key clinical symptom of progressive episodic memory loss and two distinct neuropathological features consisting of extracellular plaque deposits and neurofibrillary tangles. Just over one hundred years later, while the cause of AD is still debated, a definite diagnosis of AD can still only be made post-mortem.

1.1.1 Clinical Symptoms

The earliest symptom of AD presents with deteriorating memory loss usually noted in a decline in verbal fluency (Petersen, 2000a; Welsh et al, 1991). As the disease progresses impairment manifests itself in other symptoms including: aphasia (language disorder) apraxia (inability to articulate thoughts or physically execute learned movement), agnosia (inability to recognize objects), deterioration of higher cortical function (confusion, disorientation) and behavioural disturbances (depression, agitation, delusion), eventually leaving affected individuals unable to care for themselves (Behl, 2000; Blennow et al, 2006). However, while the clinical onset of AD occurs after the age of 65 (late-onset AD), neurodegeneration is thought to occur significantly earlier in a preclinical phase designated as MCI (Petersen, 2000a). Ten to fifteen percent of patients in this transitional zone between normal aging and AD are later diagnosed as AD with clinical dementia (Petersen, 2000b). However, a recent study with more stringent criteria for MCI measures in which patients were followed for 2 years found the conversion rate to AD to be 41% and 64% after year 1 and 2 respectively, indicating MCI as an accurate predictor of AD (Geslani et al, 2005).

1.1.2 Cognitive Measures

Since a definite diagnosis of AD can still only be made post-mortem by neuropathology, most of the cognitive measures used today are to eliminate other confounding factors or dementias following criteria established by the National Institute of Neurological and Communicative Diseases and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)(McKhann et al, 1984). An initial detailed history of the patient and a neurologic examination are conducted followed by a Mini Mental State Examination (MMSE) which is a brief but accurate examiner of cognitive function (Folstein et al, 1975). Other diagnostic criteria can include examination of changes in metabolic activity due to external factors such as drug toxicity which can contribute to dementia and neuroimaging to exclude reversible or treatable dementias (Petersen, 2000a).

1.2 NEUROPATHOLOGY

1.2.1 The Plague of the Amyloid Plaques

A diagnostic brain lesion first noted by Dr. Alzheimer, amyloid plaques are extracellular deposits of the Aβ protein in star shaped 'filamentous fibrils' (Alzheimer et al, 1995; Glenner & Wong, 1984b; Terry et al, 1964), although other proteins including proteoglycans, inflammatory molecules, metal ions, proteases, antioxidant defence molecules and cholinesterases are also components of the plaques (Atwood et al, 2002). The plaques are surrounded by dystrophic neurites and activated microglia usually associated with the central core of the plaque while reactive astrocytes are in the penumbra (Selkoe, 2001; Selkoe, 2004). A 4kDa peptide, Aβ is excised from the APP in varying amino acid species lengths (Kang et al, 1987; Kang & Muller-Hill, 1990). The amyloid plaques contain a mixture of 40- and 42-amino acid Aβ species (Iwatsubo et al, 1994) and the 42-residue form is more susceptible to aggregation (Jarrett et al, 1993). While it has been claimed that Aβ plaque load is predictive of the degree of cognitive impairment, this has recently been contended in favour of the second diagnostic brain lesion, neurofibrillary tangles, being a better predictor (Cummings et al, 1996; Lee et al, 2007).

1.2.2 Neurofibrillary Tangles

The second classic pathological hallmark observed in the first recorded case of AD is now termed neurofibrillary tangle. These tangles are thick bundles of abnormal fibers that have accumulated in the perinuclear cytoplasm of neurons in AD affected parts of the brain (entorhinal cortex, hippocampus, amygdala, frontal, temporal and parietal cortex) (Arnold et al, 1991; Selkoe, 2001). Electron microscopy detailed the fibers as paired filaments wound in helices (Kidd, 1963). Tau, a neuronal protein first noted for its involvement in microtubule assembly (Weingarten et al, 1975), was subsequently shown to be the key constituent of the neurofibrillary tangles (Grundke-Iqbal et al, 1986a; Grundke-Iqbal et al, 1986b; Kosik et al, 1986). Site specific phosphorylation regulates Tau functionality (Stoothoff & Johnson, 2005) where it has a role in modulating microtubule dynamics, neurite outgrowth, axon formation, regulating cell shape and mobility via actin cytoskeleton interactions (Shahani & Brandt, 2002). A normally soluble protein, Tau is found to be in an abnormally hyperphosphorylated form in AD making it highly insoluble and prone to aggregation (Goedert & Spillantini, 2000; Grundke-Iqbal et al, 1986b). While neurofibrillary tangles can be associated with the dystrophic neurites surrounding amyloid plaques of AD, they are also seen independent of amyloid plaques in several other neurodegenerative diseases (Goedert & Spillantini, 2000; Selkoe, 2004; Shahani & Brandt, 2002).

1.2.3 Synaptic and Neuronal Loss

Neuronal and synaptic loss is an important feature of AD shown to occur predominantly in the entorhinal cortex, hippocampus and higher associative cortex, all of which play crucial roles in memory. The entorhinal cortex, often referred to as the 'gateway', is the site of input from the associative cortex and output of this highly processed information to the hippocampus (Morrison & Hof, 1997; Witter et al, 1989). Rigorous stereological cell counts which measure volume and density of 2D sampled sections to extrapolate for the 3D structure (West & Gundersen, 1990) have demonstrated a significant neuronal loss in the entorhinal cortex, which appears to be the first area affected (Gomez-Isla et al, 1996; Kordower et al, 2001; Van Hoesen et al, 1991). In the hippocampus, the CA1 region is the most severely affected and atrophied (Hyman et al, 1984; Price et al, 2001; Simic et

al, 1997) while the superior temporal sulcus of the higher associative cortex has an almost 50% neuronal loss in severe AD cases (Gomez-Isla et al, 1997; Masliah et al, 1991).

There has been debate whether the severity of the disease correlates with the degree of neuronal loss in all of these areas. Recent studies have suggested that neuronal loss and atrophy in the entorhinal cortex occurs in MCI or mild AD but does not progress significantly further in moderate and severe AD (De Toledo-Morrell et al, 2000; Kordower et al, 2001; von Gunten et al, 2005). This conflict may be because earlier studies only compared between severe AD and control cases so that this lack of progression was overlooked (von Gunten et al, 2005). As well, the use of non-stereologic techniques and different population groups may further account for this difference.

1.2.3.1 Cholinergic Neuronal Loss – a Parkinson like specificity?

Current approved treatments for AD nearly all utilize cholinesterase inhibitors which have proven to be only marginally effective. This was based on the early discovery of marked reductions in choline-acetyltransferase (ChAT) and actylcholinesterase (AChE) activity in AD affected brains (Davies & Maloney, 1976; Perry et al, 1982) where the original source of cortical innervation was discovered to be the nucleus of Meynert (Bartus et al, 1982; Mesulam, 2004). There have been conflicting reports of neuronal damage here extending from 75% to as little as 33% loss, yet none of these investigations appear to directly examine the neuronal loss, instead basing their assumption of neuronal damage on decreases in ChAT or AChE markers (Geula & Mesulam, 1989; Geula & Mesulam, 1996; Hyman et al, 1984; Perry et al, 1982; Whitehouse et al, 1982). Additionally, during early stage AD the cholinergic fibre decrease is not present (Davies, 1999) and some groups have noted a paradoxical up regulation in ChAT activity in MCI brains, potentially as a compensatory mechanism (DeKosky et al, 2002; Ikonomovic et al, 2003). Other areas such as catecholamine innervations to the cortex (Adolfsson et al, 1979), glutamatergic pre-synaptic density (Bell et al, 2007) and disturbance of somatostatinergic and serotonergic neurons (Reinikainen et al, 1990; Rossor & Iversen, 1986; Rossor et al, 1980; Rossor et al, 1984) are present showing the pathogenesis of AD to be more ubiquitous.

1.3 FAMILIAL ALZHEIMER'S DISEASE

Approximately 25% of AD cases are believed to be due to a genetic factor, be it clear segregation as an autosomal dominant trait or increased susceptibility via risk genes (Bird, 2008; Cruts & Van Broeckhoven, 1998). The majority of AD cases are of the sporadic form developing the disease over the age of 65, while FAD is an autosomal dominant condition also termed early onset AD for occurring before the age of 65. Pathogenesis and clinical symptoms are conserved between the two forms of AD (without patient age it is difficult to distinguish early onset FAD from late stage sporadic cases), though post-mortem FAD brains demonstrate increased neuritic plaque and neurofibrillary tangle counts and shorter disease duration than sporadic AD (Mullan et al, 1993; Selkoe, 2001). Prevalence of FAD is low, however significant insight into the mechanism and pathogenesis of the disease has been garnered through identification of the genes involved (Ertekin-Taner, 2007; Harvey et al, 2003). The first gene to be identified through genetic linkage studies was the APP gene (St George-Hyslop et al, 1987), followed by the highly homologous PSEN1 and PSEN2 genes (Levy-Lahad et al, 1995; Rogaev et al, 1995; Sherrington et al, 1995). Mutations in the PSEN1 gene accounts for the majority of the mutations identified, followed by APP and a few families that have PSEN2 mutations (Cruts et al, 1998). Alternatively, two other genes, apolipoprotein E (APOE) and the recently identified sortilin-1 related receptor (SORL1) gene have been implicated in increasing the risk of developing sporadic AD (Corder et al, 1993; Poirier et al, 1993; Rogaeva et al, 2007).

1.3.1 Amyloid Precursor Protein

Identification of Aβ as the component of senile plaques in AD affected brains led to the cloning of its parents protein, APP, located on chromosome 21 (Glenner & Wong, 1984a; Kang et al, 1987). APP is composed of 770 amino acids in its longest form and the gene has 19 exons. A type I transmembrane protein, APP has a large extracellular domain (comprising of ~88% of the isoform) and a small cytoplasmic tail and is expressed ubiquitously in the body in numerous isoforms due to alternate splicing and post translational modifications (Gralle & Ferreira, 2007). The three major isoforms containing the Aβ peptide are the APP 695, 751 and 770 residues (Kitaguchi et al, 1988; Ponte et al, 1988; Tanzi et al, 1988), however it is the 695 full length isoform that is

predominantly expressed by neurons (Kang & Muller-Hill, 1990; LeBlanc et al, 1991; Tanaka et al, 1989). The importance of APP in mammals is seen by the fact that two homologues (APLP1 and APLP2) with relatively redundant function exist (Sprecher et al, 1993; Wasco et al, 1993), whereas only one APP related gene is found to be expressed in *Drosophila* (Rosen et al, 1989) and *Caenorhabditis elegans* (Daigle & Li, 1993).

1.3.1.1 APP Processing

Most transmembrane proteins are sorted through the secretory or endocytic pathway based on the sorting signal they contain (Bonifacino & Traub, 2003). As a transmembrane protein, APP is translocated to the endoplasmic reticulum (ER) and post translationally modified via N and O linked glycosylation, phosphorylation and tyrosine sulphation as it travels through the secretory pathway (Griffith et al, 1995; Zheng & Koo, 2006). During and after its journey along the secretory pathway, APP can be proteolytically processed either before, or within the Aβ peptide region, which is located in the transmembrane region of APP. This processing is conducted by three proteases: α-secretase, a member of the A Disintegrin And Metalloprotease (ADAM) family; β-secretase (BACE) and γ-secretase, a unique multimeric complex that can cleave within transmembrane regions and is composed of PS 1/2, Nicastrin, Anterior Pharynx Defective 1 (APH1) and Presenillin Enhancer 2 (PEN2) (Francis et al, 2002; Kaether et al, 2006; Kimberly et al, 2003; Lee et al, 2002).

1.3.1.1.1 Pathway A: Alpha Secretase Pathway

At the cell suface, α-secretase processes APP within the Aβ domain releasing a large soluble extracellular fragment into the lumen/extracellular space while retaining the transmembrane C-terminal fragment (Selkoe, 2001). This transmembrane C83 residue fragment is further cleaved by the γ-secretase, resulting in the release of a small secreted p3 fragment and free intracellular domain (Fig 1). α-secretase activity has been shown to occur predominantly at the plasma membrane though there is evidence that it can also occur within the secretory pathway (De Strooper et al, 1993; Sisodia, 1992).

1.3.1.1.2 Pathway B: Beta Secretase Pathway

The second 'amyloidogenic' cleavage pathway, so termed for producing the 4 kDa Aβ peptide, has generated the most interest. APP is initially cleaved by BACE after residue

596. The Aβ containing transmembrane 99 residue C-terminal fragment (CTF) is subsequently cleaved by γ-secretase to liberate Aβ (Selkoe, 2001) (Fig 1). It was previously believed that Aβ is only generated under pathogenic circumstances however it was later discovered that Aβ is in fact also secreted under normal metabolic conditions (Haass et al, 1992; Shoji et al, 1992). Yet, in FAD, missense mutations in APP slightly alter the proteolytic processing at the secretase sites causing a significant increase in Aβ production believed to then be deposited in the neuritic plaques (Citron et al, 1995). Generally, APP proteins not subject to α-secretase cleavage are internalized from the plasma membrane into endocytic compartments and cleaved by BACE. There has been some controversy regarding the membranous compartment in which Aβ is produced. It has been demonstrated to occur largely in the endosomes where APP is first internalized from the cell surface via clathrin coated pits (Kinoshita et al, 2003; Small & Gandy, 2006). This is further supported by the cellular localization of the mature BACE and γ-secretase activity in the endosome (Fukumori et al, 2006; Huse et al, 2000; Lah & Levey, 2000). However, evidence suggests that Aβ production can occur to a lesser extent early in the secretase pathway in the ER and the Golgi complex (Greenfield et al, 1999; Huse et al, 2002; Selivanova et al, 2007) resulting in an intracellular accumulation that is not always fated for secretion (Cook et al, 1997; LaFerla et al, 2007).

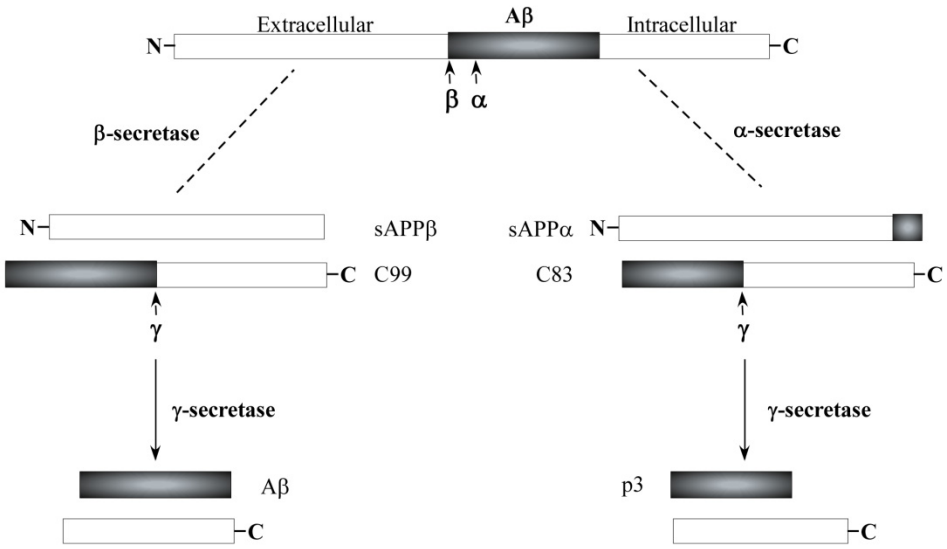
1.3.1.2 APP Function

1.3.1.2.1 The Receptor APP – Dr. Jekyll Part 1

The normal functioning of APP is still poorly understood though it appears to play a role throughout neuronal development and morphogenesis. As a type I transmembrane protein, the structure of APP has indicated that it could function as a surface receptor (Kang et al, 1987). Since its identification, a number of proteins that interact with APP have been described (Reviewed in Neve & McPhie, 2007). Notably, extracellular APP has been shown to interact with F-spondin, an extracellular matrix protein implicated in neuronal repair and development (Ho & Sudhof, 2004), and Nogo 66 receptor which regulates axonal CNS sprouting (Park et al, 2006). Both these interactions also alter the production of Aβ. Growing evidence of APP as an adhesion contact receptor has accrued from *in vitro* studies. Specific regions of APP interact with extracellular matrix proteins

Figure 1: Schematic of APP processing pathways. APP can be proteolytically processed by α-secretase to generate a C83 fragment which when further cleaved by γ-secretase results in a truncated p3 fragment. The second APP processing pathway by β-secretase results in a C99 fragment which when cleaved by γ-secretase liberates the Aβ peptide that accumulates in senile plaques of AD.

Figure 1


Extracellular
Aβ
Intracellular
N
C
β α
β-secretase
α-secretase
N
sAPPβ
sAPPα
N
C
C99
C83
C
γ
γ
γ-secretase
γ-secretase
Aβ
p3
C
C

and promote cell adhesion in cell culture (Breen et al, 1991; Ho & Sudhof, 2004; Kibbey et al, 1993; Small et al, 1999). As well, early work showed APP to form a complex with the heterotrimeric G protein, G_o, a GTP binding protein that is an important signal transducer in the brain (Murayama et al, 1996; Nishimoto et al, 1993; Okamoto et al, 1995). In fact, it has been postulated that APP may function as a G protein coupled receptor and signal through this diverse complex to regulate a number of processes including cell growth, adhesion and migration in developing neurons (Brouillet et al, 1999; Neve & McPhie, 2007; Swanson et al, 2005). Misregulation of this signalling pathway is believed to be one way by which FAD-associated mutations cause neurotoxicity (Giambarella et al, 1997; McPhie et al, 2003).

1.3.1.2.2 APP Function in neuritogenesis and synaptogenesis – Dr. Jekyll Part 2

Parallel to research examining the interacting partners of the receptor APP, mounting evidence has identified a role for APP in neuronal and synaptic development. APP is highly expressed in radial glia which direct neurons to their specific layer in the cortex during development (Trapp & Hauer, 1994) as well as in developing migratory neurons of the moth *Maduca sexta* via a G protein coupled interaction (Swanson et al, 2005). The presence of APP in neuronal migration supports APP's role in neuritogenesis. APP's neuronal growth activity has been amply demonstrated *in vitro* and *in vivo* (Masliah et al, 1992; Milward et al, 1992; Small et al, 1999), further substantiated by loss-of-function experiments showing that a reduction of APP causes decreased neuritic growth and viability (Allinquant et al, 1995; Perez et al, 1997). APP's role in neuritogenesis is thought to occur via interaction with actin regulatory proteins Fe65 and Mena which are expressed in active remodelling regions such as the growth cone and lamellipodia (Sabo et al, 2001; Sabo et al, 2003). However, the notion of APP enhancing neuritic outgrowth has been challenged since it has been demonstrated that APP can also inhibit neuritogenesis. This differential role may depend on neuronal culture, time line of assessment and plating substrate (LeBlanc et al, 1992; Young-Pearse et al, 2008). Finally, APP has been implicated in synaptogenesis. Demonstrated *in vivo*, APP and APLP2 double knockout mice have motor-neuron axons that do not form functional synapses at the right sites and circumvent their target muscle fibers (Wang et al, 2005). As well, injection of secreted APP into the brain of amnesic mice and normal rats

increase memory retention and synaptic density (Meziane et al, 1998; Roch et al, 1994). APP's role in synaptic formation has been substantiated *in vitro* (Akaaboune et al, 2000; Allinquant et al, 1995; Herard et al, 2006; Perez et al, 1997).

1.3.1.2.3 APP Function and Cell Death – Mr. Hyde

In addition to many of APP's beneficial functions, APP has had an equal history in cell death. While there have been some reports that over-expression of APP protects certain cells against specific forms of cell death (Kogel et al, 2003; Masliah et al, 1997; Nishimura et al, 2003), APP over-expression also causes toxicity, particularly certain fragments of the APP protein (Chen et al, 2000b; Copanaki et al, 2007; Lu et al, 2000; Yankner et al, 1989; Yoshikawa et al, 1992). The β-cleaved CTF of APP (C99 or C100) has been shown to cause neurotoxicity and apoptosis in cell culture (Lu et al, 2000; McPhie et al, 2001; Sopher et al, 1994; Yankner et al, 1989). *In vivo*, transgenic mice expressing this β-cleaved CTF have increased neurodegeneration and impaired memory (Berger-Sweeney et al, 1999; Nalbantoglu et al, 1997; Neve et al, 1996; Oster-Granite et al, 1996), however the pathway by which this neurotoxicity occurs is still unclear. It has been proposed that a second fragment, C31 which is released from the β-cleaved C-terminal region by caspase cleavage, may be involved (Gervais et al, 1999; Lu et al, 2000). When this caspase site was mutated in the β-cleaved CTF, it abolished the CTF dependent cell death and dysfunction (Galvan et al, 2006; Lu et al, 2000). Indeed, the C31 fragment alone causes cell death (Bertrand et al, 2001; Gervais et al, 1999; Lu et al, 2000; McPhie et al, 2001; Nishimura et al, 2002). Recently it was shown that C31 signals through an APP binding protein, APP-BP1, a cell cycle protein to cause neuronal apoptosis (Chen et al, 2003).

1.3.1.3 APP Mutations

The dawn of research on the APP gene resulted from the realization that Down's syndrome (DS) patients have similar clinical, neuropathological and biochemical features as AD patients (Glenner & Wong, 1984a; Masters et al, 1985; Menendez, 2005). The AD pathology in DS patients was thought to be due to the partial duplication of chromosome 21, drawing focus to this chromosome until the eventual identification of APP as an AD susceptibility gene (St George-Hyslop et al, 1987). The first FAD mutation in the APP gene was linked to a single missense mutation at codon 642, causing a valine-to-

isoleucine change in a London family (London mutation – Fig 2) (Goate et al, 1991). Many more have since been discovered. The Alzheimer Disease and Frontotemporal Dementia Mutation Database currently records 33 mutations in the APP gene in 80 families, 4 of which are non-pathogenic (http://www.molgen.ua.ac.be/ADMutations). These mutations include deletions, duplications and numerous missense mutations most of which are located close to the APP cleavage sites and therefore functionally affect APP processing (Selkoe & Podlisny, 2002). One of the more well studied mutations is the Swedish mutation which occurs at the BACE cleavage site. This double mutation at codon 595 and 596 results in a lysine, methionine change to an asparganine and leucine (Mullan et al, 1992) and is especially interesting because of its effects on Aβ production (Fig 2). In addition to APP mutations, duplication of the APP gene has been shown to cause early onset AD without the clinical features of DS (Cabrejo et al, 2006; Rovelet-Lecrux et al, 2006; Sleegers et al, 2006). This implies that excess APP, like DS and APP mutants, can also be inherited to cause AD neuropathology.

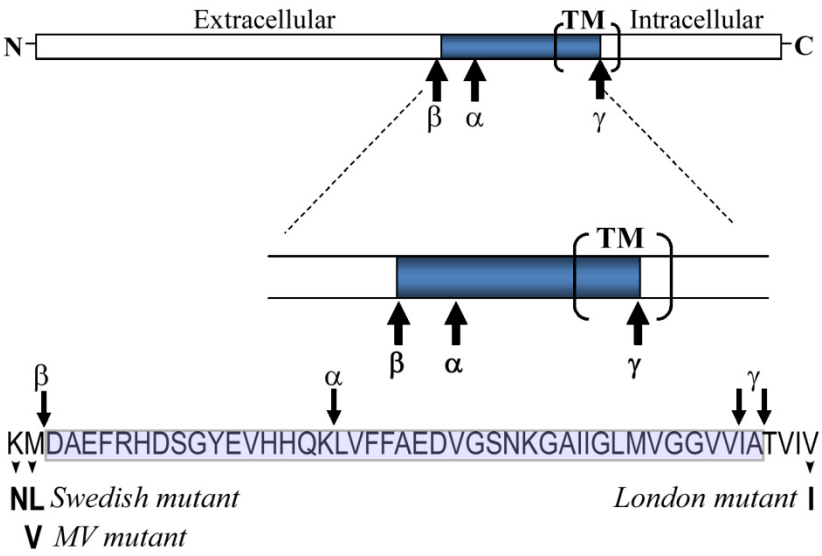
1.3.1.3.1 APP Mutations Effect on Amyloid Beta

Many of the missense mutations in the APP gene occur around or within the α, β or γ-secretase regions, which has supported the hypothesis that the FAD pathology is mainly due to alterations in Aβ production (Selkoe & Podlisny, 2002). The Swedish double missense mutation occurring at the beginning of the BACE cleavage site results in a new site that is more efficiently cleaved by BACE and subsequently has an eight to fifteen fold increase in $A\beta_{40}$ and $A\beta_{42}$ (Citron et al, 1992; Citron et al, 1995). This increase in Aβ is mirrored in other mutations occurring near the BACE cleavage site. The London mutation however which occurs near the γ-secretase cleavage region results in a shift in the ratio of Aβ toward the longer $A\beta_{42}$ species (De Jonghe et al, 2001), which is more prone to aggregation (Jarrett et al, 1993). Again, similar results were noted with other pathogenic mutations proximal to the γ-secretase cleavage region (De Jonghe et al, 2001).

Studies of animal models containing APP mutations, particularly murine transgenic models, have been important in furthering our understanding of AD neuropathology. Several transgenic mouse models have been generated (Reviewed in Gotz & Ittner, 2008), including one that contains the Swedish mutation and has been demonstrated to develop

Figure 2: Schematic of APP_{695} with mutations. Schematic diagram of the APP_{695} protein with the Aβ peptide in the shaded domain. The APP sequence containing the Aβ 1-42 region is indicated by shading within the single letter amino acid code. Letters below the code indicate the Swedish and London FAD-linked mutations and the MV mutation. βACE, α-secretase and γ-secretase sites that generate $Aβ_{40}$ and $Aβ_{42}$ respectively are also shown.

Figure 2


Extracellular
TM
Intracellular
N
C
β
α
γ
TM
β
α
γ
β
α
γ
KMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIV
NL Swedish mutant
V MV mutant
London mutant I

Aβ plaques and memory impairments, but not neurofibrillary tangles or significant neuronal loss with age (Hsiao et al, 1996; Irizarry et al, 1997). While some studies suggested a correlation between increased Aβ production and memory deficits (Chen et al, 2000a; Hsiao et al, 1996), there has been debate regarding these findings (Routtenberg, 1997; Westerman et al, 2002). These transgenic mice develop a modest memory decline at six months, but then remain relatively stable for a few more months prior to further decline (Lesne et al, 2006) and it has been argued that the onset of learning deficits does not correlate with Aβ plaque formation (Routtenberg, 1997; Westerman et al, 2002). One group has recently proposed a solution to this conundrum in the form of extracellular soluble dodecameric $A\beta_{42}$ formation (Cheng et al, 2007; Lesne et al, 2006). This 56kDa assembly is proposed to be multiple trimeric Aβ oligomers whose accumulation correlates with the pattern of memory decline noted in the Swedish transgenic mice. As well, infusion of this oligomer into young non-impaired mice transiently disrupted spatial memory only. This however was only a transient effect in a specific memory task and it remains to be seen if it correlates with human Aβ formation. While modulation of Aβ is compelling evidence for the amyloid hypothesis (which postulates that overproduction of Aβ is the initial neurotoxic insult that leads to AD pathology) (Selkoe, 2000), the FAD-associated mutations have a number of other effects including axonal dysfunction and cell death which can also contribute to AD.

1.3.1.3.2 APP Mutations Effect on Cell Death

Extensive work on the FAD-associated mutations of the APP gene has demonstrated that these mutants can cause direct or indirect cell death as well as activate caspases. Expression of FAD-linked APP mutations cause neurotoxicity in a number of cell lines including differentiated rat pheochromocytoma (PC 12), hybrid rat dorsal root ganglion and mouse neuroblastoma (F 11), T lymphocytes (T Jurkat) and kidney cells (COS clone NK1) (Eckert et al, 2001; Giambarella et al, 1997; Hashimoto et al, 2000; Luo et al, 1999; Marques et al, 2003; Niikura et al, 2000; Pellegrini et al, 1999; Yamatsuji et al, 1996a; Yamatsuji et al, 1996b; Zhao et al, 1997). These APP mutations also induce cell death or elevate cell vulnerability to age related stresses in primary neurons by multiple mechanisms (Luo et al, 1999; McPhie et al, 2003; McPhie et al, 2001; Niikura et al, 2004). Because these mutations increase the level of Aβ, it has been hypothesized that

their induced cell death is due to Aβ toxicity. However, using β–secretase inhibitors and mutations in APP that prevent the production of Aβ, a number of groups have demonstrated that FAD-associated mutants can cause an Aβ independent cell death (McPhie et al, 2001; Niikura et al, 2004; Yamatsuji et al, 1996a; Yamatsuji et al, 1996b).

G protein activation has been implicated in APP mutant cell death. G protein interfering mutants and Pertussis toxin, which inhibits G protein activation, have been shown to decrease or abolish APP mutant induced cell death in a number of cell lines (Giambarella et al, 1997; McPhie et al, 2003; Niikura et al, 2000; Niikura et al, 2004; Yamatsuji et al, 1996a). Since G proteins have a number of downstream signalling pathways, this mechanism is still unclear, but there is evidence that activation of JNK, leading to NADPH oxidase activity and the caspase cascade, may be the mechanism of APP mutant toxicity (Niikura et al, 2004). This has been supported by a number of reports implicating APP mutant dependent oxidative stress (Eckert et al, 2001; Hashimoto et al, 2000; Niikura et al, 2000; Niikura et al, 2004; Reis et al, 2007) and caspase activation (Eckert et al, 2001; Gervais et al, 1999; Hashimoto et al, 2000; Luo et al, 2002; Marques et al, 2003; McPhie et al, 2001; Niikura et al, 2000; Niikura et al, 2004).

1.3.1.3.3 APP Mutations Effect on Axonal Transport

1.3.1.3.3.1 An introduction to axonal transport

The importance of axonal transport in neurons is seen in their unique cellular morphology and polarity. Neurons usually have a long axon and numerous dendrites projecting from their cell body which require specialized trafficking of proteins to and from their designated compartments and cell body, often travelling very long distances (Stokin & Goldstein, 2006). Synthesis of proteins occur at the cell body or proximal regions of dendrites, and processing and modifications occur during transit in axons (Alberts et al, 2002; Zheng & Koo, 2006). Two forms of axonal transport exist in the neuron; fast transportation of membranous organelles, which includes APP linked vesicles, and slow transportation of cytosolic and cytoskeletal proteins. These processes require two families of molecular motors, kinesin and dynein, which travel along microtubule filaments in the anterograde (away from the cell body) and retrograde (toward the cell body) direction respectively (Hirokawa & Takemura, 2005).

APP is transported by fast anterograde transport via the kinesin motor protein (Koo et al, 1990). These kinesin transport vesicles have been shown to also contain BACE and PS1 which may explain results demonstrating synaptically released Aβ extracellular accumulations in mice (Kamal et al, 2001; Lazarov et al, 2002; Sheng et al, 2002; Sheng et al, 2003). It was initially hypothesized that APP binds directly to the kinesin light chain (KLC) subunit acting as a membrane cargo receptor for the kinesin motor (Kamal et al, 2000). However subsequent research has demonstrated that this interaction may be indirectly mediated via an adaptor protein such as JIP-1, a c-Jun N term kinase (JNK) interacting scaffolding protein, which binds both APP and the KLC (Lazarov et al, 2005; Matsuda et al, 2001; Scheinfeld et al, 2002; Sisodia, 2002).

1.3.1.3.3.2 APP over-expression and mutations disrupt axonal transport

There has been accumulating evidence that a compromise in axonal transport contributes to neurodegeneration (Lampert, 1971; Price et al, 1986; Rose et al, 2000; Roy et al, 2005; Stokin & Goldstein, 2006; Terry, 1963). In *Drosophila melanogaster,* deletion or over-expression of APP results in organelle congestion and phenotypic axonal transportation defects, similar to *Drosophila* with kinesin and dynein mutations (Gunawardena & Goldstein, 2001; Torroja et al, 1999). The authors hypothesized that this phenotype could be a result of APP binding to kinesin, therefore, over-expression of APP could result in its excess that then competes for kinesin and titrates it away from other essential pathways causing vesicle stalling and axonal transportation defects. Interestingly, similar axonal dysfunctions were noted in *Drosophila* expressing APP with the Swedish or London mutation as well as increased neuronal death in these mutants relative to wild type APP, though over-expressing APP itself also caused neuronal death (Gunawardena & Goldstein, 2001). In a mouse model of AD, young APP transgenic mice containing the Swedish mutation who had not yet developed AD neuropathology, had axonal defects consisting of large and irregularly spaced axonal varicosities (Stokin et al, 2005). This supports the proposal that axonal dysfunction is an early event that may be occurring before Aβ deposits or abnormally phosphorylated Tau. As well, these varicosities were ChAT immunoreactive and were shown by electron microscopy (EM) to contain accumulations of different vesicles, organelles and dense bodies (Stokin et al, 2005). Stokin *et al.* also observed ChAT immunoreactive swellings with comparable

morphology and diameter to those seen in the APP transgenic mouse model in FAD human brains (Stokin et al, 2005).

Since *Drosophila* with kinesin mutations exhibit vesicle stalling, Stokin *et al.* tested whether a reduction in the genetic dosage of KLC I could have a similar effect in their APP-Swd transgenic mouse. They found that a reduction in KLC I not only enhances axonal dysfunction seen previously, but also impairs APP trafficking (Stokin et al, 2005). In a different APP transgenic mouse model harboring the Swedish and London mutation, age dependent axonal swellings and spheroids were also noted (Wirths et al, 2007). Finally, in a DS mouse model, over-expression of APP caused abnormal trafficking of nerve growth factor (NGF), a neurotrophic factor that increases growth and differentiation, indicating potential disrupted trafficking (Salehi et al, 2006). Recent results suggest that these axonopathies are independent of Aβ, which is significantly increased in APP mutants (Stokin et al, 2008). Overall, a mechanism is emerging in which APP mutants as well as APP over-expression cause aberrant binding to KLC-I via JIP-1, thereby preventing it from transporting other proteins as well as impairing normal APP trafficking. PS mutants appear to have the same phenotype by the opposite mechanism as will be discussed later (See *Presenilin mutations disrupt axonal transport*)

1.3.2 Presenilin

The highly homologous PSEN1 and PSEN2 genes, on chromosome 14 and 1 respectively, code for proteins that are essential subunits of the transmembrane cleaving multiprotein complex, γ-secretase (Wolfe et al, 1999b). PSEN1 and PSEN2 encode 467 and 448 amino acid polypeptide proteins that are expressed in the brain and peripheral tissues, though PSEN1 is expressed earlier in development than PSEN2. (Lee et al, 1996). PS is believed to have 8 to 9 transmembrane domains (Spasic et al, 2006; Vetrivel et al, 2006) and a hydrophilic loop between domains 6 and 7 which undergoes a highly conserved endoproteolytic cleavage and therefore largely exists as a heterodimer (Ratovitski et al, 1997; Thinakaran et al, 1996). This processing is thought to be critical for the stability of PS (Reviewed in Vetrivel et al, 2006), though some have challenged this (Reviewed in Dillen & Annaert, 2006; Steiner et al, 1999). Within the core of PS are two conserved aspartate residues that constitute the catalytic site and therefore PS's function (Wolfe et

al, 1999b). The homologue of PS, *Sel-12,* discovered in *Caenorhabditis elegans* (Levitan & Greenwald, 1995) allowed the use of C-elegans as a model system to understand the function and interactions of PS (Baumeister et al, 1997; Levitan et al, 1996).

1.3.2.1 Presenilin Function

1.3.2.1.1 Presenilin and γ-secretase - Running with scissors

Linkage of the PSEN genes to FAD was instrumental in elucidating their physiological function. Subsequently, it was shown through yeast 2-hydrid assays that PS binds to and is also co-immunoprecipiated with APP (Waragai et al, 1997; Weidemann et al, 1997; Xia et al, 1997). It has now been established that this interaction is due to PS being a component of the multimeric γ-secretase complex which participates in the final intramembranous cleavage of APP to release the Aβ 40 to 42 residue fragments (De Strooper et al, 1998; Wolfe et al, 1999a). Three other proteins are essential components for the stability of the γ-secretase complex, Nicastrin, APH1 and PEN2 while PS provides the aspartyl catalytic site (Edbauer et al, 2003; Francis et al, 2002; Yu et al, 2000). The importance of these cofactors is seen by their co-dependence for biogenesis, maturation, stability and tight regulation (Reviewed in Vetrivel et al, 2006).

Lack of a biochemical explanation for an intramembranous protease created an initial hesitancy when describing the γ-secretase cleavage of APP. It was somewhat rescinded after the discovery that a similar cleavage occurs in a number of other proteins including the ligand activated Notch receptor, whose signalling is essential for cell fate during development (De Strooper et al, 1999; Struhl & Greenwald, 1999; Struhl & Greenwald, 2001) as well as cadherins, a family of structural molecules that play a role in cell adhesion (Marambaud et al, 2002). The relaxed sequence specificity of γ-secretase allows it to be promiscuous and to date uses over 30 type I transmembrane proteins as substrates including the ones mentioned above (Reviewed in Spasic & Annaert, 2008). Structural analysis has recently revealed that a water containing cavity allows for this intramembrane cleavage (Lazarov et al, 2006; Steiner et al, 2008).

1.3.2.1.2 Alternate Presenilin Functions

The inference that PS is a member of the γ-secretase complex was not without significant debate. Some investigators were unable to find a direct interaction between PS and APP (Thinakaran et al, 1998) giving rise to the hypothesis that PS could be involved in regulating the trafficking of membranous proteins that include APP (Selkoe, 2001). In PS1 deficient neurons maturation and trafficking of the tyrosine kinase receptor B (TrkB), required for protein growth factors that signal for survival and differentiation, and APP, is impaired (Naruse et al, 1998). As the physiological role of PS is explored, the trafficking of many other membrane proteins are being shown to be regulated by PS (Reviewed in Uemura et al, 2004; Vetrivel et al, 2006).

Yeast 2-hybrid assays have identified a number of other proteins that interact with PS without necessarily being processed by them. PS mediates the turnover of a neuron specific adhesion molecule, telencephalin, as well as α-synuclein, potentially through autophagic degradation (Esselens et al, 2004); modulates capacitative calcium entry to replenish internal stores (Green et al, 2008; Yoo et al, 2000); forms passive calcium leak channels in the ER (Tu et al, 2006) and modulates Akt/ERK signalling which are receptors that have neurotrophic properties as well as affect Tau phosphorylation (Kang et al, 2005). Loss-of-function studies further confirm the importance of PS. PS knockout mice have profound skeletal and CNS defects and do not survive long after birth (Shen et al, 1997), while conditional knockout of PS in the adult cerebral cortex mimics progressive neurodegeneration along with AD neuropathological hallmarks (Shen & Kelleher, 2007).

1.3.2.2 Presenilin Mutations

Nearly 90% of identified FAD mutations are due to missense mutations linked to the highly homologous PSEN genes (chromosome 14 and 1)(Levy-Lahad et al, 1995; Rogaev et al, 1995; Sherrington et al, 1995). Most of these mutations enhance the generation of the $A\beta_{42}$:$A\beta_{40}$ peptide ratio, which is more prone to aggregation (Borchelt et al, 1996; Murayama et al, 1999b; Scheuner et al, 1996). Currently, there are two prevailing hypotheses regarding the mechanism of action of these mutations: a gain-of-function that allows the mutants to modulate γ-secretase's cleavage location to shift the balance toward

longer Aβ species or, a loss-of-function of PS's physiological functions. A loss-of-function hypothesis is supported by conditional PS knockout mice that have neurodegeneration measured by memory, neuronal and synaptic loss and Tau hyperphosphorylation, mimicking the features of AD (Saura et al, 2004; Shen & Kelleher, 2007). This model would suggest that PS mutations that cause early onset AD may also function via this mechanism. PS mutants have been shown to have impaired γ-secretase cleavage of Notch, N-cadherin, numerous other substrates as well as reduced endoproteolysis (Bentahir et al, 2006; Marambaud et al, 2002; Murayama et al, 1999a; Song et al, 1999; Wang et al, 2006). These mutations also affect other physiological functions such as impairing PS's ability to function as a calcium leak channel in the ER (Green et al, 2008; Tu et al, 2006), contributing to the loss-of-function hypothesis. Over-expression of PS mutants causes broad spectrum apoptosis and cell death, but will not be discussed here (Hashimoto et al, 2004; Janicki & Monteiro, 1997; Weihl et al, 1999). The high frequency of PSEN1 mutations, currently 168, while 10 have been found in PSEN2, (http://www.molgen.ua.ac.be/ADMutations) indicates a possible differential function of the two proteins and therefore an alternate consequence of the respective mutations.

1.3.2.2.1 Presenilin Mutations Disrupt Axonal Transport

As discussed previously, while PS provides the catalytic site for γ-secretase, its other physiological function includes trafficking a number of membrane proteins. PS interacts with glycogen synthase kinase 3β (GSK 3β) (Takashima et al, 1998; Tesco & Tanzi, 2000) which plays a role in fast axonal transport by promoting the release of kinesin-I from membrane bound organelles through phosphorylation (Morfini et al, 2002). PS mutants have been previously shown to increase PS binding to GSK 3β in cell lines (Takashima et al, 1998; Weihl et al, 1999). *In vivo*, PS mutant knock-in mice also have increased GSK 3β resulting in impaired kinesin-I axonal transportation of membrane bound organelles (Pigino et al, 2003). In different mutant transgenic PS mice, fast axonal transportation of APP and the TrkB receptor were impaired, with an accompanying reduction in axonal kinesin-I levels. Clinically these mice also demonstrate motor dysfunction (Lazarov et al, 2007). Recent published results however do not support these findings completely. Stokin *et al.* crossed a transgenic PS mutant with a transgenic APP

mutant mouse harbouring the Swd mutation. They found that while the PS mutant causes decreased APP axonal transportation in the crossed transgenic mouse, it does not alter levels of kinesin-I or cause axonal pathology (Stokin et al, 2008). The reason for this conflict is still unclear though the fact that the Stokin *et al.* mice have a different PS mutation from the Pigino *et al.* mice may contribute to this discrepancy. However, based on previously observed axonopathies in PS1 mutant knock-in mice, the mechanism appears to involve increased PS binding to GSK 3β resulting in increased phosphorylation of kinesin-I. This promotes the release of kinesin-I from membrane organelles, and as discussed previously (See *APP mutations disrupt axonal transport*), since APP is trafficked by indirectly binding to kinesin I, its transportation along with other proteins would be impaired resulting in vesicle stalling and the phenotype noted in both PS and APP mutant models of AD.

1.3.3 Apolipoprotein E

Apolipoprotein E (ApoE), a glycoprotein that carries cholesterol and other lipids in the blood, is needed for the normal catabolism of triglyceride rich lipoproteins and is particularly high in the brain (Cedazo-Minguez, 2007; Hirsch-Reinshagen & Wellington, 2007). The ApoE gene has three expressed alleles, ε2, ε3 and ε4 of which the ε3 allele is the most common in the general population (Cedazo-Minguez, 2007; Zannis & Breslow, 1981). However, in the early 90's a group studying lipid changes in AD brains demonstrated ApoE immunoreactivity with amyloid plaques and tangles (Namba et al, 1991). Shortly after, the ε4 allele was linked as a genetic risk factor for late onset AD (Poirier et al, 1993; Strittmatter et al, 1993a) occurring in a dose dependent manner with increased risk and decreased age of onset (Corder et al, 1993). The results were confirmed in numerous other population and clinical studies (Ertekin-Taner, 2007). The group that first published the associated of APOE ε4 allele with late-onset AD also reported that the purified ε4 isoform binds Aβ with higher avidity *in vitro* (Strittmatter et al, 1993b). Subsequent research showed that under native conditions using unpurified protein, ε4 in fact has lower binding avidity than the other isoforms implying potential defective clearance of Aβ (Aleshkov et al, 1997; LaDu et al, 1994; Tokuda et al, 2000). The molecular mechanism by which the ε4 allele functions remains to be elucidated, though the prevailing hypothesis is that it has a role in Aβ aggregation and/or deposition

or deregulated Aβ clearance (Cedazo-Minguez, 2007). Several other hypotheses have been proposed regarding the isoform specific association of ApoE and AD which include: ApoE having an isoform specific role in stabilizing microtubule interactions with Tau and microtubule associated proteins (Huang et al, 1994; Strittmatter et al, 1994) as well as an isoform specific effect on neuritic outgrowth in response to injury (Holtzman et al, 1995; Nathan et al, 1995; Poirier, 1994).

1.3.4 Sortilin 1

The most recent addition to the list of potential genes that modulate AD is the Sortilin-related receptor gene, a member of the vacuolar protein sorting family. Variants in this gene have been associated with increased risk of late-onset AD in multiple independent data sets from different ethnic groups (Rogaeva et al, 2007). Unlike ApoE, in the preliminary study of SORL1, no single variant was associated with increased risk across all the data sets. However, the authors found an association between late-onset AD and two clusters of single nucleotide polymorphisms (SNPs) in two distinct regions of the SORL1 gene (Ertekin-Taner, 2007; Rogaeva et al, 2007). Other groups have subsequently confirmed the finding in more ethnic groups including Chinese and Belgium populations (Bettens et al, 2008; Meng et al, 2007; Tan et al, 2007) but there is still some conflict regarding the significance of this association (Li et al, 2008; Minster et al, 2008). Time and larger multiethnic cohorts will be needed to confirm these results. SORL1 is a neuronal protein that associates with APOE (Jacobsen et al, 2001) and is involved in the trafficking and recycling of APP within the endocytic pathway (Andersen et al, 2005; Schmidt et al, 2007). It regulates APP processing by binding to it and directing it toward the retromer recycling pathway. It has been proposed that the absence of SORL1 switches APP toward the late endosomal pathway where it is cleaved by BACE. This is supported by evidence that downregulation of the protein results in increased Aβ formation (Andersen et al, 2005; Offe et al, 2006; Schmidt et al, 2007) as well as a decrease in SORL1 expression in post-mortem AD brains (Scherzer et al, 2004).

1.4 CELL DEATH – MORE THAN A TALE OF TWO?

While typically thought of as occurring pathologically, cell death is also a necessary process for development and cellular homeostasis. Broadly divided into two

mechanisms, apoptosis and necrosis, a possible third type of cell death, autophagy has been garnering increasing attention (Degterev & Yuan, 2008). Apoptosis is characterized by an organized dismantling of the cell by caspases resulting in blebbing and apoptotic bodies, while necrosis has a swelling and bursting of organelles that trigger inflammation upon spilling into the extracellular milieu (Yuan et al, 2003). Autophagy, a controlled cell death similar to apoptosis, occurs at an underlying level under normal conditions and is characterized by double membrane enclosed vesicles of the organelles which are degraded by lysozomal enzymes (Kelekar, 2005). In addition to these three broad categories, other forms of cell death that do not fit into these morphological criteria are starting to emerge such as necroptosis. However, whether they operate under different mechanisms and require categories of their own remains to be seen (Degterev & Yuan, 2008).

1.4.1 Apoptosis

Apoptosis or cellular suicide is a tightly controlled fundamental process required for tissue maintenance, organism development and defence against cellular insults (Faleiro et al, 1997; Kerr et al, 1972; Zheng et al, 1999). The phenomena was first described in the 19th century (Vogt, 1842) but was only brought into the limelight in the early 70's when the term 'apoptosis' was coined by Kerr *et al* (1972). It is characterized by distinct controlled features including: cell shrinkage, condensed chromatin and cytoplasmic blebbing into apoptotic bodies that are phagocytosed by surrounding cells (Cohen, 1997; Kerr et al, 1972).

While being stringently controlled by both survival and death signals, apoptosis can occur by two major pathways: 1) an extrinsic pathway that involves cell surface death receptors or 2) an intrinsic pathway via cytochrome c release from the mitochondria. Both of these paths rely on the main apoptotic perpetrators, caspases. Briefly, in the extrinsic pathway, ligands bind to and activate specific cell surface death receptors which recruit a death inducing signalling complex to amplify the apoptotic signal via caspases. The intrinsic pathway can be activated both by extracellular (eg. loss of survival factors) and intracellular (eg. toxins, DNA damage) stimuli which cause the irreversible release of cytochrome c and other apoptogenic factors from the mitochondria. Cytochrome c then

binds adaptor proteins and oligomerizes into a complex that recruits and activates caspases to amplify the death signal (Reviewed in Schultz & Harrington, 2003). In both cases, the apoptotic signal is divided into an 'initiation' and 'execution' phase, however once the execution phase has been triggered the cell is fated to die (Reviewed in Movassagh & Foo, 2008). The dysregulation of this programmed cell death is believed to contribute to the pathogenesis of neurodegenerative diseases like AD (Engidawork et al, 2001).

1.4.1 Caspases

It was early studies on the nematode *Caenorhabditis elegans* (*C.elegans*) that helped genetically characterize the key components of apopototic molecular machinery – caspases (Ellis & Horvitz, 1986; Horvitz et al, 1994). Discovery of the *C.elegans* death (CED) genes, required for the activation of apoptosis, led to the identification of their mammalian homologues, the cysteine-dependent aspartate specific protease (Caspase) family (Black et al, 1989; Cerretti et al, 1992; Thornberry et al, 1992; Yuan et al, 1993). They are named for the conserved cysteine side required for catalytic activity and for their fastidious specificity to cleave their substrates after an aspartic acid residue (Alnemri et al, 1996). Having this preferred cleavage site is not enough for proteolysis of all substrates however, substrate availability is just as essential therefore limiting and controlling the vital substrates required to dismantle the apoptotic cell (Fischer et al, 2003).

Eleven caspases are encoded by the human genome and can be grouped by three criteria, either by their *in vivo* activity, the length of their prodomain or by their substrate specificity (Nicholson, 1999; Salvesen & Abrams, 2004; Thornberry et al, 1997). Loosely, Caspase 2, 8, 9, 10 are initiator or long prodomain caspases. The first to be recruited during the initiation phase of apoptosis, they activate Caspase 3, 6, 7, the effector or short prodomain caspases which go on to cleave cellular proteins. Caspase 1, 4, 5, 11 are termed the inflammatory caspases for responding to inflammatory stimuli (Reviewed in Cohen, 1997; Taylor et al, 2008). The long prodomain caspases contain a CAspase Recruitment Domain (CARD) or Death Effector Domain (DED) not seen in the short prodomain caspases. Alternatively, caspases cleave their substrates after specific tetrapeptide recognition motives which has been exploited in caspase activity assays,

inhibitors and grouping criteria (Reviewed in Boatright & Salvesen, 2003). For example, Casp6 primarily recognizes VEID while Caspase 3 recognizes and cleaves DEVD after the aspartate residue (Degterev et al, 2003). Based on this, three groups arise: Group I consists of Caspase 1, 4, 5; Group II Caspase 2, 3, 7 and Group III Caspase 6, 8, 9, 10 (Garcia-Calvo et al, 1999; Thornberry et al, 1997).

1.4.1.1 Caspase Structure and Activation

Caspases are initially synthesized as proteolytically inactive zymogens consisting of: an N-terminal prodomain, a 20kDa subunit (referred to as p20), usually a small linker region, and a 10kDa subunit (referred to as p10) (Nicholson, 1999). Cleavage of the aspartic acid residue between the p20 and p10 subunit liberates them to allow the two to make up the active heterotetrameric caspase form (Thornberry et al, 1992). Casp 6 for example can be processed by Caspase 1, 2, 3, 8, 9, 10 and the cytotoxic T lymphocyte (CTL) protease granzyme B in a number of cell lines and primary human neurons (Cohen, 1997; Guo et al, 2006; Orth et al, 1996; Park et al, 2004; Slee et al, 1999; Zheng et al, 2000). However, this cleavage is not always necessary for the initiator caspases. *In vitro* results suggest an 'induced proximity' model in which proximity of the unprocessed caspases allows their dimerization and activity (Reviewed in Boatright & Salvesen, 2003; Muzio et al, 1998).

1.5 CASPASE ACTIVITY IN AD

Prompted by the significant neuronal loss observed in diseased brains, caspases have been examined and implicated in AD. While many of the initiator caspases have been studied, Casp6 in particular has been shown to be a strong potential candidate for involvement in the pathogenesis of AD, particularly since it shows strong immunoreactivity to all the AD pathological hallmarks in severe AD as well as MCI brains (Albrecht et al, 2007; Guo et al, 2004).and will be discussed in more detail later (See *Caspase 6 activity in AD*). By immunoprecipitation and immunohistochemistry, Caspase 9 has been found to be activated in postmortem brains of AD patients (Lu et al, 2000; Rohn et al, 2002). The other major initiator caspase, Caspase 8, was strongly immunoreactive in AD affected regions of the brain and also detected by western blotting (Rohn et al, 2001; Su et al, 2002). A second group however, was unable to confirm these Caspase 8 findings by immunoblotting (Lu et al, 2000). Interestingly, it was shown that active Caspase 8 and 9

co-localize, particularly within neurons described as having a 'flame like' morphology consistent with neurofibrillary tangles. This supports the authors' model of a crosslinking of death receptors which activate both initiator caspases that subsequently activate Caspase 3, though active Caspase 3 was not demonstrated here (Rohn et al, 2002).

There has been some controversy regarding the activity of the effector Caspase 3 in AD (Gervais et al, 1999; LeBlanc, 2005; Stadelmann et al, 1999; Su et al, 2001). By immunoprecipitation-mass spectrometry and immunocytochemistry one group found active Caspase 3 in the limbic cortex of mild AD, but not severe AD brains (Gastard et al, 2003), while another found extensive active Caspase 3 immunoreactivity, particularly in a subfield of the hippocampus, entorhinal cortex and upper layers of the frontal cortex of AD brains (Su et al, 2001). This is in contrast to a number of groups that found only diffuse immunoreactivity of Caspase 3 in some AD cases, and that, mainly localized to granulovacuolar degenerating neurons (a degeneration that results in a neuronal intracytoplasmic vesicle containing a granuole) (Roth, 2001; Selznick et al, 1999; Stadelmann et al, 1999; Su et al, 2002). Finally, a recent study demonstrated increased Caspase 8 and 7 mRNA levels, but not 3 or 9 in AD cases (Matsui et al, 2006).

Of the inflammatory caspases, there are reports of increased Caspase 1 mRNA and protein levels in AD brains (Desjardins & Ledoux, 1998; Zhu et al, 1999) however that has been the extent of reports on the involvement of inflammatory caspases in AD.

1.5.1 Caspases process APP

APP was first shown to be cleaved at two predicted locations by Caspase 3 in chick motor-neurons undergoing cell death, after the authors noticed an upregulation of APP mRNA, APP protein and Aβ levels in these dying cells (Barnes et al, 1998). One site of cleavage was confirmed *in vitro* and *in vivo* through site directed mutations at aspartate 664 in the C-terminus of APP where Caspase 3, 6, 8 and 9 also cleaved it resulting in a cytotoxic C31 fragment (Gervais et al, 1999; LeBlanc et al, 1999; Lu et al, 2000; Pellegrini et al, 1999; Weidemann et al, 1999). This 3kDa C31 fragment has been shown to be toxic or make cells more susceptible to insults in primary neurons and cell lines (Dumanchin-Njock et al, 2001; LeBlanc, 2005; Lu et al, 2000; McPhie et al, 2001). Two additional Caspase 3 cleavage sites were identified *in vitro* at the N-terminus of APP,

however, it was the aspartate 664 C-terminus site that was shown to be cleaved *in vivo* and detected within the senile plaques of an AD brain (Gervais et al, 1999).

1.5.1.1 Caspase Modulation of Aβ

Dying human neurons that have active Casp6 secrete more Aβ and accumulate more intracellular Aβ which is currently believed to make cells more susceptible to cellular stress and eventually lead to cell death (LeBlanc, 1995; LeBlanc et al, 1999). Based on this and evidence from cells expressing APP cleaved C31, it was hypothesized that the C-terminus caspase cleavage modulates increased Aβ in dying cells (Gervais et al, 1999). However, a different group attempting to reproduce these results in a different cell line did not observe the same results (Soriano et al, 2001). This same group found that APP lacking the C31 fragment had decreased Aβ. They suggest that loss of the C-terminal internalization signal when expressing APP cleaved C31 results in lack of processing of APP and decreased Aβ production which explains their results. A second group that mutated the caspase cleavage sites of APP still saw an increase in apoptosis induced Aβ production (Tesco et al, 2003). This conflict is partially explained by a recently elucidated pathway by which caspases indirectly influence Aβ production. Active Caspase 3 cleaves the Golgi-localized γ-ear-containing ARF binding protein 3 (GGA3) (Tesco et al, 2007). GGA3 trafficks BACE for lysosomal degradation. Therefore, active Caspase 3 indirectly stabilizes the activity of BACE by impairing its degradation, thereby contributing to increased Aβ production and providing an explanation for the previous discrepancies. The same group noted that GGA3 levels are decreased in AD brains. Since Casp6 is activated in apoptotic neurons that have increased Aβ it would be interesting to test if Casp6 also cleaves GGA3.

1.5.2 Caspases process PS and Tau

Two other proteins involved in FAD are also caspase substrates: Tau and PS (Canu et al, 1998; Chung et al, 2001; Fasulo et al, 2000; Gamblin et al, 2003; Grunberg et al, 1998; Kim et al, 1997). Tau is cleaved by caspase 1, 3, 7 and 8 within its C-terminus *in vitro* (Gamblin et al, 2003), confirming previous results demonstrating a Caspase 3 cleavage site within Tau (Chung et al, 2001; Fasulo et al, 2000). This truncated fragment is detected in neurofibrillary tangles of an AD brain (Gamblin et al, 2003) and is cytotoxic in neuronal cells (Chung et al, 2001; Fasulo et al, 2005). Two other Casp6 cleavage sites

in Tau, either at the N terminus (Horowitz et al, 2004) or C terminus (LeBlanc et al, 1999) were discovered and confirmed to be present in tangles (Horowitz et al, 2004) or in the neuropil threads, neurofibrillary tangles and neurites of plaques of AD and MCI brains (Albrecht et al, 2007; Guo et al, 2004). Both PS proteins can be cleaved by a multitude of caspases (Grunberg et al, 1998; Kim et al, 1997; van de Craen et al, 1999), however the caspase cleaved PS fragments have not yet been confirmed in AD brains. Unlike APP, five PS missense mutations examined thus far do not sensitize PS to caspase cleavage (van de Craen et al, 1999).

1.6 CASPASE 6

Casp6 is a versatile protein that has a key role in a number of different pathways. First described in 1995, the 34 kilodalton (kDa) CED-3 homologue, then termed Mammalian Ced-3 Homologue 2 (Mch2), was added to the growing list of cysteine proteases (Fernandes-Alnemri et al, 1995) and later mapped to chromosome 4 (Bullrich et al, 1996). Casp6 role in apoptosis was solidified by the discovery that it cleaves lamin A, an essential nuclear cytoskeleton protein, resulting in the now recognized fragmentation synonymous with apoptosis (Orth et al, 1996). It has however been identified to have a number of other substrates throughout the body ranging from nuclear to cytoskeleton or cytoskeleton associated proteins. Casp6 mRNA levels have been examined in the rat where the highest expression is seen mainly in the lungs followed by the heart, kidney and muscle (Singh et al, 2002). Casp6 expression levels in humans have yet to be published though work is underway in our lab.

Casp6 knock-out mice have no reported developmental abnormalities, but these mice have never been properly characterized, nor has it been published whether other caspases are upregulated in compensation making it difficult to conclude on the importance of Casp6 in developmental neuronal cell death (Zheng et al, 1999; Zheng et al, 2000). At least in intestinal epithelial cells however, Casp6 plays an essential role in anoikis, or detachment induced cell death, which is essential to ensure intestinal epithelium homeostasis. In this rapid apoptotic response, Casp6 activation is detected minutes after cell detachment and occurs before Caspase 3 activation (Grossmann et al, 1998). In a pathological role, in a mouse model of Huntington's disease (HD), cleavage by Casp6 but

not Caspase 3 of mutant huntingtin, a protein with a polyglutamine expansion that causes HD, is required for the development of neuropathological and behavioural features of HD (Graham et al, 2006). The importance of Casp6 in human HD however has not yet been shown. Chemically induced epileptic seizures in rats cause an activation of Casp6 prior to Caspase 3 measured by western blotting, caspase activity assays and immunohistochemistry in the hippocampus (Henshall et al, 2002). Finally, ischemic injury in rat kidney (Singh et al, 2002) and human brain result in activated Casp6 (Guo et al, 2004). Of note is that ischemia in adult brain demonstrated immunoreactivity via a neoepitope antibody of active Casp6 only in the nucleus while fetal ischemic brains had active Casp6 in the nucleus, cytosol and neurites.

1.6.1 Caspase 6 Activity in AD – From cell culture to the brain

A key initiator caspase, Casp6, is thought to be an important participant in the pathogenesis of AD, demonstrated both *in vitro* and *in vivo*. Human neurons have been used as a cell model to study the mechanisms of AD. In culture, apoptotic serum-deprived human fetal neurons have increased Casp6 but not Caspase 3 activity (Guo et al, 2006; LeBlanc et al, 1999). Co-currently, these serum-deprived apoptotic neurons have increased production of the Aβ peptide, which is prevented by inhibiting Casp6 (LeBlanc, 1995; LeBlanc et al, 1999). In this cell model, Casp6 is activated by Caspase 1 (Guo et al, 2006) and microinjection of recombinant active Casp6, but not Caspase 3,7 or 8, causes a protracted cell death that can take up to 6 days in the absence of other insults, potentially mimicking the slow progressive cell death in AD (Zhang et al, 2000).

While Casp6 plays a significant role in cell culture, it has been validated in human AD brains. The active Casp6 subunit appeared to be slightly increased in the frontal, temporal, pariental and cerebellar areas of one AD brain examined (LeBlanc et al, 1999) while a two to three fold increase of active Casp6 was seen in the frontal and temporal cortex of other AD brains by western blot (Guo et al, 2004). In contrast to the first study, Guo *et al.* noted no active Casp6 in the cerebellar tissue, an area unaffected by AD. This discrepancy can be explained since the earlier study only examined one brain by western blot and cautiously interpreted their data as simply suggesting that Casp6 is present in adult brains while Guo *et al.* immunoprecipitated proteins from AD and control brains.

By immunohistochemistry, active Casp6 is also localized in neurofibrillary tangles, neuropil threads and neurites within the Aβ plaques (Guo et al, 2004). Interestingly, the authors noted that the degree of staining does not correlate with the severity or duration of the disease. In MCI brains active Casp6 immunoreactivity is detected in the same places as AD brains (Albrecht et al, 2007) indicating that Casp6 activation appears to occur early in the disease, possibly contributing to neurodegeneration and not neurotoxicity.

1.6.1.1 Caspase 6 substrates in AD

While having a number of other substrates unrelated to AD, Casp6 cleaves three important neuronal proteins involved in AD: APP (LeBlanc et al, 1999; Pellegrini et al, 1999), PS (van de Craen et al, 1999) and Tau (Gamblin et al, 2003; Horowitz et al, 2004; LeBlanc et al, 1999). Casp6 cleavage of the C-terminus of APP results in the neurotoxic C31 fragment (Dumanchin-Njock et al, 2001; Lu et al, 2000). As well, the Swd mutation of the APP gene has been shown to create a more efficient Casp6 cleavage site (Gervais et al, 1999). The preferential Casp6 site causes an increase in APP proteolysis resulting in increased Aβ production. The cleavage of PS by Casp6 has been demonstrated *in vitro* using purified protein, but its significance has yet to be demonstrated *in vivo* (van de Craen et al, 1999).

In vitro and in neuronal extracts Casp6 cleaves the cytoskeleton associated protein, Tau (Gamblin et al, 2003; Horowitz et al, 2004; LeBlanc et al, 1999). The N-terminus Casp6 cleavage site of Tau is detected in the tangles of early and late AD brains (Horowitz et al, 2004). The C-terminus Casp6 cleaved Tau is richly detected in neurofibrillary tangles, neuropil threads and neurites associated with plaques of AD brains (Guo et al, 2004). In MCI brains, the C-terminus Casp6-cleaved-Tau was present in neurofibrillary tangles and neuropil threads, but more significantly, also in non-cognitively impaired brains with low global cognitive scores (Albrecht et al, 2007). This inverse correlation of increased Tau cleaved by Casp6 immunoreactivity with decreased cognitive scores suggests that Casp6 may contribute to AD pathology via neurodegeneration early in the disease.

It was recently demonstrated that Casp6 cleaves a number of cytoskeleton associated proteins in human primary neuronal extracts (Klaiman et al, 2008). Using a proteomics approach the Casp6 substrates α-Tubulin, Drebrin, Spinophilin and α-Actinin-4 were

identified. Cleavage by Casp6 of purified or i*n vitro* translated cystoskeleton proteins confirmed these results. Additionally, the authors utilized site directed mutagenesis to identify some, but not all, of the predicted Casp6 cleavage sites in Debrin, α-Tubulin, and Spinophilin. Identifying these fragments in AD affected brain samples versus control samples can further substantiate the significance of these results in AD. *In vivo,* the neoepitope antibody to tubulin cleaved by Casp6 was immunoreactive with all the major hallmarks of AD including neurofibrillary tangles, neuropil threads and plaques (Klaiman et al, 2008). Since active Casp6 remains neuritic and does not translocate to the nucleus of neurons in severe AD brains (Guo et al, 2004), it supports the hypothesis that at least in AD, active Casp6 has a new role in neuritic degeneration.

1.7 HYPOTHESIS AND OBJECTIVE OF STUDY

The neuropathology of sporadic and FAD are similar. Based on previous studies, FAD-associated mutants cause cell death, make cells vulnerable to age related stresses and activate caspases (Eckert et al, 2003; Hashimoto et al, 2000; Luo et al, 1999; Marques et al, 2003; McPhie et al, 2003; McPhie et al, 2001; Niikura et al, 2004; Zhao et al, 1997). Casp6 in particular is activated and localized to the AD neuropathological hallmarks in sporadic AD brains (Albrecht et al, 2007; Guo et al, 2004) . Given these results, we hypothesized that Casp6 is also activated in FAD. To address this, I assessed the activity and effects of Casp6 in human fetal neurons transfected with FAD-associated mutations. We chose to use these primary neurons to address our objective because they are terminally differentiated cells that are the closest *in vitro* model to AD, a disease restricted to humans. Data obtained from human neuronal cultures have also been validated in sporadic AD brains establishing them as a credible system.

II. PREFACE

The following chapter contains a manuscript being prepared for submission to the Journal of Neuroscience. It details the work I conducted on Casp6 activity in human neurons over-expressing FAD-associated mutations and APP_{695}.

Title:

Over-expression of wild type, Swedish and London amyloid precursor protein induce Caspase-6-dependent but amyloid-beta-peptide independent disruption of the cytoskeleton in primary human neurons.

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Contribution of Authors and Acknowledgements

The work presented here was conducted almost entirely by myself, however I gratefully acknowledge *Dr. Cynthia Goodyer* (Department of Pediatrics, McGill University) and the *Birth Defects Research Laboratory* (University of Washington, Seattle) for providing us with human fetal brains as well as *Jennifer Hammond* who prepared and maintained the primary human neuronal cultures. Immunohistochemistry of the FAD Swedish brain was conducted by *Dr. Steffen Albrecht, Jennifer Hammond* and *Dr. Andrea LeBlanc* (data not shown).

The authors also thank *Dr. George Leveque* (Laval University, Quebec City, Quebec) for the full length APP cDNA, *Dr. Jannic Boehm* (University of Montreal, Montreal, Quebec) for the MV mutant construct and *Dr. Yasuo Ihara* (University of Tokyo, Japan) for the RFP-Tau construct. We also acknowledge *Guy Klaiman* and *Heather Turnan* for the DN Casp6 construct.

2.1 INTRODUCTION

Caspase 6 (Casp6) is activated in the absence of the other two effector caspases, Caspase 3 and Caspase 7, in serum-deprived human primary neurons cultures (LeBlanc et al, 1999). Microinjection of active Casp6, but not Casp3 or 7, induces cell death several days later in human neurons (Zhang et al, 2000). Casp6 is activated early in sporadic AD and is present in the three major neuropathological hallmarks of AD: neuropil threads, neurofibrillary tangles and neuritic plaques (Albrecht et al, 2007; Guo et al, 2004). Active Casp6 cleaves neuronal proteins associated with AD, such as amyloid precursor protein (APP) (Gervais et al, 1999; LeBlanc et al, 1999; Pellegrini et al, 1999; Weidemann et al, 1999), Tau (Gamblin et al, 2003; Horowitz et al, 2004; LeBlanc et al, 1999), and Presenilin 1 and 2 (van de Craen et al, 1999). Furthermore, Casp6 cleaves several cytoskeleton proteins such as α-tubulin and β-actin-regulating post-synaptic density proteins, Drebrin, Spinophillin, α-actinin-1 and -4 (Klaiman et al, 2008). Tau and α-tubulin cleaved by Casp6 (TauΔCasp6 and TubΔCasp6) are also present in neurofibrillary tangles, neuritic plaques and neuropil threads (Albrecht et al, 2007; Guo et al, 2004; Klaiman et al, 2008). Finally, Casp6 is also activated in the entorhinal cortex of aged brains that have lower global cognitive scores than their counterparts (Albrecht et al, 2007). Since the entorhinal cortex is the area first affected in AD brains (Braak & Braak, 1991), these results suggest that Casp6 could be an early instigator of AD that disrupts the architecture and consequently the function of neurons.

AD occurs either sporadically or genetically. The cause of sporadic Alzheimer is unknown however the familial forms of AD (FAD) are associated with missense mutations in the APP (St George-Hyslop et al, 1987) gene or the two homologous Presenilin genes (Levy-Lahad et al, 1995; Rogaev et al, 1995; Sherrington et al, 1995). Yet, sporadic AD and FAD show identical pathological profiles (Mullan et al, 1993). Because familial mutants increase amyloid beta peptide (Aβ) production (reviewed in Selkoe & Podlisny, 2002), Aβ is generally thought to cause AD. However, cellular stress will also overproduce Aβ in an indirect but caspase-dependent manner (Gervais et al, 1999; LeBlanc, 1995; Tesco et al, 2007; Tesco et al, 2003). Over-expression of APP missense mutations can cause cell death by multiple mechanisms including caspase activation (Eckert et al, 2003; Hashimoto et al, 2000; Marques et al, 2003; McPhie et al,

2001; Niikura et al, 2004). Expression of these mutations causes axonal dysfunction in *Drosophila* and numerous transgenic murine AD models (Gunawardena & Goldstein, 2001; Stokin et al, 2005; Wirths et al, 2007). This is consistent with reports of axonal defects, cytoskeleton abnormalities and neuronal deterioration early in AD (Lampert, 1971; Price et al, 1986; Stokin et al, 2005).

Interestingly, over-expression of wild type APP also disrupts axonal trafficking of vesicles, synaptic proteins and nerve growth factor (Gunawardena & Goldstein, 2001; Salehi et al, 2006; Torroja et al, 1999). Down's syndrome patients with a duplication of chromosome 21 which includes the APP gene, develop clinical and neuropathological features of AD (Burger & Vogel, 1973). Duplication of the APP gene can also cause AD in the absence of Down's syndrome (Cabrejo et al, 2006; Rovelet-Lecrux et al, 2006; Sleegers et al, 2006).

Given that sporadic and familial AD neuropathology is identical, we investigated whether Casp6 is also activated in over-expressed wild type or FAD APP mutants, and if it could contribute to neuronal dysfunction.

2.2 MATERIALS AND METHODS

Cell Culture

Primary human fetal neurons obtained with ethical approval from the McGill University Institutional Review Board were cultured as described previously (LeBlanc, 1995). Cultures generally contain ~90% neurons and 10% astrocytes. Mouse neuroblastoma Neuro-2a (N2a) cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in MEM (Gibco-Invitrogen, Carlbud, CA) containing nutrient supplements and 10% fetal bovine serum (HyClone-Thermo Scientific, Waltham, MA).

Cloning Strategy and Site Directed Mutagenesis of human APP

Full length APP_{695} cDNA was obtained from Dr. Georges Levesque (Laval University, Quebec) and subcloned into the bigenic eukaryotic pBudCE4.1 vector (Invitrogen, Burlington, ON) under the human cytomegalovirus (CMV) promoter using the *HindIII* and *NotI* sites. Enhanced green fluorescent protein (EGFP) had already been cloned into the pBud vector downstream of the human elongation factor 1 alpha (EF-1α) promoter as a marker of transfection (Jodoin et al, 2007). The Swedish (Swd) mutation at amino acid codon 595 and 596 (KM-NL) and the London (Lond) mutation at amino acid codon 642 (V-I) was generated by the Quik change site directed mutagenesis method (Stratagene, La Jolla, CA) using the following sense primers (substituted nucleotides indicated in bold) Swd, 5'-GATCTCTGAAGTGAA**TC**TGGATGCAGAATTCCG-3'; Lond, 5'-CATAGC GACAGTGATC**A**TCATCACCTTGGTG-3'. The histidine tagged dominant negative Casp6 C163A (DN Casp6) mutant (Hermel et al, 2004) was cloned by Heather Turnan and Guy Klaiman in the pCep4β vector. The β secretase uncleavable APP (MV) mutant was a kind gift from Dr. Jannic Boehm (University of Montreal, Quebec) (Citron et al, 1995) and was subcloned into the pBudEGFP vector *SalI* and *Xba I* restriction sites. The pDs-Red RFP-Tau (RFP-Tau) construct was a kind gift from Dr. Yasuo Ihara (University

of Tokyo, Tokyo) (Oyama et al, 2004) and the pRFP-C1 mUbiquitin (RFP-Ubq) construct was purchased from AddGene (Cambridge, MA)(Bergink et al, 2006). All constructs were confirmed by sequencing at the McGill University and Genome Quebec Innovation Centre.

Transfection

Neurons were plated on poly-L-lysine coated aclar coverslips (20 µg/mL; Sigma-Aldrich, St. Louis, MO) at a density of $3x10^6$ cells/mL and were transfected via the Helios gene gun (Bio-Rad, Mississauga, Ontario, Canada) with a pressure pulse of helium (100 psi). The cartridges used for transfection were made of 0.033 mg of DNA bound to 4.2 mg of gold microcarrier beads prepared in 1 M calcium chloride and 0.1 mL of 0.05 M spermidine (Roucou et al, 2005). Approximately 1 µg DNA coated gold beads/cartridge was delivered to three coverslips of neurons per treatment per neuronal preparation. When transfecting cells with more than one construct a ratio of 2:1 of pDS-red RFP-Tau or pRFP-C1 mUbiquitin to pBudEGFP, pBudEGFP/APP_{695} (APP_{695}), pBudEGFP/APP_{Swd} (Swd), pBudEGFP/APP_{Lond} (Lond) and pBudEGFP/APP_{MV} (MV) or 3:1 of pCep4β $Casp6_{C163A}$ (DN Casp6) to Lond was used to prepare cartridges.

N2a cells were transfected with 4 µg of pBudEGFP, full length APP_{695}, the Swd, Lond, MV and DN Casp6 mutant constructs using Lipofectamine^{2000} (Invitrogen, Carlsbad, CA) for 3hrs according the manufacturer's instructions. Transfection efficiency was assessed via a Nikon Eclipse TE2000-U fluorescent microscope (Mississauga, ON). EGFP positive cells versus the total number of cells stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) were counted yielding less than 0.01% transfection efficiency in human neurons and 75% efficiency in N2a cells. The gene gun was used to transfect the primary human neurons because they resist viral based transfections and lipid based transfections are toxic to them.

Neuronal Dropout and Cell Death Assays

The number of EGFP-positive APP_{695}, Swd, Lond, MV or DN Casp6 mutant transfected human neurons in culture was assessed live over 72 hours under fluorescent microscopy. For this analysis, the total number of transfected fluorescent neurons counted at 24 hours was standardized to 100% and subsequent neuronal counts at 48 and 72 hours were expressed as a percentage of the total number of neurons counted at 24 hours. A minimum of 50 neurons per experiment in three independent neuron preparations was assessed. Cell death was measured by assessing condensed chromatin. Twenty minutes prior to live assessment 1 μg/mL of Hoescht 33342 was added to media, cells were washed twice in PBS pH 7.5 and fresh media was added. EGFP-positive neurons displaying condensed chromatin visualized with Hoescht 33342 were counted at 48 hours. N2a cells were also assessed for cell death after being serum-deprived in MEM media for 24 hours. An average of 300 cells per experiment were assessed. Cell death was additionally confirmed in human neurons by counterstaining neurons with Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labelling (TUNEL)-Red to determine DNA fragmentation. Briefly, neurons were fixed for 20 minutes at room temperature in 4% paraformaldehyde and 4% sucrose and then permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate prior to staining with TUNEL using the In Situ Cell Detection kit I (Roche, Laval, Quebec).

Measurement of Morphological Changes in Human Neurons

Neurons displaying aberrant neuritic EGFP distribution were expressed as a percent of the total number of EGFP positive neurons at each 24 hour time point for up to 72 hours. Results were obtained by averaging neuronal counts for at least 50 EGFP positive neurons per experiment at the first 24 hour time points in three independent neuron preparations.

Caspase 6 Activity Assessment in Human Neurons

To assess Casp6 activity in APP_{695} and Swd, Lond or MV transfected neurons, neuronal preparations were pre-treated in 5 μM of z-VEID-fmk (Biomol, Plymouth meeting, PA). The Casp6 inhibitor was diluted in media and added to the cells 2 hours prior to gene gun transfection. Neurons were then assessed for EGFP-positive neuronal dropout, cell death and morphological changes as described above on a minimum of 50 cells per construct and in three independent neuron preparations. Media containing the Casp6 inhibitor was changed every 24 hours during the live assessment until the 72 hour time point.

Detergent Soluability Assay

For western blot analysis, N2a cells were plated in 6-well plates at a density of $0.8x10^6$ cells/well. Transfected cells were lysed using the non-ionic detergent Nonidet P-40 (NP-40) buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, 1% NP-40) containing a protease cocktail of 38μg/mL 4-(2-aminoethyl) benzenesulfonylfluoride (AEBSF), 0.1 μg/mL pepstatin A, 1 μg/mL *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and 0.5 μg/mL Leupeptin and harvested on ice. After micro-centrifugation at 13,000 rpm at 4°C for 10 minutes, the detergent insoluble protein pellet was frozen immediately while soluble proteins were quantified by BCA assay (Pierce, Rockford, IL). For caspase fluorogenic activity assays transfected N2a cells were lysed in caspase lysis buffer (50 mM Hepes pH7.4, 0.1% CHAPS, 0.1 mM EDTA and 1 mM DTT added fresh) containing the same protease cocktail as the NP-40 buffer and harvested on ice.

Western Blot Analyses

Proteins were separated on a 10% (APP mutant construct expression) or 15% (C163A mutant construct expression) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane. For APP_{695}, Swd, Lond and MV construct protein expression, 2 μg of protein was loaded while for the DN Casp6 mutant expression, 50 μg of protein was used.

Where indicated, blots were probed with 1:500 22C11 anti-APP (Chemicon, Bedford, MA), 1:500 Casp6 Ab4 (Neomarker, Fremont, CA) or 1:1000 anti-β-actin (Clone AC-15, Sigma-Aldrich, St. Louis, MO) antibodies. Immunodetection was conducted with HRP-conjugated goat anti-mouse (Jackson Immunoresearch, West Grove, PA) or sheep anti-rabbit (GE Healthcare/Amersham, Piscataway, NJ) secondary antibodies and chemiluminescent development with Milipore ECL (Bedford, MA) or Amersham ECL Plus (Piscataway, NJ) western blotting detection system.

Caspase Fluorogenic Activity Assay

For caspase fluorogenic activity assays, N2a cells were incubated 24 hours post-transfection in serum free MEM media (Gibco-Invitrogen, Carlsbad, CA) for 3 hours prior to harvesting. Transfected N2a soluble protein extracts were assayed for Casp6 and Casp3 activity using preferred fluorogenic peptide substrates as previously described. Five µg of neuronal protein extract (in triplicates) were incubated with either z-VEID-AFC for Casp6 activity or z-DEVD-AFC (Biomol, Plymouth meeting, PA) for Casp3 activity in Stennicke's reaction buffer (20 mM PIPES, 10 mM DTT, 1 mM EDTA, 30 mM NaCl, 0.1% CHAPS and 10% sucrose at pH7.2). Cleavage was measured by release of the fluorogenic Ac, N-acetyl coumarin (AFC) at 2 minute intervals over an hour at 37°C by the BioRad (Hercules, CA) Fluoromark apparatus. AFC excitation was at 390 nm and emission was at 538 nm. A standard curve of 0-50 µM free AFC release was used to calculate release of the fluorogenic moiety in the protein preparations. The rate of cleavage was calculated based on the linear phase of the assay. Specific activity is expressed as nanomoles of AFC release per minute per µg of protein.

Statistical Evaluations

Statistical analysis was performed with a one or two way analysis of variance (ANOVA) and Tukey or Bonferroni post-hoc test using the Statview software (SAS Institute Inc, Cary, NC) or Graphpad Prism software (La Jolla, CA).

2.3 RESULTS

Swedish and London FAD-associated APP mutants have increased Caspase-6-like activity in serum-deprived N2a cells

Both the Swd and Lond mutations are autosomal dominant FAD-associated missense mutations in the APP gene (Fig 2). The Swd is a double mutation resulting in a lysine, methionine change to an asparganine and leucine occurring at the BACE cleavage site. The Lond is a single missense mutation causing a valine-to-isoleucine change near the γ-secretase recognition sequence. To assess if APP_{695}, Swd and Lond mutants activate Casp6, their cDNAs were cloned into the pBud bigenic vector.

Exogenous protein expression of the transfected constructs was examined in the mouse neuroblastoma N2a cell line. Western blots were probed with an APP antibody specific to amino acid 66 to 81 of the APP N-terminus to ensure that none of the FAD-associated point mutations would interfere with detection (Fig 3A). Although APP was expressed in these cells, we loaded a low amount of protein (2 μg/lane) so that endogenous levels did not impede detection of exogenous protein expression. There was no detectable expression of APP in mock and EGFP transfected N2a cells at any of the time points. APP_{695}, Swd and Lond mutant constructs were all stably expressed at high levels at all the time points, but wild typeAPP_{695} levels were slightly higher than those of the APP mutants. Capitalizing on caspases' adherence to recognizing and cleaving their substrates within specific amino acid residues, caspase activity was measured using synthetic fluorogenic peptide substrates. VEIDase (Casp6 like activity) and DEVDase (Casp3 like activity) caspase activity assays in 24 hour post transfected N2a cells did not reveal any caspase activity (Fig 3B). Twenty-four hours post-transfection, cells were serum-deprived for 3 hours to potentially mimic a cellular stress that could occur in aging brains. In this situation the Swd and Lond mutants induced VEIDase, but not DEVDase activity. Cell death analysis was conducted by assessing condensed chromatin via Hoechst staining in transfected cells serum-deprived for 24 hours (Fig 3C) because more than 3 hours of stress is needed for a phenotype to develop. While Swd and Lond mutants

induced VEIDase activity, they did not induce cell death in serum-deprived N2a cells. Collectively, these results show that FAD associated APP mutants elicit Casp6-like activity in stressed N2a cells but do not cause cell death.

Caspase 6 dependent EGFP beading in neurites of APP_{695}, Swedish or London mutant transfected primary human neurons

To assess if wild type APP_{695} and the Swd or Lond APP mutants activate Casp6 in a terminally differentiated cell type, primary human neurons were transfected. By live fluorescent microscopy, EGFP was homogeneously distributed in the cell body and neurites of transfected human neurons (Fig 4A inset). When APP_{695}, the Swd or the Lond mutants were co-expressed with EGFP for 48 or 72 hours, EGFP beaded in the neurites of transfected neurons (Fig 4B-D). Morphologically, the beading of this cytosolic protein occurred at uneven intervals along the neurites and in varying sizes (Fig 4C inset). A Hoechst stain was used to differentiate the nuclei of the cells from the beads and to determine the viability of transfected neuron cell bodies (Fig 4A-D).

Quantitative analysis demonstrated that when APP_{695}, the Swd or Lond mutant was over-expressed for 48 and 72 hours, approximately 50% of neurons developed a beaded morphology while the remaining neurons still had normal EGFP distribution (Fig 4E). In contrast, EGFP expression in the absence of APP or APP mutant over-expression showed beading in less than 10% of transfected neurons. Together, these results indicate that APP_{695} and APP mutant over-expression cause the aggregation of cytosolic EGFP in the neurites of human neurons.

To investigate whether neuritic EGFP beading in APP transfected neurons was due to Casp6, APP_{695}, Swd and Lond mutant transfected neurons were treated with 5μM of the Casp6 inhibitor, z-VEID-fmk. A cell permeable irreversible inhibitor, z-VEID-fmk uses the specific recognition sequence of Casp6 to covalently bind its active site, thereby preventing Casp6 access to other substrates. Treatment with the Casp6 inhibitor

abrogated EGFP beading morphology in APP_{695}, Swd and Lond over-expressing neurons for up to 72 hours (Fig 4E). Indeed, treated neurons transfected with APP_{695} consistently had no neuritic beading at the 24 and 48 hour time points as did treated vector transfected neurons at the 72 hour time point, hence the lack of visible error bars on the graph. To confirm that the EGFP beading was truly dependent on Casp6, we co-transfected the Lond mutant with DN Casp6. We first assessed endogenous Casp6 and exogenously expressed DN Casp6 in transfected N2a cells (Fig 4F). Endogenous Casp6 was detected as a 31 kDa protein in the N2a cells. DN Casp6 was detected at 36 kDa in N2a cells transfected for up to 72 hours. These results indicate that endogenous Casp6 is present mostly as a p20p10 Casp6 lacking the pro-domain in N2a cells and that the DN Casp6 is efficiently expressed at all time points. Co-transfection of the DN Casp6 with the Lond mutant in human neurons prevented EGFP beading morphology (Fig 4G) confirming that beading is Casp6 dependent. Therefore, APP_{695}, Swd and Lond mutants induce EGFP neuritic beading in a Casp6 dependent manner.

APP_{695}, Swedish and London mutants cause Caspase 6 dependent EGFP positive neuron dropout and cell death in human neurons

Primary human neurons were transfected with APP_{695}, Swd and Lond mutants and monitored live by fluorescent microscopy every 24 hours for up to 72 hours during which the total number of EGFP expressing neurons at each time point was counted. Results were expressed relative to the 24 hour time point (Fig 5A). EGFP vector transfected neurons maintained almost the same number of fluorescent neurons over the 72 hour period; however, neurons over-expressing APP_{695} had a 50% loss of fluorescent neurons after 48 hours in culture (Fig 5A). Similarly, the number of EGFP-positive Swd and Lond mutant transfected neurons decreased by 44% and 36%. After 72 hours in culture, there was an even more significant EGFP positive neuronal dropout. Only 22% of APP_{695} transfected neurons are EGFP positive while 19% and 22% of Swd and Lond mutant transfected neurons are EGFP positive at the 72 hour time point.

The loss of EGFP-positive neurons could be due to a decrease in EGFP expression and not necessarily due to the death of the neurons, therefore, we observed condensed chromatin with the cell permeable dye Hoechst 33342 at 48 hours post-transfection as a measure of cell death. Fifty percent of the neurons over-expressing APP_{695} had condensed chromatin after 48 hours (Fig 5B). Swd and the Lond mutant transfected neurons had 51% and 39% cell death, respectively. Additionally, a sample of transfected neurons from each construct was counter-stained with TUNEL-Red to determine DNA fragmentation. Neurons transfected with APP_{695}, the Swd or Lond mutant that had condensed chromatin visualized by Hoechst 33342 also had fragmented DNA (Fig 5C). The background level of cell death with the pBud-EGFP vector alone in neurons was 14.2 $\pm$ 1.4%. However, un-transfected cells had cell death of 5.8 $\pm$ 0.5% (data not shown) which is not statistically different from pBud-EGFP vector transfected cells indicating that EGFP is not cytotoxic in neurons expressing it for up to 48 hours.

Treatment with 5μM of the Casp6 inhibitor z-VEID-fmk prevented EGFP positive neuronal dropout in neurons over-expressing APP_{695} and the Lond mutant, but not the Swd mutant, at the 48 hour time point (Fig 5A). By 72 hours, the Casp6 inhibitor attenuated neuronal dropout levels of APP_{695}, Swd and Lond mutant transfected neurons. Quantification of cell death by condensed chromatin demonstrated that the Casp6 inhibitor also completely prevented cell death in APP_{695}, Swd and Lond mutant transfected neurons (Fig 5B). The DN Casp6 co-expressed with the Lond mutant inhibited EGFP positive neuronal dropout (Fig 5D). Likewise, the 40% cell death induced by the Lond mutant was abolished by co-expression of DN Casp6 (Fig 5E). Collectively, these results indicate that over-expression of the FAD mutations and APP_{695} cause Casp6-dependent loss of EGFP-positive neurons and neuronal cell death.

Cell death, but not neuritic beading and EGFP positive neuronal loss is dependent on Aß in APP transfected neurons

To determine whether the observed neuritic beading and cell death through over-expression of APP_{695} may be due to Aβ, we over-expressed a mutant form of APP with a single methionine to valine mutation (MV mutant) that eliminates cleavage of the β-secretase site at the +1 position of Aβ (Citron et al, 1995) (Fig 2). The MV mutant was well expressed from 24 to 48hrs post-transfection (Fig 3A).

Cell death in MV mutant transfected neurons, measured by condensed chromatin, was equivalent to the EGFP vector transfected neurons and was significantly lower than the APP over-expressing neurons at 48 hours post-transfection (Fig 6A). However, co-expression of EGFP and the MV mutant resulted in a 19% drop of EGFP-positive neurons 48 hours post-transfection (Fig 6B). While this was significantly different from vector transfected neurons, the EGFP-positive neuronal dropout in the MV mutant was still less than EGFP-positive neuronal dropout in APP_{695} over-expressing neurons.

Neurons over-expressing the MV mutant also developed EGFP neuritic beading. Quantification of the EGFP beading demonstrated that while beading in APP_{695} transfected neurons was significantly higher than EGFP transfected neurons, surprisingly, 30% of neurons expressing the MV mutant displayed EGFP neuritic beading (Fig 6C&D). The aberrant EGFP distribution 48 hours after transfection was similar to the beading seen in APP_{695} and APP mutant transfected neurons. Together, these results indicate that the Aβ generated from the APP is responsible for cell death but not EGFP beading.

To determine whether the EGFP-positive neuronal dropout in MV mutant transfected neurons was due to Casp6, neurons were treated with z-VEID-fmk. The Casp6 inhibitor had no effect on EGFP positive neuronal dropout in MV mutant transfected neurons at the

48 hour time point, however the Casp6 inhibitor prevented EGFP positive neuronal dropout in APP_{695} transfected neurons (Fig 6B). Treatment with the Casp6 inhibitor prevented aberrant EGFP beading in the MV mutant and APP_{695} over-expressing neurons at the 48 hour time point (Fig 6C). Despite this, at the 24 and 48 hour time point, MV mutant transfected neurons still had significantly more EGFP neuritic beading than APP_{695} transfected neurons. These results indicate that cell death in APP_{695} is Aβ dependent, but EGFP positive neuronal dropout and neuritic beading is not. Additionally, neuritic beading in the MV mutant is Casp6 dependent, but EGFP positive neuronal dropout is Casp6 independent.

Beading of Tau and Ubiquitin proteins when co-expressed with APP_{695}, Swedish, London and MV FAD-associated mutants in neurons

Tau is a cytoskeleton-associated protein that is abundant in neurons. To assess whether beading of the cytosolic EGFP protein was an indication of neuritic cytoskeletal impairment, an RFP-Tau fusion vector was co-transfected with APP_{695}, Swd, Lond or MV mutations. RFP-Tau was normally expressed in the axon of the transfected neuron (Fig 7A). pBud-EGFP and RFP-Tau transfected neurons showed a homogenous distribution of Tau and EGFP in the axons and cell body (Fig 7B). Neurons co-expressing Tau, APP_{695} and EGFP had an abnormal beaded distribution of Tau and EGFP as early as 24 hours post-transfection (Fig 7C). Swd, Lond and MV mutant transfected neurons co-expressing RFP-Tau and EGFP also had neuritic beaded Tau and EGFP morphology (Fig 7D-F). Merged images of EGFP and RFP-Tau demonstrated a complete overlap of the beading in APP_{695}, Swd, Lond and MV mutant transfected neurons (Fig 7C-F). Therefore, abnormally distributed EGFP in APP_{695}, Swd, Lond and MV mutant transfected neurons appeared to co-localize with beaded Tau.

The Ubiquitin (Ubq) protein is a ubiquitously expressed regulatory protein used to facilitate proteasome-meditated degradation of proteins. To determine whether the EGFP neuritic beads contain Ubq or Ubq-tagged proteins, an RFP-Ubq fusion vector was co-

expressed with the Lond or MV mutations. Like the RFP-Tau, RFP-Ubq was evenly distributed in transfected neurons (Fig 8A). However, co-expression of RFP-Ubq with the Lond or MV mutant caused Ubq to accumulate as beads in the neurites (Fig 8B-C). The RFP-Ubq positive beads appeared to co-localize with the EGFP beads. Together, the results indicate that the beads are made of several proteins including EGFP, Tau and Ubq.

2.4 DISCUSSION

From our previous work on Casp6 in primary human neurons and sporadic AD brains, we proposed the hypothesis that Casp6 is an instigator of the cognitive impairment and neuropathological changes of AD (LeBlanc, 2008). Support for this hypothesis must be provided from the genetic forms of AD since both the sporadic and familial AD have almost identical clinico-pathological features. We concluded that both FAD-associated mutants and APP_{695} over-expression result in the activation of Casp6 based on several observations. First, over-expression of the Swd or Lond APP mutants, but not wildtype APP_{695} resulted in VEIDase activation in serum-deprived N2a cells. Second, in primary human neurons, both a low concentration of the Casp6 peptide inhibitor, z-VEID-fmk, and co-expression of DN Casp6 abolished neuritic morphological changes and cell death induced by overexpression of wildtype APP_{695} or the Swd or Lond mutants. Third, active Casp6 and TauΔCasp6 localized to the neuropil threads, neurofibrillary tangles and neuritic plaques of an FAD Swd human brain. Our results are consistent with the Swd mutant induced Casp6 activity and cell death in N2a cells and primary rat cortical neurons (McPhie et al, 2001; Ramalho et al, 2006). We previously observed pre-clinical Casp6 activation in aged individuals with normal but lower cognitive scores than their counterparts; and Casp6 dependent cleavage of a number of cytoskeleton proteins in primary human neurons including TauΔCasp6 and α-TubulinΔCasp6, which were present in the neuropathological hallmarks of sporadic AD (Albrecht et al, 2007; Klaiman et al, 2008). As well, there is a Casp6 dependent increase in Aβ in stressed primary human neurons (LeBlanc et al, 1999). The present findings that FAD mutant and APP_{695} over-expression induces Casp6 activation provides an etiological link between the familial and sporadic forms of AD.

Swd or Lond mutants or APP_{695} over-expression cause Casp6 dependent but Aβ independent disruption of protein distribution in the neurites of primary human neurons. Indeed, EGFP, Tau and Ubiquitin agglomerated as round beads in the neurites of FAD mutant or APP_{695} transfected neurons, whereas these proteins normally distribute homogeneously in the cell soma and neurites in the absence of FAD mutant or APP_{695} over-expression. Apparent co-localization of the Tau and Ubiquitin with EGFP indicated that Casp6 activity results transport deficits of these proteins. Our results are reminiscent of several observations made in APP transgenic animal models and in AD. Axonopathy and transport deficits due to Swd or Lond mutants or APP_{695} over-expression have been demonstrated in rat hippocampal neurons, AD primary neurons, murine and *Drosophila* neurons (Gunawardena & Goldstein, 2001; Lampert, 1971; Nishimura et al, 1998; Salehi et al, 2006; Stokin et al, 2005). These defects were characterized by swellings containing abnormal accumulations of microtubule-associated and molecular motor proteins or mitochondria, organelles and vesicles as well as severe degeneration of neurites. Since Casp6 cleaves a number of cytoskeleton proteins (Klaiman et al, 2008), our results indicate that aberrant distribution of EGFP, Tau and Ubiquitin may be evidence of neuritic defects and transport deficits due to restructuring of the neurites by Casp6. Our findings that Casp6 dependent morphological alternations of neurites are not the result of Aβ production, since the Aβ-deficient MV mutant also gives rise to protein neuritic beading, is consistent with recent published results that showed that axonal defects induced by APP_{695} over-expression are not dependent on Aβ in an AD murine model (Stokin et al, 2008).

Wildtype and mutant APP_{695} over-expression in human neurons also induced Casp6 and Aβ dependent condensed chromatin. However, the MV mutant did not result in condensed chromatin in transfected cells despite aberrantly altering neuritic morphology. The observed cell death is consistent with numerous studies with Swd or Lond mutant induced cell death in COS-NK1, PC12, NT2N, rat and mouse primary cortical neurons (Luo et al, 1999; McPhie et al, 2001; Niikura et al, 2004; Yamatsuji et al, 1996b; Zhao et al, 1997). Our present results infer that Aβ is downstream of Casp6 activation in human neurons and are corroborated by previous studies that showed Casp6 dependent over-production of Aβ in serum-deprived human neurons (LeBlanc, 1995; LeBlanc et al,

1999). Additionally, Tesco *et al.* recently demonstrated a mechanism by which active caspases indirectly stabilize the activity of BACE by impairing its degradation, thereby contributing to increased Aβ production (Tesco et al, 2007). This same mechanism may potentially be utilized by Casp6. Therefore, these results imply that elimination of Aβ in AD brains may not necessarily restore neuronal function if morphological aberrations in the neurites that cause neuronal dysfunction occur prior to Aβ accumulations.

Despite the absence of condensed chromatin, the MV mutant transfected neurons showed a slight Casp6 independent, EGFP-positive neuronal dropout suggesting that either, the MV mutant has an alternate neurotoxic effect causing a different form of cell death (Yuan et al, 2003), or, the EGFP-positive dropout is due to inhibition of EGFP expression. Given that the MV mutant induces Casp6 dependent protein beading in neurons, it is likely that over-expression of the MV mutant has a detrimental effect on neurons that may induce a non-apoptotic or non-necrotic type of cell death. Morphologically apoptotic neurons are not noted in AD brains with abundant active Casp6 and active Casp6 remains neuritic in these AD neurons (Guo et al, 2004). Further evaluation of the sub-cellular organization of the Casp6 positive AD neurites and neurons by electron microscopy will be required to determine if the cell death observed in primary human neuron cultures is replicated in AD.

Collectively, our results demonstrated that over-expression of the Swd or Lond FAD-associated mutations or wild type APP_{695} cause a Casp6 dependent morphological neuritic alteration and cell death in primary human neurons, potentially placing Casp6 upstream of Aβ. These findings also provides an etiological link between sporadic and FAD which would suggest that if Casp6 activity dismantles the cytoskeleton of neurons, an inhibitor could be a relevant treatment against AD.

Figure 3: Swd and Lond APP mutants induce Casp6 activity in N2a cells. A. Western blot analysis from 2µg N2a protein cell extracts transfected with EGFP, APP_{695}, Swd, Lond and MV mutants for 24, 48 and 72 hours. **B.** Fluorogenic caspase VEIDase or DEVDase activity assay using 5 µg cytosolic extract from mock, EGFP, APP_{695}, Swd and Lond mutant. Transfected N2a cells were treated with (open bars) or without serum (closed bars) for 3 hours. Data represents mean ± SEM of two independent experiments in triplicates *$p<0.05$, statistically significant difference between EGFP and Swd or Lond transfected cells. **C.** Percentage of cell death measured by condensed chromatin in N2a cells transfected with EGFP, APP_{695}, Swd and Lond mutants after 24 hours of serum deprivation. Data represents mean ± SEM of two independent experiments with an average of 300 cells counted per experiment.

Figure 3

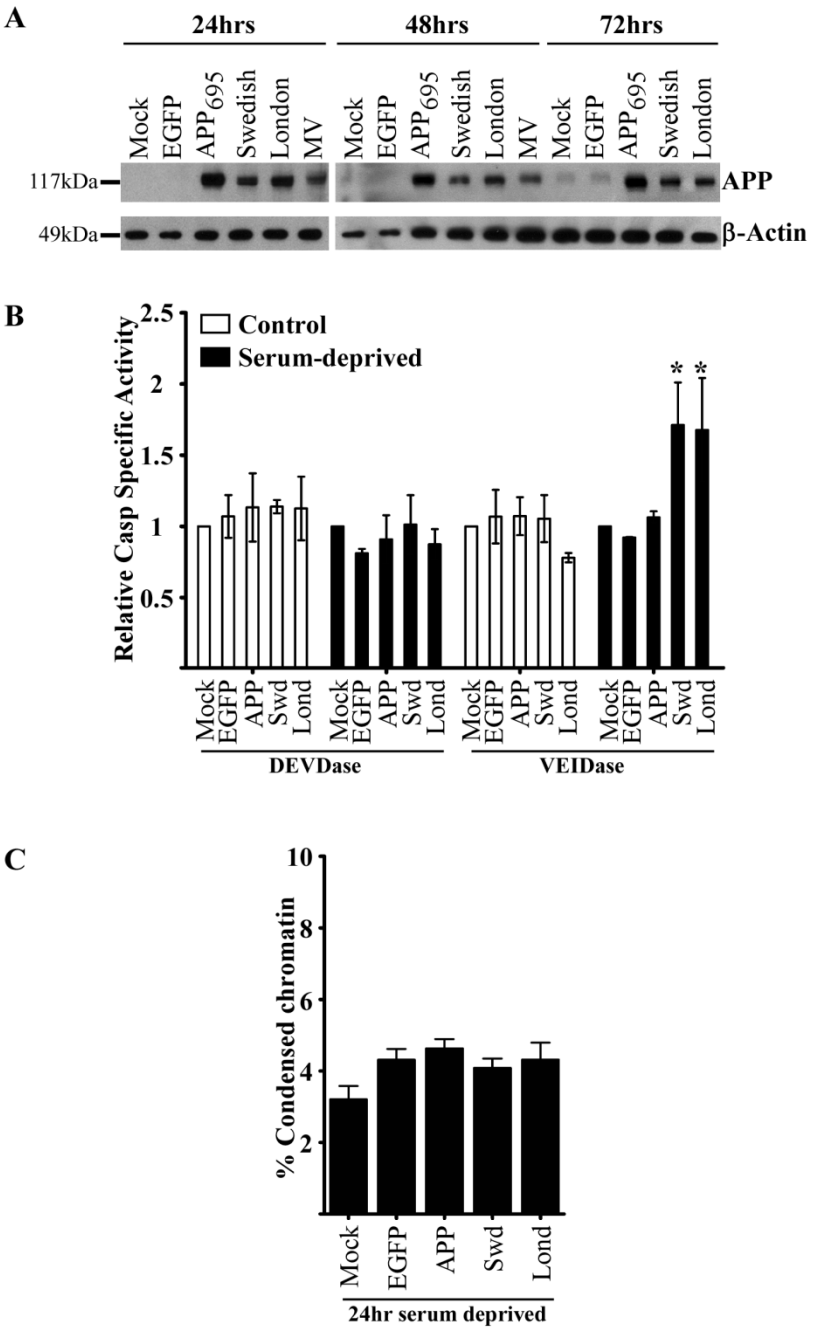

A
24hrs
48hrs
72hrs
Mock
EGFP
APP695
Swedish
London
MV
117kDa
APP
49kDa
β-Actin
B
Control
Serum-deprived
Relative Casp Specific Activity
2.5
2
1.5
1
0.5
Mock
EGFP
APP
Swd
Lond
DEVDase
VEIDase
C
% Condensed chromatin
10
8
6
4
2
24hr serum deprived

Figure 4: APP linked mutations cause Casp6 dependent neuritic beading Fluorescent micrograph of human neurons 48hrs post-transfection expressing EGFP alone **(A)**, APP_{695} **(B)**, Swd **(C)** and Lond **(D)** mutation. For **A-D** Hoechst stain with arrow head indicates viability of transfected neuron. **A** and **C** inset show higher magnification of diffuse and EGFP beaded neurites. Each picture was taken at a 20x magnification **E.** Percentage of EGFP beaded cells transfected with EGFP alone, APP_{695}, Swd and Lond constructs 48 and 72 hours post-transfection (open bars) and treated with Casp6 inhibitor z-VEID-fmk (closed bars). Data represents mean of three independent experiments ± SEM *$p<0.01$, statistically significant difference between EGFP and APP_{695}/ Swd/ Lond. #$p<0.001$ statistically significant difference between APP_{695} ± VEID, Swd ± VEID, Lond ± VEID. **F.** Western blot of Casp6 with the Neomarker antibody from N2a cells transfected and expressing for 24, 48 and 72 hours mock, EGFP, Lond and DN Casp6. Recombinant active Casp6 (R-Casp6) from bacterial extracts was used as a positive control. **G.** Percentage of beaded cells in neurons co-transfected with Lond mutation and DN Casp6 after 24 and 48 hours of expression (closed bars). Data represents mean of three independent experiments ± SEM *$p<0.05$, statistically significant difference between EGFP and Lond + DN Casp6.

Figure 4

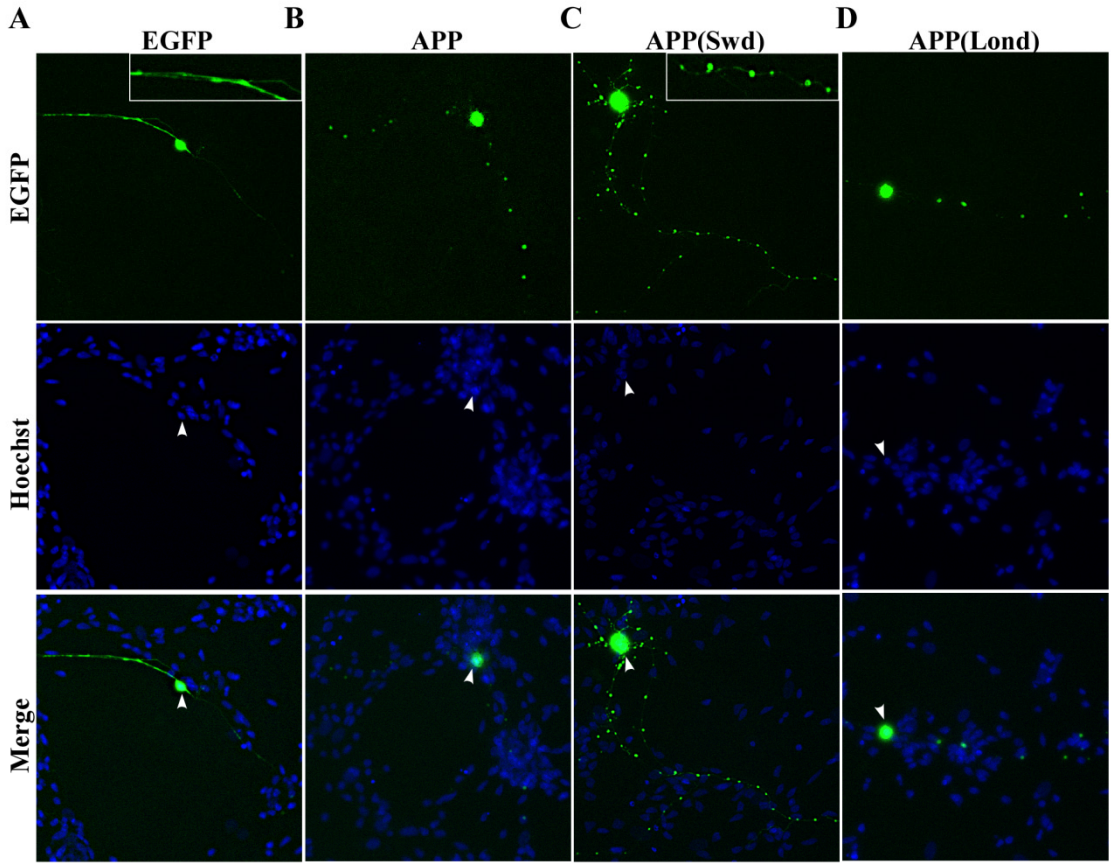
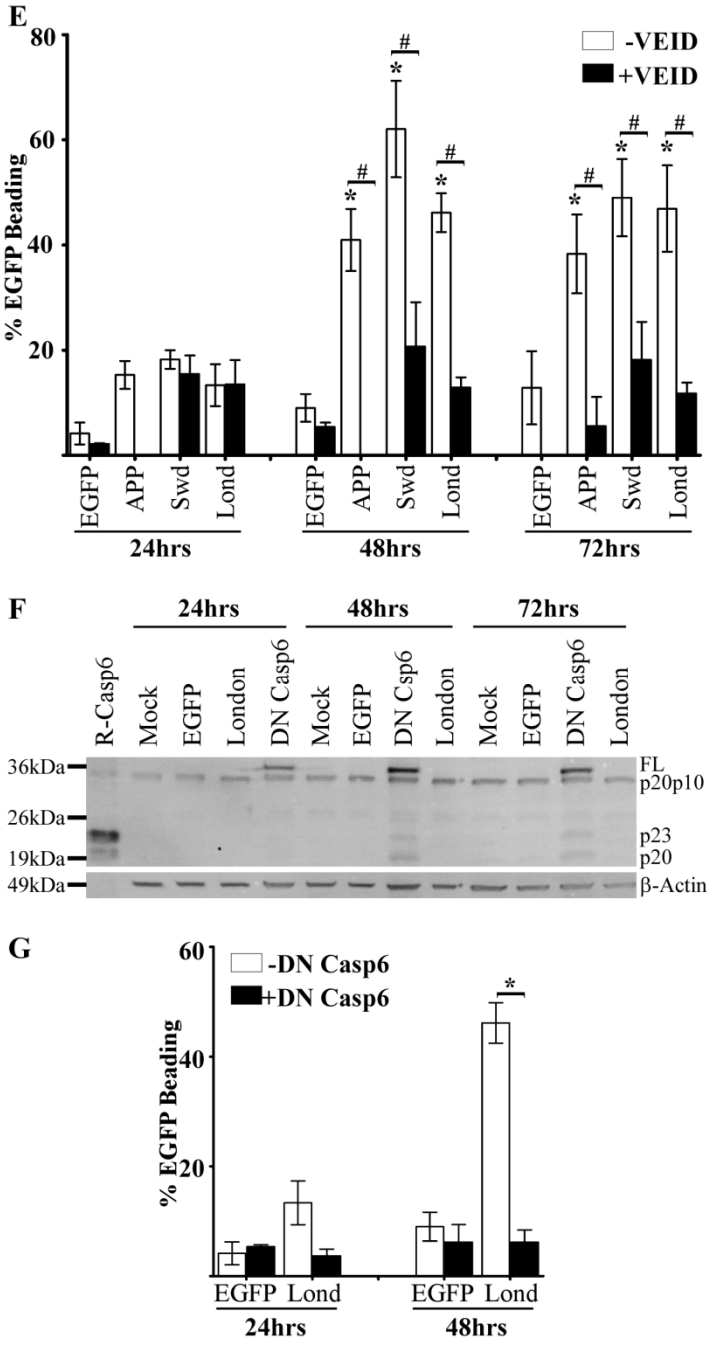


Figure 4


E
% EGFP Beading
-VEID
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APP
Swd
Lond
24hrs
48hrs
72hrs
F
R-Casp6
Mock
EGFP
London
DN Casp6
DN Csp6
36kDa
26kDa
19kDa
49kDa
FL
p20p10
p23
p20
β-Actin
G
-DN Casp6
+DN Casp6

Figure 5: Casp6 dependent EGFP-positive neuronal dropout and cell death by APP linked mutations **A.** Percentage of neurons expressing EGFP over 48 and 72 hours (open bars) and treated with Csp6 inhibitor z-VEID-fmk (closed bars) in EGFP alone, APP_{695}, Swd and Lond transfected neurons. Quantification normalized to 100% at 24 hours (solid line). Data represents mean of three independent experiments ± SEM *$p<0.001$ statistically significant difference between EGFP and APP_{695}/ Swd/ Lond. **$p<0.01$ statistically significant difference between VEID treated EGFP and APP_{695}/ Swd/ Lond. #$p<0.05$ statistically significant difference between APP_{695} ± VEID, Swd ± VEID, Lond ± VEID. **B.** Percentage of cell death after 48 hours of expression measured by condensed chromatin in neurons transfected with EGFP, APP_{695}, Swd and Lond mutants and treated without (open bars) or with z-VEID-fmk Casp6 inhibitor (closed bars). Data represents mean ± SEM of three independent experiments *$p<0.05$ statistically significant difference between EGFP and APP_{695}/ Swd/ Lond. #$p<0.05$ statistically significant difference between APP_{695} ± VEID, Swd ± VEID, Lond ± VEID. **C.** Lond mutant transfected cells expressing EGFP with and without condensed chromatin and counterstained with TUNEL Each picture was taken at a 60x magnification **D.** Percent EGFP neuronal dropout and **E.** cell death in neurons co-transfected with Lond mutation and DN Casp6 after 48 hours of expression (closed bars). Data represents mean of three independent experiments ± SEM *$p<0.05$ (EGFP dropout) *$p<0.001$ (cell death), statistically significant difference between EGFP and Lond + DN Casp6.

Figure 5

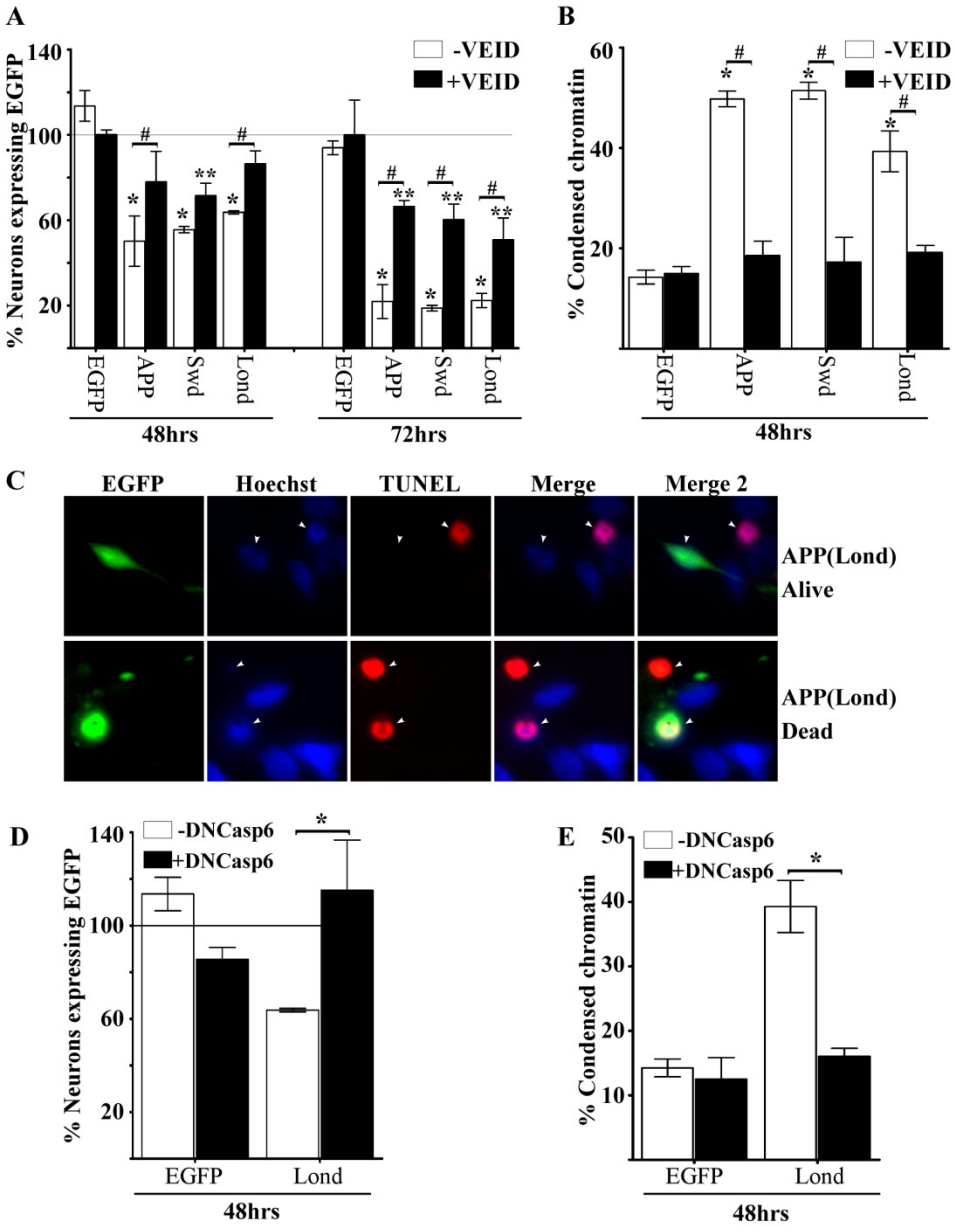

A
% Neurons expressing EGFP
-VEID
+VEID
EGFP
APP
Swd
Lond
48hrs
72hrs
B
% Condensed chromatin
48hrs
C
EGFP
Hoechst
TUNEL
Merge
Merge 2
APP(Lond)
Alive
APP(Lond)
Dead
D
-DNCasp6
+DNCasp6
% Neurons expressing EGFP
48hrs
E
% Condensed chromatin
48hrs

Figure 6: Aβ dependent cell death but not EGFP positive neuronal dropout or beading A. Cell death assessed by condensed chromatin in neurons transfected with EGFP alone, APP_{695} and MV mutant for 48hrs. **B.** EGFP positive neuronal dropout in neurons transfected with EGFP alone, APP_{695} and MV mutant for 48 hours without (open bars) and with (closed bars) Casp6 inhibitor z-VEID-fmk. **C.** Percentage of EGFP beaded cells transfected with EGFP alone, APP_{695} and MV mutant 24 and 48 hours post-transfection (open bars) and treated with Casp6 inhibitor z-VEID-fmk (closed bars). **D.** MV mutant co-expressing EGFP has neuritic EGFP beading 48 hrs post-transfection. Hoechst stain with arrow head indicates viability of transfected neuron. Each picture was taken at a 20x magnification. For **A**, data represents mean of three independent experiments ± SEM *$p<0.05$, statistically significant difference between EGFP and APP_{695}. For **B** and **C**, data represents mean of three independent experiments ± SEM *$p<0.01$, statistically significant difference between EGFP and APP_{695}/ MV; **$p<0.01$ statistically significant difference between VEID treated EGFP and MV; #$p<0.01$ statistically significant difference between APP_{695} and MV; ##$p<0.05$ statistically significant difference between VEID treated APP_{695} and MV.

Figure 6

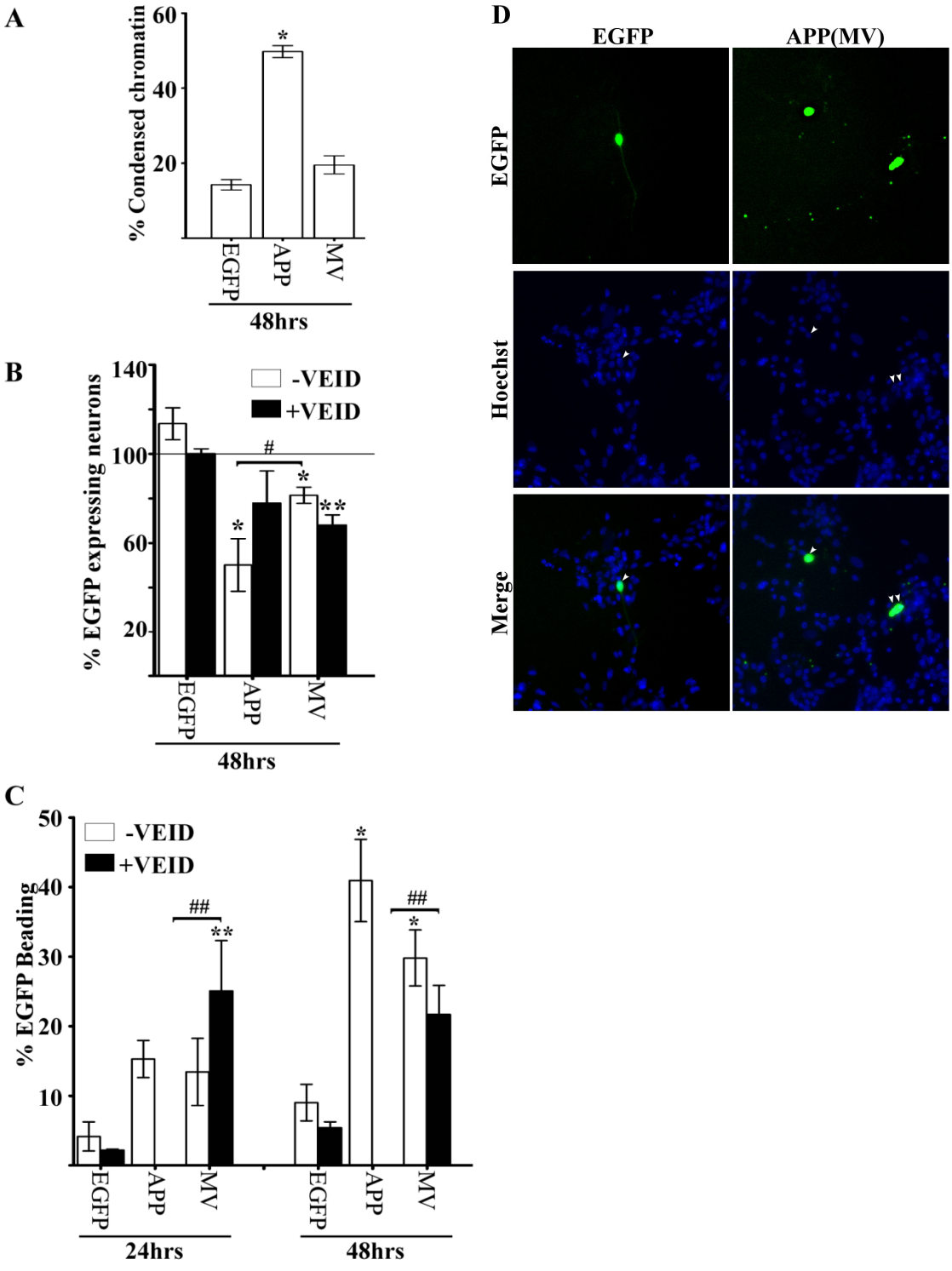

A
% Condensed chromatin
60
40
20
*
EGFP
APP
MV
48hrs
B
% EGFP expressing neurons
140
100
60
20
-VEID
+VEID
#
*
*
**
EGFP
APP
MV
48hrs
C
% EGFP Beading
50
40
30
20
10
-VEID
+VEID
##
**
*
##
*
EGFP
APP
MV
EGFP
APP
MV
24hrs
48hrs
D
EGFP
APP(MV)
EGFP
Hoechst
Merge

Figure 7: APP linked mutations cause Tau to bead. Fluorescent micrograph of human neurons 48 hours post-transfection expressing RFP-Tau alone **(A),** EGFP + RFP-Tau **(B)**, APP_{695} + RFP-Tau **(C),** Swd + RFP-Tau **(D)**, Lond + RFP-Tau **(E)** and MV + RFP-Tau **(F)** mutations. For **A-F** Hoechst stain with arrow heads indicate viability of transfected neuron cell body and ***a** – astrocyte, ***n** – neuron. Each picture was taken at a 20x magnification.

Figure 7

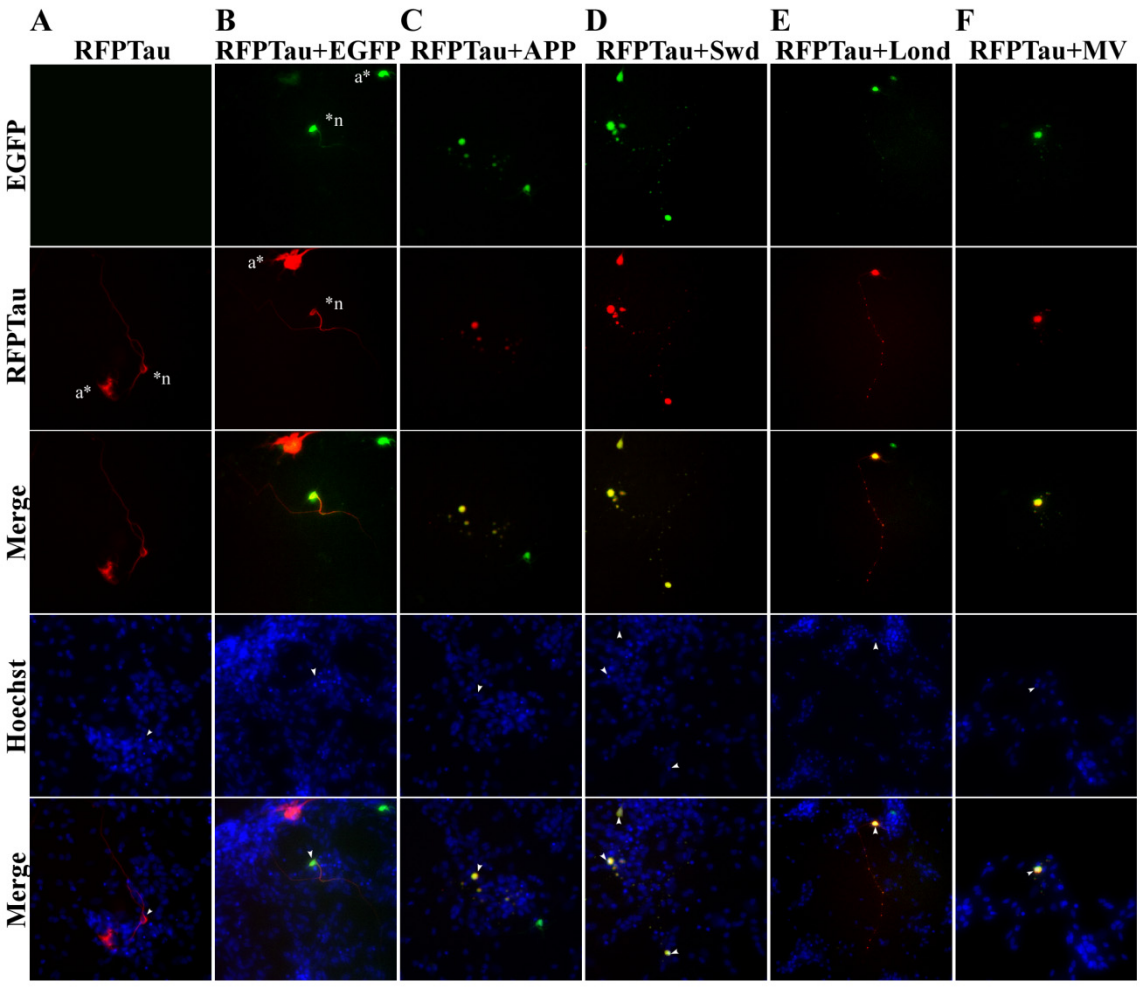
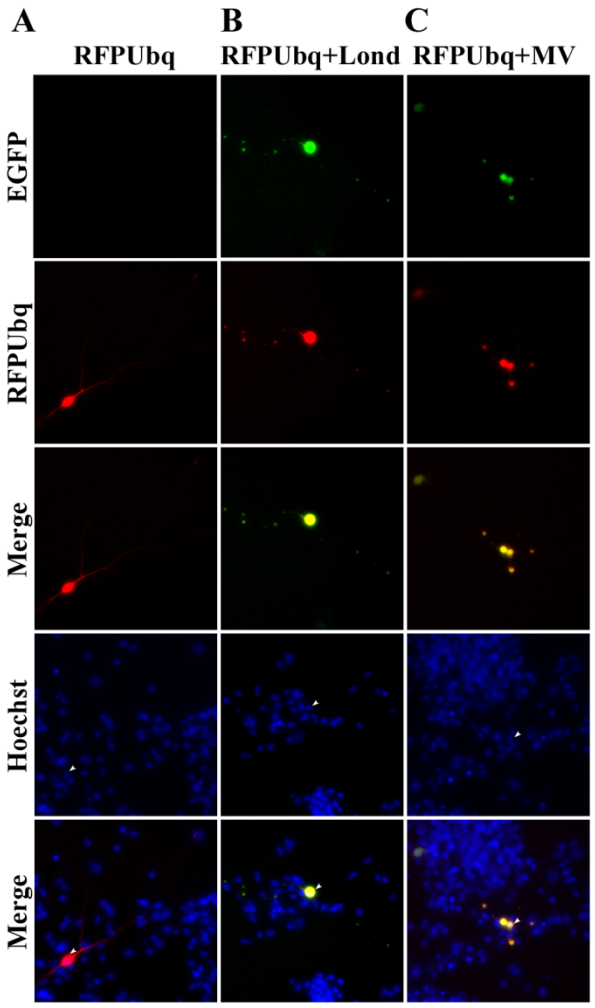

A
RFPTau
B
RFPTau+EGFP
C
RFPTau+APP
D
RFPTau+Swd
E
RFPTau+Lond
F
RFPTau+MV
EGFP
RFPTau
Merge
Hoechst
Merge
a*
*n

Figure 8: APP linked mutations cause Ubiquitin to bead. Fluorescent micrograph of human neurons 48 hours post-transfection expressing RFP-Ubq alone **(A),** Lond + RFP-Ubq **(B)** and MV + RFP-Ubq **(C).** For **A-C** Hoechst stain with arrow heads indicate viability of transfected neuron. Each picture was taken at a 20x magnification.

Figure 8


A
B
C
RFPUbq
RFPUbq+Lond
RFPUbq+MV
EGFP
RFPUbq
Merge
Hoechst
Merge

III. GENERAL DISCUSSION

The neuropathology of sporadic and familial AD are identical in that they both develop senile plaques, neurofibrillary tangles, synaptic loss and neurodegeneration (Mullan et al, 1993). While missense mutations in three genes have been identified in FAD, a link between the etiology of the two forms of AD has still not been established. It has previously been shown that there is an increase in active Casp6 in AD affected parts of the brains and that active Casp6 associates with neuropathological hallmarks at all stages of sporadic AD (Albrecht et al, 2007; Guo et al, 2004). This study presents the first evidence that FAD-associated mutations as well as APP over-expression activate Casp6 in primary human neurons resulting in morphological changes assessed by aberrant EGFP, Tau and Ubiquitin distribution as well as neuronal cell death.

3.1 FAD mutations cause potential axonal dysfunction

Axonal dysfunction has been examined as an early event in AD. Reports of cytoskeleton defects in both sporadic and familial AD (Lampert, 1971; Price et al, 1986; Rose et al, 2000; Stokin et al, 2005; Terry, 1963) as well as *Drosophila* and murine AD models (Gunawardena & Goldstein, 2001; Lazarov et al, 2007; Minoshima & Cross, 2008; Pigino et al, 2003; Pigino et al, 2001; Stokin et al, 2005; Wirths et al, 2007) support the hypothesis that axonal disruption may be a key event in the widespread neurodegeneration of AD.

Over-expression of the Swd and/or Lond mutants in *Drosophila* and transgenic murine AD models result in vesicular stalling, varicosities and swellings containing abnormal accumulations of organelles and vesicles (Gunawardena & Goldstein, 2001; Stokin et al, 2005; Wirths et al, 2007). Our investigation of Swd or Lond mutant over-expression in primary human neurons demonstrates similar protein trafficking abnormalities. EGFP, a cytosolic protein, beads in almost 50% of neurons co-expressing either of the FAD mutants. While a cytosolic protein should be diffuse and able to migrate throughout the neurites, cytoskeleton degeneration could cause organelle and vesicular stalling. Inclusions which form as a result of this effectively serve as roadblocks that prevent the normal trafficking of other proteins, including cytosolic ones. Possible cytoskeleton impairment in our model is supported by axonal beading of the cytoskeleton associated protein Tau when co-expressed with the FAD mutants. Tau is not only aberrantly

distributed, but an overlaid view of EGFP and Tau demonstrates an apparent co-localization of the beads which needs to be confirmed by confocal microscopy.

Cellular stresses can cause the misfolding of proteins which can form toxic protein aggregates and cause inactivation of functional proteins (Gao & Hu, 2008). Ubiquitin, a regulatory protein used to tag misfolded proteins for proteosomal-mediated degradation is found to accumulate in the plaques and tangles of AD brains (Perry et al, 1987; Tabaton et al, 1991; Upadhya & Hegde, 2007). In our study, we found that monomeric Ubiquitin beads when co-expressed with the Swd or Lond mutants and, similar to Tau, these beads appear to co-localize with the beaded EGFP. The Ubiquitin beads therefore can be indicative of misfolded proteins marked for degradation which overwhelmed the ubiquitin-proteosomal system and formed clusters of aggregates; or, they may indicate vesicle bound protein stalling due to cytoskeleton abnormalities in which trafficked Ubiquitin incidentally accumulates. Live assessment of vesicular movement and expression of other cytoskeleton proteins such as Neurofilament or Tubulin can help clarify this as well as help determine whether the neuritic beading is reversible once the stress is prevented. Regardless, the beading of Ubiquitin we observed in human neurons over-expressing FAD mutants parallel observations in human sporadic AD brains, but remains to be assessed in human FAD brains.

3.2 Caspase 6 dependent morphological changes and cell death

Our study demonstrates that morphological changes and cell death due to FAD mutants or APP over-expression is Casp6 dependent in human neurons. Casp6 activity thus far has only been shown in sporadic AD brains (Guo et al, 2004) and indirectly, in FAD mutant over-expressing mouse cortical neurons (McPhie et al, 2001) and N2a cells (Ramalho et al, 2006). This is the first direct evidence of Casp6 activity in a human model used to study FAD, providing a potential bridge for the etiology of the two disease types. In stressed human neurons, Casp6 activity causes an indirect increase in the Aβ peptide and cleaves Tau, proteins which contribute to the neuropathological hallmarks of AD (LeBlanc et al, 1999). Casp6 activation by FAD mutations could result in the same phenotype, albeit by different mechanisms.

Our study shows the first morphological implications of Casp6 activation. Caspases have been mainly connected to cell death. Casp6's cleavage of lamin A and other nuclear matrix proteins established its role in apoptosis (Orth et al, 1996). However, in neurons of sporadic AD, active Casp6 remains neuritic and does not translocate to the nucleus (Guo et al, 2004). As well, it was recently shown that Casp6 cleaves a number of cytoskeleton proteins including Tau and Tubulin which are localized at the neuropathological hallmarks of sporadic AD (Klaiman et al, 2008). We propose that the downstream repercussions of this cleavage can be manifested in aberrant protein trafficking along the affected neurites.

The mechanism of action by which Casp6 can cause such dysfunction is still unknown. Kinases phosphorylate molecular motor subunits and modulate fast axonal transport. Caspase 3 proteolytically cleaves and activates an isoform of protein kinase C which inhibits kinesin dependent fast axonal transport measured by membrane and synaptic trafficking in a squid neurotoxic model of Parkinson's disease (Morfini et al, 2007). A similar role could be proposed for Casp6 in AD. For example, inhibition of cyclin dependent kinase 5 (CDK5) results in activation of GSK 3 which phosphorylates kinesin I thereby promoting detachment of kinesin from the vesicles it is transporting (Morfini et al, 2004). This could be a pathway to examine for Casp6 activity as it has already been shown to be impaired by PS mutants (toxic gain-of-function) (Pigino et al, 2003). Interestingly, GSK 3 also regulates Tau via site specific phosphorylation and is highly immunoreactive in granulovacuolar degeneration in AD neurons (Leroy et al, 2002). However, since Casp6 cleaves cytoskeleton proteins, its function could be restricted to only dismantling the tracks, such as Tau and Tubulin, along which the cargo vesicles run. Meanwhile Caspase 3 attacks the train or the kinesin motor proteins. As well, in primary fetal human neurons, it is the inflammatory Caspase 1 that activates Casp6 (Guo et al, 2006). Takeuchi *et al.* showed that activated microglia, which are part of the inflammatory cascade, can cause neuritic beading (Takeuchi et al, 2005). This could be another mechanism exploited by Casp6 to cause neurodegeneration in AD, particularly since inflammation and activated microglia have been implicated in the disease (Reviewed in Eikelenboom et al, 2006).

3.3 APP_{695} over-expression also causes morphological changes and cell death

In our human neuronal model, over-expressing APP_{695} caused EGFP neuritic beading and cell death equivalent to that of the FAD mutants. While FAD has been linked to missense mutations in APP, a number of families who have duplications of regions of chromosome 21 that includes the APP gene have early onset AD (Cabrejo et al, 2006; Rovelet-Lecrux et al, 2006), and in two patients, only the APP gene was duplicated (Sleegers et al, 2006). Further support that specifically APP over-expression can cause AD is seen in a patient with a duplication of a region of chromosome 21 that does not contain the APP gene who had DS but not AD (Prasher et al, 1998). Understanding the effects of APP over-expression in AD is only now being recognized, however it has long been studied via a different model, DS. Patients with DS develop many of the clinical symptoms of early onset AD as well as the neuropathological features. In a murine model of AD, it was shown that specifically APP over-expression inhibited the trafficking of a number of molecules, while over-expressing APP in *Drosophila* resulted in vesicular stalling and cell death (Gunawardena & Goldstein, 2001; Salehi et al, 2006). Our results therefore corroborate evidence that increased APP amounts can cause axonal dysfunction, which in human neurons, is Casp6 dependent. Unfortunately, due to the limitations of single cell analysis, we are unable to assess the level of APP_{695} over-expression.

3.4 The MV mutant may not be a suitable model to assess the effects of Aβ

The MV mutation, first examined by Citron *el al.* causes a significant reduction, but not abolishment of the Aβ peptide (Citron et al, 1995). It has subsequently been used by a number of groups to assess the effect of Aβ in loss-of-function experiments. In our model, the MV mutant inhibited cell death, but not neuritic beading or EGFP-positive neuronal loss in comparison to control and APP over-expressing neurons. Many groups have only used cell death as an assessment of Aβ toxicity and using this alone, our results would confirm their findings that Aβ is the source of neurotoxicity. However, neurons over-expressing the MV mutant continue to have EGFP-positive neuronal dropout in a Casp6 independent manner. This indicates that either 1) cell death is occurring in an apoptotic/necrotic-independent manner not detected by condensed chromatin 2) EGFP-positive neuronal dropout is not a measure of cell death but of decreased protein expression or dysfunction 3) the MV mutant is functioning by a different mechanism,

potentially a gain-of-function, not comparable to APP over-expression. Additionally, our assessment of neuronal morphology in the MV mutant is also contrary to results demonstrating Aβ dependent toxicity. EGFP neuritic beading continued to occur in a Casp6 dependent manner in the MV mutant, equivalent to that of APP over-expressing neurons. Therefore, this suggests that Casp6 may be upstream of Aβ in terms of dysfunction and it is equally possible that the MV mutant may not be a suitable model to assess the effects of Aβ because of alternate toxic effects. In the MV mutant, the C31 toxic fragment is still produced as well as other fragments capable of neurotoxicity (Lu et al, 2000; McPhie et al, 2001; Yankner et al, 1989; Yoshikawa et al, 1992). Our results then will have to be confirmed via a different approach. The literature has amply demonstrated an Aβ dependent toxicity in a number of cell lines though it is dependent on a numerous factors including length of the Aβ species, oligomerization, solubility, intra versus extracellular aggregation and concentration of synthetic Aβ peptides used (Cappai & Barnham, 2008; Jarrett et al, 1993; Lesne et al, 2006; Li et al, 2007; Pike et al, 1993; Yankner et al, 1990). These have supported the amyloid hypothesis, however, it is starting to emerge that Aβ itself may not necessarily be responsible for neurotoxicity (McPhie et al, 2001; Niikura et al, 2004; Yamatsuji et al, 1996a; Yamatsuji et al, 1996b). Some groups have proposed that axonal dysfunction also occurs in an Aβ independent manner in sporadic AD and a murine AD model (Stokin et al, 2008; Tabaton et al, 1989).

IV. CONCLUSION

My results demonstrate that FAD-associated APP mutations and APP over-expression cause Casp6 dependent morphological changes and cell death in human neurons. We assessed morphological changes via aberrant distribution of the EGFP, Tau and Ubiquitin protein all of which bead when co-expressed with the Swd or Lond mutant or with APP_{695}. As well, over-expression of APP_{695} or the FAD mutants caused increased cell death as assessed by condensed chromatin. Our observations of morphological changes and cell death were shown to occur in a Casp6 dependent manner. While cell death appeared to be Aβ independent, the morphological changes were not, implying that Casp6 may be upstream of Aβ, but these results need to be confirmed by an alternate experiment. Given that Casp6 is active, cleaves a number of cytoskeleton proteins and

is localized at the neuropathological hallmarks of sporadic AD, we provide a potential link between the pathophysiology of the two forms of AD and a model system in which to study it.

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APPENDIX

Human Ethics Approval

McGill

Faculty of Medicine
3655 Promenade Sir William Osler
Montreal, QC H3G 1Y6

Faculté de médecine
3655, Promenade Sir William Osler
Montréal, QC, H3G 1Y6

Fax/Télécopieur: (514) 398-3595

January 15. 2008

Dr. Andréa LeBlanc
Jewish General Hospital
Dept. of Neurology & Neurosurgery
3755 Cote Ste-Catherine
Montreal, Quebec
H3T 1E2

Dear Dr. LeBlanc:

We are writing in response to your request for continuing review for the study A01-M82-99 entitled "Role of caspases in human neuronal cell death and in Alzheimer's Disease."

The progress report was reviewed and we are pleased to inform you that full Board re-approval for the study was provided on **January 14, 2008,** valid until **January 13, 2009**. The certification of annual review has been enclosed.

We ask you to take note of the investigator's responsibility to assure that the current protocol and consent document are deposited on an annual basis with the Research Ethics Board of each hospital where patient enrollment or data collection is conducted.

Should any modification or unanticipated development occur prior to the next review, please advise the IRB promptly.

Yours sincerely,

Roberta Palmour

Roberta Palmour, PhD
Co-Chair
Institutional Review Board

cc: A01-M83-99
Ms. L. Martin – JGH

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DATE OF I.R.B. APPROVAL

JAN 14 2008

Faculty of Medicine
McGill University

McGill Faculty of Medicine
Institutional Review Board
-Continuing Review-

Principal Investigator: Andrea leBlanc Department/Institution: Neurol. & Neurosurg.
IRB Review Number: A01-M82-99 Study Number (if any): _____ Review Interval: _____
Title of Research Study: Role of caspases in human neuronal cell death and in Alzheimer's disease..
Date of initial IRB approval: Jan 12, 2000 Date of previous continuing review (if applicable): Jan 12, 2007

INTERIM REPORT (PLEASE CHECK OR SPECIFY)

Current Status of Study:

Active Study: ACTIVE On Hold: _____ Closed to Enrolment: _____

Interim Analysis: _____ Final Analysis: _____ Study Not Activated*: _____

*If the study has not become active at McGill, please provide correspondence to explain; enclosed: _____

McGill hospital(s) where study has received approval of local Research Ethics Board(s) (if applicable):

JGH: X MCH: _ MGH: _ MNH/MNI: _

RVH: _ SMH: _ Other: _ _____

McGill hospital(s) where study has not received approval of local Research Ethics Board(s) (if applicable): N/A

If study sponsorship or financial support has changed, please provide correspondence to explain; enclosed: Grant renewed by CIHR on Oct. 1, 2006 (5 year renewal)

Number of subjects to be enrolled at McGill: N/A Number of McGill subjects enrolled to date: N/A

Number of McGill subjects enrolled since last review: N/A Have McGill subjects withdrawn from the study?: N/A

Has the study been revised since the last review?: No Have the study revisions been approved by the IRB?: N/A

Has the consent form been revised since the last review?: No Date of the current consent form: _____

Are there new data since the last review that could influence a subject's willingness to provide continuing consent?: N/A

Have there been any serious adverse experiences (SAEs)?: N/A

Have all serious adverse experiences (SAEs) and safety reports relevant to the study been reported to the IRB?: N/A

SIGNATURES:

Principal Investigator: Andrea LeBlanc Date: 19/12/2007

IRB Chair: Roberta Palmour Date: Jan 14, 2008