The localization of c-Abl in Alzheimer's disease

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The two major hallmarks of Alzheimer's disease (AD) are amyloid plaques and neurofibrillary tangles (NFTs). Evidence suggests that the main component of amyloid plaques, β-amyloid peptide (Aβ) facilitates tau pathology via activation of specific kinases. Both glycogen synthase kinase-3β (GSK-3β) and cyclin-dependent kinase 5 (cdk5) have been demonstrated to be activated by Aβ and contribute to tau hyperphosphorylation.

Recently, c-Abl has been implicated in Aβ-facilitated tau pathology by *in vitro* model systems. Alvarez et al. reported that c-Abl could be activated by Aβ in primary cultured neurons (Alvarez et al. 2004), and Derkinderen et al. found a novel phosphorylation site in paired helical filament tau (Tyr 394) that could be phosphorylated by c-Abl (Derkinderen et al. 2005). Moreover, Aβ has been shown to bind integrin receptors on the cell surface and transduce a signal from the extracellular space to the cell interior, regulating the cytoskeleton and/or gene transcription (Caltagarone, Jing et al. 2007). c-Abl can also be activated by integrin activation. Therefore, we hypothesize that c-Abl is associated with Aβ-facilitated tau phosphorylation via integrin binding and activation, contributing to the generation of AD pathology. We tested this hypothesis by examining the expression and distribution of c-Abl in the human hippocampus and by characterizing c-Abl interacting proteins in AD brain.

We discovered that the activation state of c-Abl was altered during AD progression and c-Abl was associated with phospho-tau during AD. Preliminary co-immunoprecipitation data

also suggested a possible association of c-Abl with another integrin signaling protein, paxillin. This study is the first to examine the expression and localization of c-Abl in healthy control and AD hippocampus, which contributes to our understanding of the functional role for c-Abl in AD pathogenesis. Interestingly, c-Abl was localized to granulovacuolar degeneration bodies (GVDs) during late-stage AD, a novel discovery that identifies a new protein component of GVDs in AD.

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1.0 INTRODUCTION

1.1 ALZHEIMER'S DISEASE (AD)

1.1.1 Epidemiology

AD is the most common form of dementia in the older population. AD accounts for about 50–75% of all senile dementia cases, with the greatest proportion in older age groups (American Psychiatric Association 1997). It afflicts about 5–10% of those people over 65 years old and almost half of those over 85 years old (Evans, Funkenstein et al. 1989). By 2007, 5 million people were afflicted with AD in the USA alone. There is no significant difference between distinct ethnicities or geographic populations, although some studies claimed that Asians have a lower prevalence of AD, which might be caused by different criteria and data collecting methods (Osuntokun, Hendrie et al. 1992). As the percentage of the population over age 65 years increases, the prevalence of the disease will increase even higher in the future (Ray, Ashall et al. 1998).

AD is ultimately fatal, typically 6–10 years after onset. About 20% of the mortality is directly related to the dementia. Some deaths indirectly related to AD are contributed to infection, behavioral disturbances and terminal complications. AD is considered the fourth leading cause of death in the US (Reiman and Caselli 1999).

There are two forms of AD: familial and sporadic. The pathogenesis of these two forms are similar, except that familiar AD is early onset, usually between 16 and 65 years of age, while, sporadic AD is late onset and usually occurs in people over the age of 65. The risk factors discussed below predominately focus on the sporadic form of AD, though may facilitate earlier disease onset or more rapid progression in familial forms of the disease.

Age is a definite risk factor for AD. The prevalence rates for AD increases 2 fold about every 5 years after the age of 65: from 1-2% in the 65-74 years old age group to 25% and even more in the ≥ 85 years old age group (Hendrie 1998). Another well established risk factor for AD is genetic susceptibility. About 10% of persons with AD have a family history of the disorder in one or more first-degree relatives (Rosenberg 1997). The risk of developing AD is greater for individuals with more than one first-degree relative with AD or who have family members with AD onset before the age of seventy (Reiman and Caselli 1999). The prevalence of AD also shows gender difference. Females have a higher risk of development of AD, especially in the elderly population (Azad, Al Bugami et al. 2007).

Current evidence suggests that vascular factors, such as midlife hypertension, diabetes, and cerebrovascular disease, contribute to the pathogenesis and clinical expression of AD (Qiu, Winblad et al. 2005). Interestingly, psychosocial factors such as mental, physical, and social activities have a beneficial effect on cognition and may postpone the onset of AD (Fratiglioni, Paillard-Borg et al. 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to decrease the risk of AD, supporting the involvement of inflammation in AD pathogenesis. Clinical trials with NSAIDs have indicated some level of efficacy in AD patients, and may become an adjunct or combination drug therapy approach during the next decade (Tschape and Hartmann 2006).

1.1.2 Clinical features

AD is characterized by a gradual and continuing decline of cognitive abilities, especially in memory. Memory decline initially manifests as a loss of episodic memory including failure to recall recent events and autobiographical activities (LaFerla, Green et al. 2007). Typically, patients start to lose the ability to express speech, called aphasic symptoms, including disability in naming, with vague and empty speech at the earliest stage of disease, then difficulty recognizing words in the more advanced stages, and inability to speak (mutism) in the most advanced stage. AD also includes impairments in the performance of routine tasks like cooking, dressing, and driving impairments, called apraxic symptoms. Patients lose the inability to recognize objects and familiar faces, called agnosic symptoms; as well as demonstrating impaired executive function included disability in judgment, reasoning, and to plan, execute, monitor, and revise certain activities (McKhann, Drachman et al. 1984).

In addition to these cognitive impairments, most persons with AD develop additional behavioral disturbances, which are often the greatest source of distress in patients and families. These disturbances include theft, unfaithfulness, and the misidentification of familiar people, hallucinations, wandering and pacing, repetitive behaviors, verbal and physical aggression, sleep disturbances, depression, or inappropriate social actions, and declining personal hygiene (American Psychiatric Association 1994).

Late in AD progression, individuals are severely confused, bedridden, incontinent, and unable to feed themselves. The duration of illness commonly lasts about 6–10 years with a high variation between individuals (McKhann, Drachman et al. 1984). In most patients, death is caused by intervening illnesses (e.g. pneumonia and sepsis), behavioral disturbances (e.g.

automobile collisions, falls, and other accidents), or terminal complications (e.g. inanition and aspiration) (Reiman and Caselli 1999).

1.1.3 Diagnosis

Standardized diagnostic criteria for AD were developed in a conference held by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) in 1984 (McKhann, Drachman et al. 1984). These criteria were revised and improved in The Diagnostic and Statistical Manual of Mental Disorders (4th edition) (American Psychiatric Association 1994). Using more standardized criteria, the neuropathological evidence of AD at autopsy showed the accuracy of antemortem diagnosis for AD becomes about 70–96% (Berg and Morris).

The most useful and routine evaluations of AD include information about the onset, progression, and types of symptoms, as well as physical and neurological examinations, cognitive evaluation, and the patients medication history (Reiman and Caselli 1999). Noteworthy, it is important to rule out potentially reversible conditions that could cause or aggravate cognitive impairment, such as drugs (most commonly medications with sedative-hypnotic and anticholinergic effects), chronic meningo-encephalopathy, endocrine disorders (chemistry profiles), infection (complete blood counts and erythrocyte sedimentation rate test), nutritional deficiencies (test serum TSH level and serum B_{12} and folate levels), and severe anemia (complete blood counts) (Berg and Morris); (American Psychiatric Association 1997). Additional procedures used for AD diagnosis include an electrocardiogram, chest X-ray, and urinalysis. Computed tomography (CT) or magnetic resonance imaging (MRI) is routinely indicated to rule out structural abnormalities, such as brain trauma or other diseases induced

local atrophy (Miller, Cummings et al.). Although brain imaging techniques, such as CT, MRI, PET and SPECT showed a significant difference between AD patients and normal controls (Duara; Geaney and Abou-Saleh 1990; Minoshima, Frey et al. 1995; Fox, Freeborough et al. 1996), these techniques are not routinely used to make the clinical diagnosis, since such changes are also observed in patients with other diseases (Duara). Notewoethy, a groundbreaking study at University of Pittsburgh reported that Pittsburgh Compound-B (PiB) specifically binds to beta-amyloid deposits in the brains of living patients with Alzheimer's disease and can be detected by PET. The finding will ultimately aid in the early diagnosis of Alzheimer's, help clinicians monitor the progression of the disease, and further the development of potential treatments.

For patients who satisfy the criteria for AD with onset prior to the age of 60 and with a familial history consistent with autosomal dominant transmission pattern, a positive genetic test for presenilin 1, presenilin 2, and amyloid precursor protein mutations could potentially confirm the diagnosis of AD (Reiman and Caselli 1999). Currently, genetic testing is not recommended to predict the risk of developing AD in a cognitively normal person in order to avoid unnecessary emotional burden.

1.1.4 Neuropathology

The first case of AD, described by Dr. Alois Alzheimer in 1907, was of a female patient suffering from dementia (Alzheimer 1907). Two remarkable pathological alterations were found in this patient's brain using silver stains. Later studies identified that the first lesion was extracellular deposit consisting of aggregates of β-amyloid peptide (Aβ), called amyloid plaques (Masters, Simms et al. 1985). The second lesion was extra- or intra-cellular inclusion called neurofibrillary tangles (NFTs), subsequently shown to be composed of aggregates of the

abnormally hyperphosphorylated microtubule associated protein tau (Grundke-Iqbal, Iqbal et al. 1986). The presence of these two hallmarks during postmortem examination is still required for a neuropathologic diagnosis of AD. Other pathological changes include diffuse plaques, amyloid angiopathy, neuropil threads, Hirano bodies, and granulovacuolar degeneration (Small and Cappai 2006). These changes eventually cause severe synaptic dysfunction and neuronal loss, leading to significant atrophy of the brain of a patient with AD compared to the brain of an age-matched, non-demented individual with normal aging atrophy (LaFerla, Green et al. 2007).

1.1.4.1 Plaques

Plaques are insoluble extracellular deposits primarily composed of aggregated Aβ peptide. Plaques can be described as 'diffuse' or 'senile' (neuritic). The classical senile plaques, also called neuritic plaques, are 50–200 mm spherical structures in diameter, with a dense central core composed of aggregated fibrillar Aβ (Iwatsubo, Odaka et al. 1994), surrounded by dystrophic neurites often containing paired helical filaments and by reactive astrocytes and microglia (Mandybur and Chuirazzi 1990); (Wisniewski and Terry 1976). Neuritic plaques are predominantly distributed in the hippocampus and cortex, especially the third layer of the frontotemporal cortex in AD. Senile plaques are positively stained by hematoxylin and eosin, Congo red, thioflavin S and silver stain. The plaques distribute in increased density in brain regions following AD progression with a big variation among individuals. Surprisingly, plaques are not correlated with the severity of cognitive impairment (Reisberg 1983; Bierer, Hof et al. 1995).

In AD, diffuse plaques, which are far more abundant than senile plaques in most brains, are amorphous, roughly spherical, and contain less dense deposits of Aβ (Tagliavini, Giaccone et

al. 1988) that are not stained by β-sheet specific dyes such as Congo Red and Thioflavin-S. Diffuse plaques do not contain dystrophic neurites or activated microglial cells. They may be often observed in the brain of normal aged individuals. Diffuse plaques can be stained by modified Bielchowsky stain (Suenaga, Hirano et al. 1990) or the silver methenamine method. Separate processes are believed to be responsible for the formation of the dense-core and diffuse plaques (Armstrong 1998; Dickson and Vickers 2001; Wegiel, Bobinski et al. 2001; van Groen, Liu et al. 2003)..

1.1.4.2 Neurofibrillary tangles (NFT)

NFTs are intracellular deposits that consist primarily of the microtubule-associated protein tau within dystrophic neurons (Small and Cappai 2006). NFT could also be extracellular when neurons die and are eliminated leaving only the NFTs, called "ghost tangles" with a shape typically like a flame. During AD, tau is hyperphosphorylated and dissociated from microtubules, spontaneously forms insoluble paired-helical filaments and eventually NFT. Electron microscopy reveals that NFT ultrastructurally are composed of bundles of filament with 10-13 nm in diameter, called paired helix filaments (PHF) with a β-sheet structure (Reisberg 1983). They can be stained by silver and Congo red. Neurofibrillary tangles are primarily found in the cerebral cortex and hippocampus, especially in the hippocampal pyramidal neurons and small pyramidal neurons in the outer laminae of the frontotemporal cortex. They are not observed in the cerebellum, spinal cord, peripheral nervous system or extraneuronal tissues, which are not affected by AD. The density of NFT in specific layers of entorhinal cortex and cerebral cortex is correlated with the severity of memory impairment and dementia of AD patients (Bierer, Hof et al. 1995).

1.1.4.3 Granulovacuolar degeneration bodies (GVD)

GVDs were first described by Simchowicz (Simchowicz, 1911) as a cytoplasmic membrane-bounded intracellular vacuole containing a centrally located argentophilic and hematoxyphilic positive dense granule (Anderton 1997). Histochemical and ultrastructural studies have suggested that GVD may be a special type of autophagosome, a double-membrane vesicle inside the cell, also called autophagic vacuole, which mediates the sequestration of organelles and long-lived proteins (macroautophagy) (Xie and Klionsky 2007). It is most often detected in hippocampal pyramidal neurons. Although GVD has also been described in other neurodegenerative diseases and in aged control subjects, the number of affected neurons is higher in AD (Anderton 1997). Thus, it is considered a marker for AD. Prior immunohistochemical studies have shown that the granules of GVD react with antibodies to phospho-tau, ubiquitin and neurofilaments (Anderton 1997).

1.1.4.4 Hirano bodies

Hirano bodies are rod-shaped structures up to 30 mm long and 8 mm wide that are found in or adjacent to hippocampal pyramidal neurons. They increase in number in aging brains and are also more often observed in AD (Anderton 1997). Fluorescent probe labeling and high voltage electron microscopy indicate that Hirano bodies is composed of F-actin (Galloway, Perry et al. 1987). Cytoskeletal proteins, principally those proteins associated with microfilaments, i.e. tropomyosin, a-actinin and vinculin (Galloway et al. 1987a), tau and neurofilament proteins, have also been reported to stain Hirano bodies (Galloway et al.1987b; Schmidt et al. 1989).

1.1.4.5 Synaptic loss and neuronal cell death

Synaptic dysfunction is considered as one of the earliest manifestations of AD pathology. It is prominent in AD brains and more strongly correlates with cognitive alterations than cell loss or plaque load (Terry 2000). Many mouse models of AD show synapse loss before plaque formation and neuronal loss (Hsia, Masliah et al. 1999; Oddo, Caccamo et al. 2003). Deficits of neurotransmitters, decreased synapse density synaptic contacts (Rutten, Van der Kolk et al. 2005) and altered electrophysiological parameters have been observed in human or transgenic animal brains (Selkoe 2002).

The earliest synaptic dystrophy appears as alterations in the morphology of the individual spines and the density of spines, showing a progressive thinning out of these small structures that normally cover virtually every part of the mature dendritic tree. Irregular swellings are observed in the dendrites especially at branch points and in the cell body itself. Electron microscopic studies indicate that such degeneration change is accompanied by loss of the normal microtubular cytoskeleton, which is progressively replaced by PHF (Scheibel 1979).

Progressive loss of spines and dendritic tree leads to a decreased membrane surface area available for synaptic afferents. Each neuron thereby receives less information, and the processing capability of the areas involved consequently diminish. Such changes have been found in multiple brain areas, causing progressive decline of sensorimotor and cognitive function, resulting in amnesic dysfunction (Reisberg 1983). Because of the redundant nature of brain connections, no obvious change in the individual's mentation or behavior is observed at the early stage of AD (Palop, Chin et al. 2006). However, once a critical point is reached and passed, the loss of cognitive function begins to manifest and will progress until death.

1.1.4.6 Cell loss

The progressive synaptic dystrophy eventually causes neuronal degeneration and cell death that can be mediated by apoptosis or necrosis (Lassmann, Fischer et al. 1993). In the case of pyramidal cells, the classic triangular shape is often replaced by an irregular pear-shaped outline, greater in size than the healthy mature cell. Macroscopically, the brain may appear normal in early stages of AD. Over time the disease causes an atrophy of the hemispheres following the progression of AD. The brain weight in AD patients may be reduced by more than one third compared to the brain of age matched healthy control subjects due to reduction of grey and white matter, although white matter atrophy is believed as a secondary and late event. The grey matter atrophy is typically located at temporal lobes, limbic system including hippocampus and frontal lobes. The ventricle space is widened as a consequence of brain atrophy. However, why AD brain atrophy specifically locates in these areas is yet clear.

1.1.5 Genetics of AD

AD can be divided into two genetic types: familial AD with an autosomal dominant transmission pattern; and sporadic AD, which shows modest familial clustering and probably results from both genetic and environmental factors (Lendon, Ashall et al. 1997). There are no pathological or clinical differences between familial AD and sporadic AD, except that familial AD generally has an earlier onset. Therefore determination of how gene mutations may cause familial AD could be helpful to understand the pathogenesis of both forms. The gene mutations identified in familial AD also provide possibilities to generate transgenic mouse models of AD.

Although only about 10% of AD cases are familial, most understanding of the mechanisms of AD pathogenesis are inspired from the study and gene screen of the genome of

these familial cases. Mutations in three genes have been found to induce the onset of familial AD including β-amyloid precursor protein (APP), presenilin-1 and presenilin-2. Mutations in these genes account for about half of AD cases with dementia onset prior to the age of 60 and autosomal dominant pattern transmission of familial history (Rosenberg 1997). There are several susceptibility genes have been considered as genetic risk factors for AD, including Apolipoprotein ε4 allele, which contributes to up to 50% of familial and sporadic AD cases with dementia onset after the age of 60. One or more genes on chromosome 12 remain to be identified.

1.1.5.1 APP mutations

APP is a widely expressed cell transmembrane type 1 protein, generating Aβ peptide by proteolysis. The gene that encodes APP is located on chromosome 21. The mutations of APP gene account for about 5–10% of familial AD (Goate 1998). Most of them enhance Aβ aggregation or increase Aβ production, especially the 42 amino acid form of Aβ (Aβ42). Mutation of the 670:671 codons on APP gene (lysine to asparagine and methionine to leucine), also called as Swedish mutation, leads to increased cleavage of APP by the β-secretase, also called BACE1 (β -site of APP cleaving enzyme) (Sinha and Lieberburg 1999). Thr714Ile (Austrian) mutation affects γ-secretase cleavage and dramatically increases Aβ42/Aβ40 ratio *in vitro* (Kumar-Singh, Dewachter et al. 2000). Missense mutations of the 717 (valine to isoleucine) also cause increased production of Aβ (Selkoe 1996; Zubenko 1997). Transgenic mice with APP mutations of the 717 and 670:671 codons develop neuritic plaques and cognitive deficits. Additionally, increased dosage of the APP gene also results in AD (Cabrejo, Guyant-Marechal et al. 2006; Rovelet-Lecrux, Hannequin et al. 2006). Mutations within the APP promoter that

increase APP expression have been linked to AD (Selkoe 1996). Similarly, triplication of chromosome 21 in Down syndrome leads to Aβ accumulation earlier than normal aging and typically develop early onset AD (Gyure, Durham et al. 2001; Mori, Spooner et al. 2002). Several mutations within the Aβ sequence of APP have also been identified, such as the Dutch, Flemish, Italian, Arctic and Tottori mutations (Wakutani, Watanabe et al. 2004), although among these, only Tottori mutation cause FAD [Wakutani, 2004 #1]. These mutations generally cause Aβ aggregation or protofibril formation (Demeester, Mertens et al. 2001; Nilsberth, Westlind-Danielsson et al. 2001; Murakami, Irie et al. 2002). Additionally, these mutations also cause Aβ to be more resistant to the degradation by neprilysin-catalyzed proteolysis (Tsubuki, Takaki et al. 2003).

1.1.5.2 Presenilin mutations

The more common gene mutations responsible for the majority of autosomal dominant early onset AD cases are in the presenilin-1 (PS1) gene (Haass 1997; Lendon, Ashall et al. 1997). Mutations of the PS1 gene on chromosome 14 account for more than 50% of early-onset, autosomal dominant AD, and some intermediate onset, autosomal cases (Haltia, Viitanen et al. 1994; Sherrington, Rogaev et al. 1995; Zubenko 1997). Forty-seven PS1 mutations have been described. The gene on chromosome 1 encoding PS2, a homolog of PS1, also have two mutations linked to AD. Presenilin 1 and 2 are membrane proteins that are highly homologous with each other, and mediate the cell surface signaling of a variety of receptors via intramembrane proteolysis (Zubenko 1997). Interestingly, mutations in PS-1 and PS-2, such as the PS1M146V mutation, increase the ratio of Aβ42 to Aβ40 and generate more pathogenic Aβ42 that is more likely aggregated than Aβ40 (Jarrett, Berger et al. 1993; Jarrett, Berger et al.

1993; Younkin 1998). Thus, alterations in the ratio of Aβ42 to Aβ40 appear to be the primary mechanism by which presenilin mutations lead to AD.

1.1.5.3 APOE polymorphisms

The apolipoprotein E (*APOE*) gene is the only gene that has been linked to sporadic AD. Spliced variants of the APOE gene appear to account for about 50% of late onset AD cases (Bullido, Artiga et al. 1998). The gene has three major alleles, ε2, ε3, and ε4. The ε2 allele frequency is lower in AD cases than in controls, indicating that e2 allele is protective; in contrast, the e4 allele is substantially overrepresented among AD patients when compared to control populations (Strittmatter and Roses 1996). The risk for AD within persons with two copies of the ε4 allele increases 3.5 fold compared to persons without ε4 allele (Corder, Saunders et al. 1993). ApoE protein is an apoprotein that regulates cholesterol uptake and release. It is the major lipid carrier in the CNS, synthesized by astrocytes and secreted into the CSF. ApoE3 and apoE2 play critical functions in normal lipid homeostasis, repairing injured neurons, maintaining synaptodendritic connections, and scavenging toxins, but apoE4 does not. ApoE4 protein binds both Aβ and plaques *in vivo.* It may increase Aβ production, increase Aβ deposition in plaques and impair its clearance (Mahley, Weisgraber et al. 2006). People who carry apoE4 have a higher plaque burden. ApoE4 also has toxicity independent on Aβ, such as affecting the phosphorylation and aggregation of tau. Furthermore, a polymorphism in the transcriptional regulatory region of the *APOE* gene was found to increase risk for AD (Bullido, Artiga et al. 1998). The ApoE2 allele may lower risk for AD by binding to tau and preventing hyperphosphorylation of tau during AD (Strittmatter, Weisgraber et al. 1994).

1.1.5.4 Others genetic risk factors of AD

Recently, a new genetic variant of the sortilin-related receptor 1 (SORL1) gene, located on chromosome 11, has been identified in AD (Rogaeva, Meng et al. 2007). SORL1 regulates trafficking of APP from the plasma membrane into recycling endosomes. APP that is not cleaved at the plasma membrane and not diverted into recycling endosomes by SORL1 is internalized into the early/late endosome system 58, where APP can be cleaved and generate Aβ. Genetic variants in SORL1 increase APP accumulation and Aβ-production in endosomes, causing an increased risk for late-onset AD (Rogaeva, Meng et al. 2007). In addition, one or more genes on chromosome 12 have been linked to AD but remain to be identified.

1.1.6 Pathogenic mechanisms of AD

1.1.6.1 DNA damage

In 1987, Robison and colleagues demonstrated that alkylation damage is inefficiently repaired in patients with AD (Robison, Munzer et al. 1987). Since then, a large body of data has been reported that accumulated oxidative DNA damage exists in the cells of patients with AD (Kadioglu, Sardas et al. 2004). Prior studies have shown that there is increased DNA damage and decreased DNA repair in patients with AD. Several DNA base adducts, such as 8-hydroxyguanine (8-OHG), 8-hydroxyadenine (8-OHA), 5-hydroxycytosine (5-OHC), and 5-hydroxyuracil show elevations in late-stage AD brain in both nuclear and mitochondrial DNA (mtDNA) isolated from vulnerable regions of AD brain compared to age-matched normal control subjects. However, Markesbery and coworkers showed that in mild cognitive impairment, the earlier manifestation of AD, these DNA base adducts were elevated, indicating that oxidative DNA damage occurs in early stage of AD, not merely a secondary event (Lovell and Markesbery

2007). Two DNA repair pathways, base excision repair and nonhomologous end joining, are adversely affected in AD (Shackelford 2006; Fishel, Vasko et al. 2007). Base excision repair has evolved to handle the numerous minor alterations often caused by cellular metabolic activity, including spontaneous modification, oxidation and loss of bases. It is particularly important in postmitotic tissues such as brain, where simple base modifications are more likely to occur than is major damage to DNA (Vyjayanti and Rao 2006); (Ren and de Ortiz 2002). Nonhomologous end joining is required when DNA is damaged by ionizing radiation or chemicals and that cause a double-strand break (Dronkert and Kanaar 2001; Thompson and Schild 2002).

1.1.6.2 Cell cycle reentry

The neuronal degeneration of AD occurs in specific regions of the brain, including the hippocampus, frontal cortex and temporal cortex, that function in learning, memory and language. Mature neurons are arrested in the cell cycle G0 phase to permit the ongoing morphological alteration and synaptic remodeling. Thus, downregulation of G0 arrest proteins or induction of G1 entry proteins will result in the unbalance between cell cycle and differentiation control.

Cell cycle progression is controlled by regulating activity of a series of CDKs through the coordinated expression of cyclins and cyclin dependent kinase inhibitors (CDKI) (Bowser and Smith 2002). Cyclin D1 is a critical regulator of the cell cycle transition from G0 to G1 phase. Accumulation of the cyclin D1/cdk4 complex leads to activation of kinases that phosphorylate and inactivate retinoblastoma protein (Rb), releasing its inhibition of E2F and allowing the transcription of E2F-target genes, such as cyclin E, cyclin A and cdc2. Cyclin E forms a complex with cdc2 and promotes G1 to S phase transition. Both cyclin E and cdc2 have been shown to be upregulated in cell culture models of neuronal cell death (Yu, Caltagarone et al. 2005). The

activity of cyclin D1/cdk4 complex and cyclin E/cdc2 complex is regulated by CDKI, p27 and p21, respectively. Increased levels of p27 and phosphorylated p27 (Thr187) are present in the cytoplasm of vulnerable neuronal populations in AD brain (Ogawa, Lee et al. 2003). Phosphorylated p27 (Thr187) colocalizes with dystrophic neurites, neurofibrillary tangles and neuropil threads.

In AD, cyclin D and cdk4 show aberrant expression in the hippocampus and subiculum, but not in the cerebellum (Busser, Geldmacher et al. 1998; Jordan-Sciutto, Malaiyandi et al. 2002). Prior study in our lab demonstrated an increased phosphorylated Rb protein (ppRb) and E2F1 immunoreactivity in AD brain compared to control brain, and ppRb was predominantly localized within the nucleus while E2F1 was in the cytoplasm (Jordan-Sciutto, Malaiyandi et al. 2002). Both ppRb and E2F1 were found in the cells surrounding a subset of Aβ–containing plaques. *In vitro* cell models exhibit similar ppRb and E2F1 distributions in PC12 cells upon Aβ treatment (Jordan-Sciutto, Rhodes et al. 2001). Increased levels of Cyclin D1, cyclin E and ppRb have been demonstrated in the early stage of AD, however, their levels are decreased by late stage AD (Hoozemans, Stieler et al. 2006).

1.1.6.3 Mitochondria

Mitochondrial function declines with age. Lower energy production could compromise the ability to maintain cellular homeostasis in response to toxic insult. Impaired energy metabolism might promote neuronal cell death and contribute to age-related diseases (Mattson, Pedersen et al. 1999). Significant decreases in glucose uptake and utilization, as well as reductions in the activities of certain mitochondrial enzymes were observed in AD patients (Beal

1995; Yankner 1996). These changes could be due to accumulation of Aβ in mitochondria (Manczak, Anekonda et al. 2006).

Decreasing the metabolic rates of cultured cells enhances the Aβ generation from APP metabolism, which requires γ-secretase. An active γ-secretase complex consist of presenilin, nicastrin, APH-1 and PEN-2 has been located in the mitochondria (Hansson, Frykman et al. 2004). Aβ can inhibit mitochondrial function at micromolar concentrations in cultured cells (Shearman, Ragan et al. 1994). Progressive accumulation of intracellular Aβ in mitochondria might cause diminished enzymatic activity of respiratory chain complexes III and IV, and a reduced rate of oxygen consumption (Caspersen, Wang et al. 2005). Aβ also could lead to damage of the mitochondrial genome. In AD, a threefold elevation in damage to mitochondrial DNA has been reported (Wang, Xiong et al. 2005). The mutations of two mitochondrial genome genes encoding tRNAGln and cytochrome *c* oxidase have been identified in AD with a higher frequency than in controls (Grundke-Iqbal, Iqbal et al. 1989; Mori, Spooner et al. 2002).

1.1.6.4 Oxidative stress and excitoxicity

Oxidative damage increases with age, and markers of oxidation are further elevated in the AD brain, particularly in affected cortical regions (Mattson 1994; Beal 1995; Yankner 1996). Oxidative stress could result from impaired mitochondrial function, because lower respiration efficiency could lead to increased reactive oxygen species as free electrons are donated to molecular oxygen (Yankner 1996). Alternatively, oxidation could result from microglial release of reactive oxygen species or from excitotoxic-like stress. Both Aβ and tau undergo oxidation in AD, which makes both proteins more prone to aggregate *in vitro* (Mattson 1994; Beal 1995; Yankner 1996). Thus, it is plausible that oxidative damage accumulates normally with age and

promotes the initiation of AD. The lipophilic antioxidant a-tocopherol (vitamin E) has some palliative effects on AD symptoms (Sano, Ernesto et al. 1997).

1.1.6.5 Inflammation

The two primary mediators of inflammation in the CNS, astrocytes and microglia, are extensively activated in the AD brain. A large number of inflammatory mediators and components of the complement cascade are elevated in the AD brain and co-localize with both plaques and tangles. Activated inflammatory response localizes around dense core amyloid plaques, but much less extensive around diffuse plaques (Rogers, Webster et al. 1996; McRae, Dahlstrom et al. 1997). The fibrilar state of Aβ appears to be important for microglial activation, since the neuroinflammatory response and the microglial activation in AD are observed in late AD stage (Braak IV/V) when fibrillar Aβ begins to be present. Also, Aβ induced microglial activation is reduced by β-sheet breakers like minocyclin and tetracyclin (Takata, Kitamura et al. 2003; Familian, Boshuizen et al. 2006). Increased levels of cytokines, complement proteins and acute phase proteins can be detected in Aβ plaques (Akiyama, Barger et al. 2000). It is generally believed that these inflammatory proteins are synthesized locally in the brain, since there is no apparent influx of leukocytes and immunoglobulin from blood vessels (Eikelenboom, Zhan et al. 1994). Microglia function as macrophages in the brain, involved in the maintenance of homeostasis but they are rapidly activated in response to neuronal damage and stress (Ling and Wong 1993). In AD brain, one potential function of microglia is to be recruited and activated to remove the Aβ, which is supported by the *in vitro* study in cultured microglia (Paresce, Chung et al. 1997). Aβ recruits microglia along a concentration gradient in culture, and microglia can phagocytose Aβ. However, at the same time, fibrillar Aβ is a substrate for microglial

immobilization via binding with the scavenger receptor, a microglial cell-surface receptor. Thus, deposition of Aβ could lead to the activation, recruitment and immobilization of microglia. Aβ induces microglial proliferation and increases expression and release of neurotoxic substances, including reactive oxygen species, pro-inflammatory cytokines, excitotoxins, and proteases that participate in neuronal cell death. In addition, Aβ also can induce an inflammatory response in the brain by directly initiating the complement cascade via binding complement factor C1q. Therefore, the role of inflammation in neurodegenerative disorders has been called a "double-edged sword" (Wyss-Coray and Mucke 2002). Several epidemiological studies and one limited clinical trial have demonstrated that chronic use of nonsteroidal anti-inflammatory drugs reduces the risk of AD, supporting the notion that inflammation is associated with AD pathogenesis (Rogers, Webster et al. 1996). Histocompatibility antigens were found to be associated with altered age of onset or risk for sporadic AD (Curran, Middleton et al. 1997; Payami, Schellenberg et al. 1997), raising the possibility that genetic factors that alter the capacity of inflammatory response could influence AD risk.

In summary, the pathogenic mechanisms of AD are complicated. None of them alone is sufficient to lead to the disease. They may play a role in scenario and cause serial insults. The "two-hit hypothesis" has been proposed that at the beginning, neurons recruit permanent compensatory changes to against the first insult, and keep themselves still functioning normally or in a slightly compromised status; however, such neuronal compensatory potential will be depleted by further insults, and eventually leading to neuronal cell death (Zhu, Lee et al. 2007).

1.1.7 Current therapeutics and drug targets

Reductions in multiple neurotransmitter systems have been found in AD, including acetylcholine, serotonin, norepinephrine, dopamine, glutamate, GABA, somatostatin, and corticotropin-releasing factor (Zubenko 1997). Currently approved drugs for the treatment of AD in US include two classes of drugs, the acetyl cholinesterase inhibitors tacrine, donepizil, rivastigmine, and galantamine, which interfere with the degradation of acetylcholine as well as the N-methyl-D-aspartate receptor antagonist memantine (Findeis 2007). However, the effects of these treatments are relatively modest. They enhance the remaining cognitive function of patients, but cannot provide significant delay of disease progression, possibly because neither class of drug actually targets the underlying pathogenic mechanism of AD.

Based on the studies of APP/Aβ pathways, experimental therapies have been developed to prevent the production of Aβ, the formation of toxic forms of Aβ, and prevent the toxic effects of Aβ. Antibodies raised against Aβ peptide bind to the *in vitro*-formed beta-amyloid assemblies, leading to disaggregation of the fibrils and inhibition of the neurotoxic effects of beta-amyloid on PC12 cells (Solomon, Koppel et al. 1997). Immunotherapy approaches have been well studied based on active or passive vaccination with Aβ. Active vaccination with Aβ in transgenic mice essentially prevented the development of beta-amyloid-plaque formation and markedly reduced the extent and progression of AD neuropathology (Schenk, Barbour et al. 1999; Morgan, Diamond et al. 2000), raising the possibility of immunization with amyloid-beta in preventing and treating AD. These effects include restoring cognitive deficits which also have been observed by the direct administration of antibodies against Aβ during subsequent experiments (i.e. passive vaccination) (DeMattos, Bales et al. 2001; Dodart, Bales et al. 2002). Because of

robust effects in animal models, this approach has entered clinical trials. Unfortunately, this first trial was halted because of a small but significant occurrence of meningoencephalitis (Orgogozo, Gilman et al. 2003). This raised concerns about the safety of immunotherapy approaches. However, efforts continue with immunotherapy approaches. Recent approaches include the use of premanufactured antibodies which might provide more uniform results across patients by avoiding the variability of response to a vaccine (Findeis 2007).

Another well established approach is developing antagonists of Aβ aggregation. Numerous studies demonstrated a range of compounds that inhibit the transition of monomeric Aβ to toxic oligomers and/or polymers. Some of these compounds have entered clinical trials, such as 3-aminopropane-1-sulfonate also named Alzhemed™ (Aisen 2005), the peptidic compound Apan™ at Praecis Pharmaceuticals (Findeis 2002), and scyllo-inositol at Elan Pharmaceuticals (McLaurin, Golomb et al. 2000).

The earliest inhibitors of Aβ production were discovered using cell-based pharmacological screens before the pathways for Aβ generation through APP processing were understood. Interestingly, while β-secretase is considered as the rate-limiting protease for Aβ production (Lin, Koelsch et al. 2000), these pharmacological screens showed the inhibitors of γ-secretase uniformly reduced Aβ production. Compounds that selectively inhibit APP processing or selectively lower the level of Aβ42 are particularly useful in order to avoid side effects induced by inhibiting other essential substrates of γ-secretase, including Notch (Findeis 2007).

In addition to preventing Aβ production and toxicity, experimental therapies have been developed based on current understanding of tauopathy in AD, mainly through inhibiting tau phosphorylation, such as glycogen synthase kinase-3 (GSK-3) inhibitor, Lithium, or inhibitors for other signal pathways that contribute to tau phosphorylation; or through enhancing tau

dephosphorylation by inhibiting PP2A's regulators to increase the activity of PP2A, the main phosphatase of tau.

Table 1 Current and experimental therapies of AD. *The most up-to-date information on clinical trials and ultimate FDA approval for AD therapy was obtained from www.clinicaltrials.gov*

Mechanism	**Product**	**Company**	**Clinical stage**
Acetyl cholinesterase inhibitors	tacrine		approved
	donepizil		approved
	rivastigmine		approved
	galantamine		approved
NMDA receptor antagonist	Memantine (Ebixa™, Namenda™)	Merz	approved
Nicotinic agonists	AZD3480 (ispronicline, TC-1734)	AstraZeneca Pharmaceuticals LP	Phase II/IIa/IIb
Increase the amount of choline	MKC-231	Mitsubishi Tanabe/Targacept	Phase II/IIa/IIb
Partial 5-HT4 receptor agonist	PRX-0314	EPIX	Phase I
GABAB receptor antagonist	SGS-742	Novartis/ Saegis	Phase II/IIa/IIb
Passive immunization against Aβ	bapineuzumab	Wyeth/Elan	Phase III
	humanised m266	Lilly	Phase I
	AAB-002	Wyeth/Elan	Phase 0
	RN1219	Pfizer/Rinat	Phase I
Active immunization against Aβ	anti-Aβ fragment	Novartis/Cytos	Phase I
	ACC-001	Wyeth/Elan	Phase I
Antagonists of Aβ aggregation	Alzhemed™	Neurochem	Phase III
	Apan™	Praecis Pharmaceuticals	
	scyllo-inositol	Elan Pharmaceuticals	
Inhibits oligomer formation	PBT2	Prana Biotech	Phase II/IIa/IIb
γ-Secretase modulators	Flurizan	Myriad Genetics	Phase III
	E2012	Eisai	Phase I
	Docosahexanoic acid (DHA)	Martek Biosciences Corporation	Phase III

γ-Secretase inhibitors	LY450139	Lilly	Phase II
	GSI-953	Wyeth	Phase I
GSK-3β inhibitor	Lithium		approved
Other:			
Anti-inflammatory	Rofecoxib (Vioxx™)	Merck	Phase III
	Naproxen (Aleve™, Anaprox™, Naprosyn™)	Proctor & Gamble, Roche, Syntex Roche Ireland	Phase III
	Ibuprofen (Advil™, Motrin™, Nuprin™)		
	Dapsone (Avlosulfon)	Immune Network Research Ltd.	
High affinity HMG-CoA reductase inhibitor	Simvastatin (Zocor®)	Merck	
increasing insulin sensitivity	Rosiglitazone	GlaxoSmithKline	
Nerve Growth Factor	Nerve Growth Factor Therapy		
Neotrophic Agents	NeoTrofin	NeoTherapeutics, Inc.	
hormone	Estrogen (Premarin™)		
cerebrospinal fluid shunt	COGNIShunt™		
Lowering cholesterol	Atorvastatin (LipitorTM)	Pfizer	

1.2 β-AMYLOID PEPTIDE IN AD PATHOGENESIS

1.2.1 Metabolism, assembly and clearance of β-amyloid peptide

Recently, the research about mild cognitive impairment, considered as a transition state from "normal brain aging" to AD has gained increased attention. It appears to be a continuous process caused primarily by aging-dependent accumulation of Aβ in the brain. Therefore,

understanding the metabolism and clearance of Aβ and manipulating the Aβ levels in the brain, might be able to prevent and cure the disease, or at least attenuate the speed of brain aging. Under normal conditions, Aβ is constantly produced from its precursor and immediately catabolized, whereas upon aging and pathological situations, the metabolism of Aβ is dysregulated and leads to pathological deposition.

1.2.1.1 Biogenesis of β-amyloid peptide

Aβ is produced by endoproteolysis of the amyloid precursor protein (APP) through the sequential cleavage of APP by groups of enzymes termed α-, β- and γ-secretases. Three enzymes with α-secretase activity have been identified, ADAM9 (Wyss-Coray and Mucke 2002), ADAM10 (Markesbery 1997) and ADAM17 (Steiner, Winkler et al. 2002), all belonging to the ADAM (A Disintegrin And Metalloproteinase protein), a family of peptidase proteins (Allinson, Parkin et al. 2003). β-site APP-cleaving enzyme 1 (BACE1) has been identified as the β-secretase, which is a type I integral membrane protein belonging to the pepsin family of aspartyl proteases (Hussain, Powell et al. 1999; Sinha, Anderson et al. 1999; Vassar, Bennett et al. 1999). The γ-secretase has been identified as a complex of enzymes composed of presenilin 1 or 2, (PS1 and PS2), nicastrin, anterior pharynx defective and presenilin enhancer 2 (Wolfe, Xia et al. 1999; Yu, Nishimura et al. 2000; Levitan, Lee et al. 2001; Francis, McGrath et al. 2002; Steiner, Winkler et al. 2002). APP is type I transmembrane protein, with its N-terminus outside of the membrane and the C-terminus inside. The proteolysis of APP shares common characteristics with other type I transmembrane proteins, like Notch. First, APP is cleaved by the α-secretase or β-secretase at a position 83 or 99 amino acids from the C- terminus respectively, producing a large N-terminal domain (sAPPα or sAPPβ) secreted into the extracellular space (Kojro and

Fahrenholz 2005). The remaining C-terminal fragment is retained in the membrane and subsequently cleaved by γ-secretase, producing an intracellular domain of APP (AICD) (Pinnix, Musunuru et al. 2001). Cleavage of APP by α-secretase occurs within the Aβ region, thereby inhibiting generation of Aβ. Alternatively, the amyloidogenic pathway, β-secretase mediated APP cleavage leads to Aβ generation. β-secretase is described as the rate-limiting protease for the production of Aβ. Subsequent cleavage by the γ-secretase releases an intact Aβ peptide with a length of 40 or 42 amino acids. The majority of Aβ peptide is Aβ40, whereas Aβ42 usually only accounts for about 10% of total Aβ. However, Aβ42 is the predominant isoform found in cerebral plaques (Jarrett, Berger et al. 1993; Younkin 1998), probably because it is more hydrophobic and more prone to fibril formation than Aβ40 (Jarrett, Berger et al. 1993). Mutations in genes known to cause autosomal dominant AD, APP, PS1 and PS2, generally manifest by altering the metabolism or enhance stability of Aβ. These genetic mutations have been used to generate transgenic mouse models of AD.

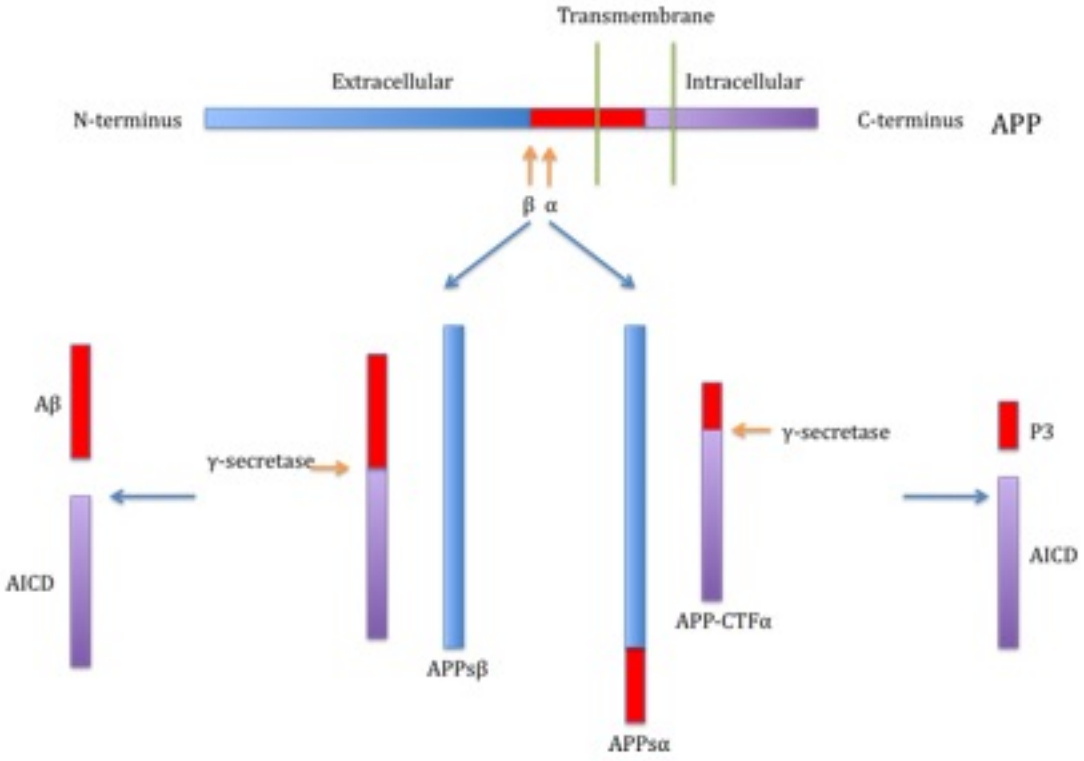


Figure 1 Aβ is generated by APP processing.

Aβ is produced by endoproteolysis of the amyloid precursor protein (APP) through the sequential cleavage of APP by groups of enzymes termed α-, β- and γ-secretases. APP is type I transmembrane protein, with its N-terminus outside of the plasma membrane and C-terminus inside. First, APP is cleaved by the α-secretase or β-secretase at a position 83 or 99 amino acids from the C- terminus respectively, producing a large N-terminal domain (sAPPα or sAPPβ, blue) secreted into the extracellular space (Kojro and Fahrenholz 2005). The remaining C-terminal fragment is retained in the membrane and subsequently cleaved by the γ-secretase, producing an intracellular domain of APP (AICD, purple) (Haass, Hung et al. 1993). Cleavage of APP by α-secretase occurs within the Aβ region (red), thereby inhibiting generation of Aβ. Alternatively, the amyloidogenic pathway, β-secretase mediated APP cleavage leads to Aβ generation. Subsequent cleavage by the γ-secretase releases an intact Aβ peptide with a length of 40 or 42 amino acids.

1.2.1.2 Catabolism and clearance of β-amyloid peptide

Under normal conditions, Aβ is constantly generated, but it can either be catalyzed or transported out of the brain rapidly before it can be deposited. Although all the gene mutations found in familial AD cause an increased production of Aβ42, there is little evidence supporting the up-regulation of Aβ generation upon aging prior to Aβ deposition in the brain (Lemere, Lopera et al. 1996). Thus, down-regulation of Aβ clearance might be the primary cause for sporadic AD.

While the enzymes and pathways contributing to the production of Aβ are well studied, much less is known regarding Aβ catabolism. Biochemical and molecular biological approaches predicted that neprilysin is likely to be the primary Aβ degrading enzyme in brain (Takaki, Iwata et al. 2000; Shirotani, Tsubuki et al. 2001). Neprilysin is a member of neutral endopeptidase family. It belongs to the type II transmembrane protein family with C-terminus containing the active site facing the extracellular side of membranes (Roques, Noble et al. 1993; Turner, Isaac et al. 2001). This topology is suited for the degradation of extracytoplasmic peptides such as Aβ. After synthesis in the soma, neprilysin is axonally transported to presynaptic terminals, where it likely degrades Aβ (Fukami, Watanabe et al. 2002).

The ability to degrade Aβ was significantly reduced by about 50% in neprilysin knockout mouse brains (Saido and Iwata 2006). Neprilysin mRNA levels have been shown to be significantly reduced in the specific areas of hippocampus vulnerable to AD pathology in sporadic AD patients at early stage of AD compared to age-matched normal controls (Yasojima, Akiyama et al. 2001; Yasojima, McGeer et al. 2001). Protein and activity levels of neprilysin are also down-regulated in animal models (Yasojima, Akiyama et al. 2001; Yasojima, McGeer et al. 2001) (Iwata, Takaki et al. 2002). This evidence supports a close association of neprilysin with

the pathogenesis of AD. Single nucleotide polymorphisms of human neprilysin gene have been found, however their relationship with sporadic AD is not clear (Iwata, Higuchi et al. 2005).

Fortunately, the neprilysin knockout mice show normal characteristics in reproduction, development and adult anatomy (Lu, Gerard et al. 1995), because of the redundancy in the neutral endopeptidase family (Kiryu-Seo, Sasaki et al. 2000; Turner, Isaac et al. 2001). Recently, somatostatin, which binds with a G-protein-coupled receptor, has been shown to increase brain neprilysin activity by altering neprilysin expression and localization, therefore resulting in a more efficient Aβ42 degradation, indicating a new pharmacological target for regulating Aβ levels in the brain (Saido and Iwata 2006).

In addition to neprilysin, there are a number of other Aβ-degrading enzyme candidates, including endothelin-converting enzymes and insulin-degrading enzyme (Saido and Iwata 2006).

1.2.1.3 Assembly of β-amyloid peptide

Aggregates of Aβ fibril are the main component of senile plaques in AD brain. *In vitro* studies such as electron microscopy and time-lapse atomic force microscopy analyses have shown that a number of intermediates are involved in Aβ fibril assembly before the formation of mature Aβ fibril, including oligomers and protofibrils. Mature Aβ fibrils are observed in the electron microscope as 6- to 10-nm diameter fibrils with cross-β core structure (Kirschner, Abraham et al. 1986), which commonly appear long, straight, and unbranched. In contrast, protofibrils are much shorter, more flexible and narrow (5 nm) assemblies with a beaded morphology (Teplow, Lazo et al. 2006). Oligomers are different sizes of Aβ assemblies, co-existing with monomers and larger aggregates such as protofibrils and fibrils during the formation of fibrils (Urbanc, Cruz et al. 2006). Ross and Poirier defined these Aβ aggregation

forms: “oligomeric aggregates are small globular assemblies, protofibrils are soluble fibril-shaped structures, thinner and shorter than a mature fibril and fibrillar aggregates are stable, insoluble and highly structured aggregates” (Ross and Poirier 2005).

Fibrillization of Aβ proceeds in two phases: a rate-limiting nucleation step in which a “seed” for further aggregation is formed, followed by an extension phase in which the addition of monomers bind to the ends of the fibrils (Harper and Lansbury 1997; Teplow 1998). *In vitro* biophysical studies have revealed that Aβ42 nucleates easier than Aβ40 (Harper and Lansbury 1997), and thus forms fibrils with a significantly higher rates than Aβ40. Aβ42 fibrils are more toxic than that of Aβ40 (Teplow, Lazo et al. 2006). Transgenic mice overexpressing high levels of Aβ42 without APP expression exhibit massive amyloid pathology, in contrast to transgenic Aβ40 mice (McGowan, Pickford et al. 2005), suggesting the importance of Aβ42 in Aβ fibril formation. Interestingly, the Arctic APP mutation (E693G) is the only mutation in the Aβ peptide that can cause a classical AD phenotype by itself alone, through accelerating protofibril formation, not by increasing fibrillization (Nilsberth, Westlind-Danielsson et al. 2001; Lashuel, Hartley et al. 2003).

Transgenic mice expressing Aβ exhibit neurological deficits prior to amyloid deposition, suggesting that “soluble” Aβ assemblies were neurotoxic (Teplow, Lazo et al. 2006). Both *in vitro* and *in vivo* evidence has implicated the soluble Aβ oligomers as the predominant neurotoxic species (Glabe 2005). Oligomeric forms of Aβ were detected in the brain and cerebrospinal fluid of AD patients, and the levels of one type of oligomer, termed Aβ-derived diffusible ligands, are dramatically higher in AD patients compared to age-matched controls, supporting the idea that Aβ oligomers is the predominant neurotoxic species in AD brain (Klein, Stine et al. 2004).

1.2.2 Aβ peptide is considered as a causative factor for AD pathogenesis

Overwhelming evidence suggests that the major causative factor of AD is abnormal processing of APP by β-secretase and γ –secretase, causing a increased production of Aβ. In addition, an impaired Aβ clearance leads to the accumulation of toxic aggregates (Hardy and Selkoe 2002).

1.2.2.1 Aβ cascade hypothesis

The most commonly accepted theory of AD pathogenesis is that Aβ plays a critical role in the development of AD by initiating a cascade of molecular events that ultimately leads to neuropathology changes and clinical symptoms (Selkoe 1991; Hardy and Higgins 1992). The cascade begins with the cleavage of APP into Aβ40 or Aβ42 (Zubenko 1997), which aggregate into insoluble fibrils and therefore result in the loss of synapses and neurons through multiple mechanisms as mentioned before in section 1.1.6, such as excitotoxicity, cytoskeletal abnormality and inflammation (Zubenko 1997). In sporadic late-onset AD, increased production and a reduction of Aβ clearance causes an age-dependent accumulation of Aβ, coupled to an age-dependent reduced cellular protective ability, eventually leading to the development of AD (American Psychiatric Association 1994).

1.2.2.2 Intra- versus extra- Aβ

Aβ is produced intracellularly in multiple subcellular compartments, including the endoplasmic reticulum (ER), Golgi and lysosomes. It is secreted into the extracellular space and ultimately deposited and forms plaques in the extracellular space in AD brain. There exists a

balance between extra- and intracellular Aβ pools. Strong evidence suggests that extracellular Aβ is toxic for cells *in vitro* (Cuello 2005). Recently, intracellular Aβ has been paid increased attention and is currently considered as an early event in AD pathogenesis before extracellular Aβ deposition (Wirths, Multhaup et al. 2004). A transgenic mouse model carrying four familial AD mutations produces very high levels of Aβ and exhibits intraneuronal rather than extracellular Aβ aggregates (Casas, Sergeant et al. 2004). Interestingly, these mice exhibit more severe neuronal loss than models with predominantly extracellular deposits, suggesting that intracellular Aβ is more toxic compared to extracellular Aβ.

Intracellular Aβ42 is predominantly accumulated in multivesicular bodies (MVBs) of neurons in AD brain, where it is associated with synaptic pathology and causes cognitive deficits (Takahashi, Milner et al. 2002). Electrophysiological studies indicate that intraneuronal Aβ led to a profound deficit in long term potentiation (LTP), a form of synaptic plasticity underlying learning and memory (Morris, Anderson et al. 1986). The triple transgenic AD mouse model develops intraneuronal accumulation of Aβ at the time that cognitive deficits are first detected (Billings, Oddo et al. 2005). The removal of intraneuronal Aβ with immunotherapy restores cognition to similar levels of control non-transgenic mice (Billings, Oddo et al. 2005).

1.2.2.3 The molecular mechanisms of Aβ toxicity

One mechanism whereby Aβ contributes to neuronal cell death is activation of the unfolded protein response (UPR) by disturbing Ca^{2+} homeostasis. Aβ induces release of Ca^{2+} from the endoplasmic reticulum (Demuro, Mina et al. 2005), which may severely impair the function of the ER in protein synthesis and folding. Misfolded proteins in the ER trigger the UPR, a cellular stress response that intends to protect the cell against the toxicity of misfolded

proteins (Forman, Lee et al. 2003; Rutkowski and Kaufman 2004). Immunohistochemistry for phosphorylated pancreatic ER kinase (pPERK) and phosphorylated eukaryotic initiation factor 2a (p-eIF2a), a substrate for PERK, indicates that the activation of the UPR occurs only in AD neurons and not in non-demented control cases (Hoozemans, Veerhuis et al. 2005). Data from Scheper's lab show that oligomeric Aβ, but not fibrillar Aβ induces ER stress at a relatively early stage of AD progression (Hoozemans, Chafekar et al. 2006).

Prior study suggests that Aβ triggers apoptotic death in neurons by altering the balance of proapoptotic and antiapoptotic proteins (Caricasole, Copani et al. 2003). Aβ increases the expression of the proapoptotic protein bax, and downregulates the antiapoptotic protein bcl2 (Paradis, Douillard et al. 1996; Tamagno, Robino et al. 2003). Tamagno and co-workers showed that Aβ evokes a classical type of neuronal apoptosis including the activation of caspase 3, recruitment of p53, releasing of cytochrome c from the mitochondria into the cytosol and poly-ADP ribose polymerase cleavage (Tamagno, Robino et al. 2003). The neuronal Aβ induced apoptosis involves increased expression of p53, activation of p38MAPK and JNK, phosphorylation and inactivation of bcl2, eventually causing neuronal cell death (Shoji, Iwakami et al. 2000).

Some studies indicate that Aβ induces necrotic cell death although the molecular mechanism is not clear yet. Necrotic neural cell death has been found in AD brains bearing presenilin-1 E280A mutation (Velez-Pardo, Arroyave et al. 2001). When nerve growth factor (NGF) treated PC12 cells were exposed to 20 μM Aβ(25–35), electron microscopy data showed immediate cytosolic vacuolization, Golgi-apparatus breakdown and neurite disintegration followed by a final cell lysis. Furthermore, Aβ induces necrotic cell death and abnormal cell permeability without any DNA damage. All these data suggests a necrotic death rather than an

apoptotic one (Behl, Davis et al. 1994). In addition, the interleukin-1β converting enzyme (ICE) cascade activation and caspase activation contribute to Aβ induced necrotic cell death and AD pathogenesis (Suzuki 1997).

1.2.2.4 Aβ induced synaptic dysfunction

Synaptic dysfunction is considered as one of the first manifestations of AD pathology. It is observed before plaque formation, indicating that this may be induced by non-fibrillar Aβ. long-term potentiation (LTP), a form of synaptic plasticity widely used as a cellular model of learning and memory mechanisms, is inhibited by Aβ, and this may contribute to early synaptic dysfunction in AD (Cullen, Suh et al. 1997; Lambert, Barlow et al. 1998). A large body of convincing evidence showed that highly mobile soluble oligomers of Aβ rapidly and potently inhibit LTP in the hippocampus both *in vivo* and *in vitro* (Walsh and Selkoe 2004). Oligomeric Aβ was shown to colocalize with clusters of PSD-95, a marker for post-synaptic terminals, on dendritic arbors of hippocampal cultures (Koenigsknecht and Landreth 2004). Aβ reduced the levels of proteins involved in synaptic transmission such as PSD-95 and the glutamate receptor subunit GluR1 (Almeida, Tampellini et al. 2005) and activity of proteins involved in dendritic spine dynamics like the kinase PAK and the spine regulatory protein drebrin (Zhao, Ma et al. 2006). Aβ may directly affect the presence of receptors in the synaptic cleft, such as NMDA (*N*-methyl-D-aspartate) receptors through regulating its endocytosis (Snyder, Nong et al. 2005). These changes suppress normal activity-dependent physiological activation of these cellular pathways by synaptically released glutamate acting through NMDA receptor and mGluR5 (metabotropic glutamate receptors, subtype 5) receptor, resulting in inhibition of high-frequency stimulation-induced LTP.

Soluble Aβ oligomers are assumed to be generated by misprocessing under pathological conditions and therefore may not expected to have 'physiological' receptors. However, the high potency of these oligomers to rapidly inhibit LTP induction indicates oligomeric Aβ interacts selectively with specific binding domains of receptors (Rowan, Klyubin et al. 2007). It has been reported that integrins can interact with Aβ (Sabo, Lambert et al. 1995). In neurons, α5β1 integrin or α2β1 integrin binds and mediates Aβ toxicity. The binding is through the amino acid sequence RHD of Aβ, which is structurally close to the general integrin recognition sequence RGD (Ghiso, Rostagno et al. 1992; Sabo, Lambert et al. 1995). Noteworthy, integrin α5β1 does not bind with fibrillar Aβ (Matter, Zhang et al. 1998). It has been demonstrated that integrin α5β1 mediates the internalization and degradation of nonfibrillar Aβ. Therefore it decreases the formation of the insoluble fibrillar Aβ deposits and protects from Aβ toxicity (Matter, Zhang et al. 1998). Grace and co-workers have demonstrated that Aβ-induced dystrophy is mediated by the aberrant activation of focal adhesion (FA) proteins (Grace and Busciglio 2003). FAs are integrin-based structures that mediates cell-substrate adhesion, and APP is colocalized with integrins in neurons, suggesting that APP may bring Aβ fibrils into physical contact with integrin receptors (Grace and Busciglio 2003).

Prior studies suggested that integrin α5 and type-1 tumour necrosis factor α receptor (TNF-R1) are essential for Aβ induced LTP inhibition. Soluble oligomers of Aβ rather than insoluble fibrils bound to TNF-R1 directly through the intracellular death domain of TNF-R1 (Li, Yang et al. 2004). A possible mechanism is that Aβ oligomers bind α5 integrins and trigger either tumour necrosis factor α (TNFα) release or Aβ internalization, leading to TNF-R1 activation. This will activate stress kinases and downstream production of nitric oxide, superoxide and other mediators, resulting in LTP inhibition (Rowan, Klyubin et al. 2007). These

findings support the hypothesis that soluble oligomers of Aβ are important for the synaptic dysfunction and loss in AD, although it cannot exclude the possible contribution of amyloid fibrils in neurodegeneration.

1.3 TAUOPATHY IN AD PATHOGENESIS

1.3.1 Background of tau

Tau is a cytosolic protein predominantly expressed in neurons. It is particularly abundant in the axons of neurons, although it is also associated with the cell membrane (Arrasate, Perez et al. 2000; Maas, Eidenmuller et al. 2000). It promotes microtubule assembly and stabilization (Drubin and Kirschner 1986). Tau plays an important role in neurogenesis, axonal maintenance and axonal transport. Tau is considered as a microtubule-associated protein, since it was isolated from purified microtubules. Specific microtubule-binding domains (MBDs) in tau mediate its binding to microtubules. These domains are three or four repeated sequences of 31 or 32 residues, a highly conserved tubulin binding motif, in the C-terminus of tau (Lee, Cowan et al. 1988; Goedert, Spillantini et al. 1989; Himmler 1989). Around these MBDs, there are two proline-rich regions that affect the ability of tau to bind microtubules if phosphorylated. Serine and threonine residues present in these proline-rich regions are modified by multiple protein kinases.

There are six major isoforms of tau expressed in the adult human brain, derived from a single gene by alternative splicing. The proportion of these tau isoforms and their phosphorylation status differ during development and in adult brains (Goedert, Spillantini et al.

1989; Kosik, Orecchio et al. 1989), such that tau phosphorylation is dramatically reduced in adult brain (Garver, Harris et al. 1994; Matsuo, Shin et al. 1994). Moreover, different neurons express different tau isoforms. These six tau isoforms contain three or four tubulin-binding repeats, and have or lack either one or two 29 amino-acid length inserts at the N−terminus of tau. Each of these six isoforms likely has precise and distinctive physiological roles, although their functions are generally similar.

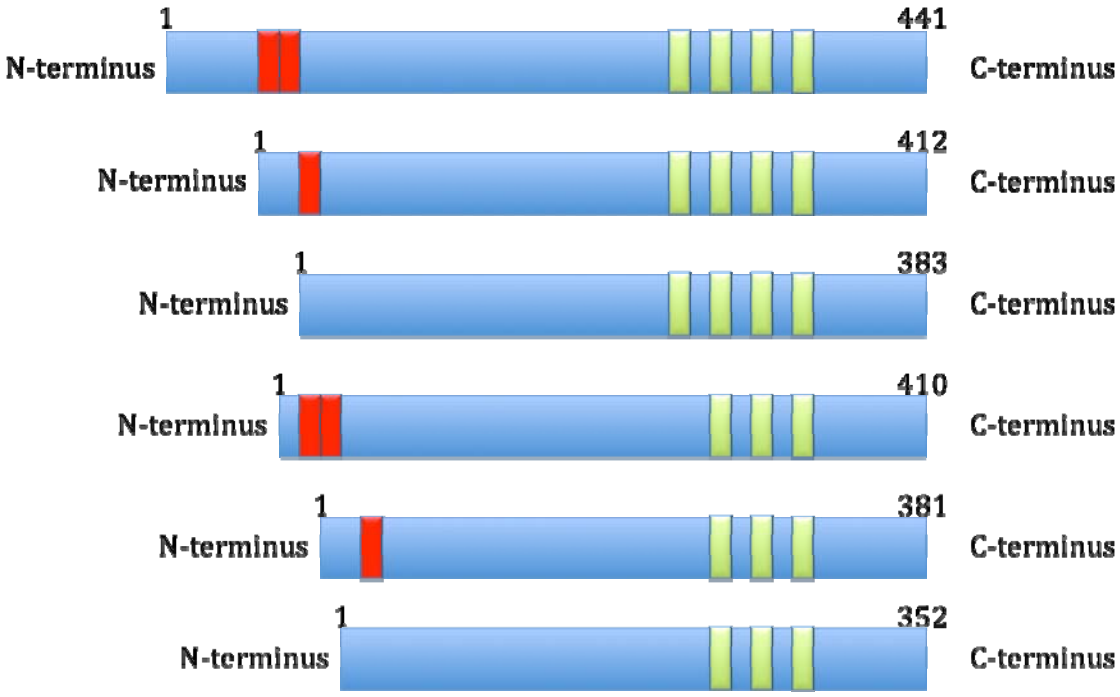


Figure 2 tau isoforms

These six tau isoforms contain three or four tubulin-binding repeats (yellow), and have or lack either one or two 29 amino-acid length inserts (red) at the N-terminus of tau.

The primary function of tau is to stabilize microtubules. Tubulin-binding repeats of tau, the positively charged proline-rich regions, bind tightly to specific pockets in β-tubulin at the negatively charged inner surface of the microtubule (Kar, Fan et al. 2003; Amos 2004).

β-tubulin pockets of adjacent protofilaments may be occupied by the different repeats within the same microtubule binding domain of tau, causing the crosslinking of three or four dimers (Amos 2004) and further stabilization of the microtubule. Tau contributes to the maintenance of an appropriate morphology of neurons and to the regulation of axonal transport, and thus, when abnormal, contributes to synaptic dysfunction and neurodegeneration (Roy, Zhang et al. 2005; Trojanowski, Smith et al. 2005). Under normal physiological conditions, the phosphorylation state of tau, determined by the actions of kinases and phosphatases, keeps tau in a constant dynamic equilibrium, binding (promoted by tau dephosphorylation) and detaching (promoted by tau phosphorylation) from the microtubule. Although tau bound on the microtubule physically perturbs the movement of vesicles and other cargo along the axon, tau is essential to microtubule integrity. Thus, relatively frequent on and off cycles of tau may be required for effective axonal transport. Besides this primary function, prior studies have indicated that tau may also be associated with other structures and enzymes, such as the plasma membrane (Brandt, Leger et al. 1995; Maas, Eidenmuller et al. 2000), RNA (Kampers, Pangalos et al. 1999), presenilin (Takashima, Murayama et al. 1998; Buee, Bussiere et al. 2000) and the src-family tyrosine kinase Fyn (Lee 2005). However the function of these specific tau interactions is not known.

Tau is degraded primarily by the ubiquitin-proteasome system including Hsc70, CHIP and UbcH5B. A significant decrease in proteasome activity was observed in AD patients (Keller, Hanni et al. 2000). Moreover, tau protein present in PHFs is ubiquitinated and immunohistochemistry studies have demonstrated that intracellular neurofibrillar tangles (NFTs) and Pick bodies are CHIP- and ubiquitin-positive (Petrucelli, Dickson et al. 2004), indicating that inhibition of proteasome activity results in tauopathy formation in AD brain. In addition, tau also can be degraded by proteases, such as the calcium-activated protease calpain (Litersky and

Johnson 1995), Cathepsin D and puromycin-sensitive aminopeptidase (PSA) (Karsten, Sang et al. 2006). Tau also can be cleavaged by caspases, generating a truncated tau that is more toxic and prone to aggregation than wild-type tau (Rohn, Rissman et al. 2002; Gamblin, Chen et al. 2003), suggesting that cleavage of tau by caspases in the AD brain may contribute to the formation of NFTs. Finally, phosphorylated tau interacts with HSP-27, which facilitates degradation of pathogenic hyperphosphorylated tau by an unknown mechanism (Kosik and Shimura 2005).

To date, over 30 different gene mutations in tau have been identified in patients with familial frontotemporal dementia with Parkinsonism linked to chromosome-17 (FTDP-17). All cases of FTDP-17 exhibit filamentous inclusions composed of hyperphosphorylated tau. Mutations of the tau gene (*MAPT*) are considered as the causative factor of FTDP-17 (von Bergen, Barghorn et al. 2001; Goedert and Jakes 2005). Importantly, these genetic studies provide solid evidence that tau dysfunction is sufficient to trigger neurodegeneration and dementia even in the absence of other pathogenic insults, such as amyloid plaques. In comparison to wild type tau, the mutant forms are easier to be phosphorylated and less efficiently dephosphorylated. Therefore, they are more prone to fibrillization (Alonso Adel, Mederlyova et al. 2004); or have impaired MT binding properties (Hong, Zhukareva et al. 1998; Dayanandan, Van Slegtenhorst et al. 1999). Furthermore, intronic and coding-region mutations in exon 10 of *MAPT* (N279K, L284L, ΔN296, N296N, N296H, S305N and S305S) alter the alternative splicing of tau, leading to an increased ratio of four-repeat to three-repeat isoforms and perturbing the normal one-to-one ratio of the 3R to 4R tau isoforms. Therefore, an imbalance among tau isoforms is involved in the development of tauopathy.

Approximately 20 years ago tau was identified as the main component of paired helical filaments (PHFs) (Grundke-Iqbal, Iqbal et al. 1986; Kosik, Joachim et al. 1986; Wood, Mirra et al. 1986). Purified tau was able to assemble into fibrillar polymers *in vitro* that resemble PHFs ex vivo (Montejo de Garcini, Serrano et al. 1986). Besides AD, aberrant polymers of phosphorylated tau have also been detected in other neurological diseases, including Pick's disease, frontotemporal dementia linked to chromosome 17 (FTDP-17), corticobasal degeneration, progressive supranuclear palsy, Guam Parkinsonism dementia complex, dementia with argyrophilic grains, Niemann-Pick disease type C and dementia pugilistica (Buee, Bussiere et al. 2000). These diseases are collectively named tauopathies. In AD, approximate 95% of total phosphorylated tau is found in neuronal processes known as neuropil threads or dystrophic neuritis (Mitchell, Nissanov et al. 2000).

1.3.1.1 Tau phosphorylation

Among the multiple post-translation modulations of tau, the most studied is serine/threonine phosphorylation. Over 18% of the residues within the largest isoform of tau, that contains 441 amino acids, are serine and threonine and 79 of these are putative serine or threonine phosphorylation sites (Grundke-Iqbal, Iqbal et al. 1986; Ihara, Nukina et al. 1986; Baudier, Lee et al. 1987). A number of these sites have also been demonstrated to be phosphorylated within NFTs in the AD brain (Lovestone and Reynolds 1997). These sites can be divided into two main groups: those that can be phosphorylated by proline-directed kinases like GSK-3, cyclin dependent kinase 5 (cdk5), p38, JNK or cdc2; and those that can be phosphorylated by non-proline directed kinases like PKA, PKC, CaM kinase II, MARK kinases (Baudier, Lee et al. 1987; Correas, Diaz-Nido et al. 1992; Drewes, Lichtenberg-Kraag et al. 1992; Hanger, Hughes et al. 1992; Scott, Spreen et al. 1993; Goedert, Hasegawa et al. 1997;

Imahori and Uchida 1997; Lucas, Hernandez et al. 2001) or CKII, mainly close to acidic residues in exon 2 and 3 (Correas, Diaz-Nido et al. 1992). Recently, a novel tau-tubulin kinase, member of the casein kinase I superfamily (Sato, Cerny et al. 2006) has been found not only to modify tau residues 198, 199, 28 and 422 but also facilitate tau aggregation. Increased expression or activation of such serine/threonine kinases including GSK-3β, CDK5, Akt, MAPK and JNK have also been detected in AD brains (Baker, Litvan et al. 1999; Houlden, Baker et al. 2001; Jiang, Tang et al. 2003; Evans, Fung et al. 2004; Hernandez, Perez et al. 2004). Of these many kinases, GSK-3β and CDK5 are considered to play primary roles (Glatz, Rujescu et al. 2006), and are therefore referred to as tau kinases-1 and -2, respectively. Multiple signal pathways have been reported to contribute to tau phosphorylation, including Wnt, PDPK-1/p70S6kinase/mTor, PI3kinase/PKB/GSK-3, α7 nicotinic receptor and insulin.

In addition to serine/threonine modifications, phosphorylation at tau tyrosines also has been reported. The nonreceptor tyrosine kinase, Fyn, member of src family, modifies tau at tyrosine 19 at early stages of development (Lee, Thangavel et al. 2004). Missense mutations of tau, present in FTDP-17, could increase the interaction of tau and Fyn (Bhaskar, Yen et al. 2005), facilitating the phosphorylation of tau by Fyn. Most recently, it was reported that not only Fyn can phosphorylate tau, but also c-abl, which modifies tau at tyrosine 394 (Derkinderen, Scales et al. 2005).

1.3.1.2 Tau Dephosphorylation

Compared to tau phosphorylation, far fewer studies have examined the influence of phosphatases on tauopathy (Mawal-Dewan, Henley et al. 1994; Liu, Iqbal et al. 2005). Several phosphatases have been found to dephosphorylate tau protein, such as protein phosphatase (PP) 1, PP2A, PP2B (calcineurin) and PP2C (Goedert, Cohen et al. 1992; Geddes, Hughes et al. 1993;

Szucs, Ledesma et al. 1994; Yamamoto, Hasegawa et al. 1995). PP2A is the phosphatase that acts on most phosphorylation sites (Goedert, Jakes et al. 1995; Gong, Lidsky et al. 2000), thus the importance of PP2A as a major tau phosphatase has been well studied. PP2A interacts with tau directly through its tubulin binding region (Sontag, Nunbhakdi-Craig et al. 1999). Mutations in that region impair the capacity of PP2A to bind to tau, and lead to an increase in tau phosphorylation, which has been observed in some FTDP-17 patients bearing such mutations (Goedert, Satumtira et al. 2000). Interestingly, the mRNA and protein level of PP2A and its activity are decreased in AD brains, suggesting that reduced tau dephosphorylation by PP2A may also contribute to tau pathological lesion in AD (Vogelsberg-Ragaglia, Schuck et al. 2001; Sontag, Luangpirom et al. 2004). Expression of dominant negative PP2A mutant in transgenic mice leads to hyperphosphorylation, ubiquitination and aggregation of tau (Kins, Crameri et al. 2001). However, NFTs failed to develop in these transgenic mice, suggesting that reducing phosphatase activity alone may not be sufficient to induce NFT formation in AD (Kins, Crameri et al. 2001). Other phosphatases including PP2B and PP1 also dephosphorylate abnormally hyperphosphorylated tau isolated from AD brain tissue (Afagh, Cummings et al. 1996; Wyss-Coray and Mucke 2002), however their contribution to tau dephosphorylation within the brain seems to be much less than that of PP2A (Liu, Iqbal et al. 2005). In addition, tau hyperphosphorylation causes conformational changes of tau that inhibit phosphatase function, while such aberrant conformations and inhibited phosphatase activity could be partially reversed by chaperones like Pin-1 (Butterfield, Abdul et al. 2006).

1.3.1.3 Mechanism for pathological tau phosphorylation

The process of tau mediated dynamic microtubule stabilization is tightly regulated during development via phosphorylation (Bramblett, Goedert et al. 1993; Brion, Smith et al. 1993;

Goedert, Jakes et al. 1993). Tau is phosphorylated at multiple sites during embryonic and early postnatal periods, which likely allows for rapid microtubule remodeling, thereby facilitating axonal plasticity required for the establishment or diminishment of neuronal connections. In contrast, in the normal adult brain, tau phosphorylation is markedly reduced. However, phosphoepitopes of tau that only appear during development under normal conditions, reappear in AD abnormally (Lovestone and Reynolds 1997).

Prior studies demonstrated that aggregates of β-amyloid induce tau phosphorylation (Busciglio, Lorenzo et al. 1995) by activating GSK-3β (Takashima, Noguchi et al. 1993) and/or downregulating PP2A activity, and as consequence, phosphorylated tau protein polymerizes into PHF and later on aggregates into NFT. Therefore, the gene encoding PS-1, whose mutations have been identified in FAD, could facilitate tau pathology by enhancing β-amyloid production. On the other hand, PS-1 downregulates tau hyperphosphorylation by inhibiting PKB (Weihl, Ghadge et al. 1999) and activating PI3K, thus inhibiting GSK-3 activity (Baki, Shioi et al. 2004). Mutant PS-1 loses these functions, resulting in tau hyperphosphorylation. Thirdly, PS-1 might contribute to tau hyperphosphorylation by activating tau kinases such as protein kinase C or calmodulin kinases, since PS-1 mutations result in an increase in cytoplasmic calcium by losing the ability to control the calcium leakage from ER. Double transgenic mice carry the same mutant PS1 allele and tau P301L mutation, but no APP_{swe} overexpression, develop the same extent tau pathology as the 3xTg-AD mice with a much slower time frame, indicating the importance of facilitating role of Aβ for tau pathology (Oddo, Caccamo et al. 2003).

In addition, tau hyperphosphorylation in AD can be induced by activation of NMDA receptors that activates ERK/MAPK signal pathways (Li, Sengupta et al. 2004; Amadoro, Ciotti et al. 2006). Changes in the pathways involving Wnt (Jope and Johnson 2004) or insulin (Planel,

Miyasaka et al. 2004) also could result in abnormal tau phosphorylation. Interestingly, tau phosphorylation is modulated by ApoE ε4 isoform, a risk factor of late-onset AD (Schmechel, Saunders et al. 1993; Strittmatter, Saunders et al. 1993), which stimulates phosphatidylinositol 3-kinase and then protein kinase B/GSK-3β (Beffert, Morfini et al. 2002; Ohkubo, Lee et al. 2003) through reelin receptors. In addition to these, tau phosphorylation also can be modulated by hibernation, learning or hormone level, such as the elevation of corticosterone levels in rodents caused by neuronal stress (Hanson, Levin et al. 1997).

During AD, tau may act as a common mediator of neurodegeneration for various upstream pathological events, including Aβ, as well as oxidative stress and inflammation, which trigger or contribute to an abnormal detachment of tau from the microtubule independently or in combination (Rapoport, Dawson et al. 2002; Andersen 2004; Liu, Lee et al. 2005; Moreira, Smith et al. 2005; King, Kan et al. 2006). For example, it has been suggested that oxidative stress could be responsible for detrimental covalent modifications of tau (Horiguchi, Uryu et al. 2003), which are likely to cause misfolding, hyperphosphorylation and aggregation, and thereby contribute to abnormal disengagement of tau from the microtubule, as well as to the formation of tau inclusion.

1.3.1.4 Tauopathy & Aβ

Amyloid plaques and neurofibrillary tangles (NFTs) are the two main pathological lesions of AD brain. Aβ-immunoreactive material was frequently present in NFT-containing neurons (Grundke-Iqbal, Iqbal et al. 1989), indicating that these two pathologies might be linked (Blurton-Jones and Laferla 2006). Tau has been reported to be necessary for Aβ-induced neuronal toxicity and cognitive dysfunction, as neurons harvested from tau knockout mice are

resistant to Aβ-induced death (Rapoport, Dawson et al. 2002), suggesting that either a direct or indirect interaction between Aβ and tau may be important for the development of AD dementia (Roberson, Scearce-Levie et al. 2007). Therefore, exploration of the possible mechanism linking Aβ and tau pathology may greatly improve our understanding of AD. Mounting evidence supports the notion that Aβ may directly or indirectly interact with tau to accelerate NFT formation. Aβ facilitates tau phosphorylation, aggregation, mis-localization, and accumulation by affecting multiple molecular and cellular pathways, including activating specific kinases, triggering inflammatory responses and pro-inflammatory cytokines, inhibiting the proteasome, or indirectly causing axonal transport deficits. These two main pathological changes of AD play a role in a scenario that eventually leads to AD.

Among the potential mechanisms mentioned above, the most important pathway by which Aβ facilitates tau pathology is enhancing tau hyperphosphorylation by activating protein kinases. For example, neuronal exposure to Aβ induces activation of GSK-3β and a corresponding increase in tau phosphorylation *in vitro* (Takashima, Noguchi et al. 1993; Busciglio, Lorenzo et al. 1995; Alvarez, Toro et al. 1999). Furthermore, blocking the two major tau kinases, cdk5 or GSK-3β, or antisense knockdown of GSK, prevent Aβ-induced tau phosphorylation and protect neurons against Aβ, suggesting that tau phosphorylation is critical for Aβ-mediated toxicity (Takashima, Noguchi et al. 1993; Takashima, Honda et al. 1998; Alvarez, Toro et al. 1999). Interestingly, mutating serine-422 to alanine prevents the Aβ-induced aggregation of tau, suggesting that phosphorylation of tau at specific residues is critical (Ferrari, Hoerndli et al. 2003; Pennanen and Gotz 2005). *In vivo* evidence also supports the influence of Aβ on kinase activity and tau phosphorylation and aggregation. For example, mutant APP transgenic mice show an increased activation of tau kinases around Aβ plaques, such as

SAPK/JNK and p38 MAPK (Hwang, Cho et al. 2004; Puig, Gomez-Isla et al. 2004). Surprisingly, APP transgenic mice exhibit phospho-tau containing dystrophic neurites associated with Aβ plaques, but fail to develop significant tau hyperphosphorylation and NFT pathology, which might be caused by either the species differences between mouse and human tau or other critical proteins such as tau kinases, or the limited life-span of mice. In order to better mimic tau pathology, researchers generated double- or triple- transgenic models crossing FTDP-17 linked mutations in tau and other AD related gene mutations like PS1 and APP (Lewis, McGowan et al. 2000; Gotz, Chen et al. 2001; Lewis, Dickson et al. 2001; Oddo, Caccamo et al. 2003). For example, Lewis and colleagues crossed a mutant tau transgenic mouse line with the Tg2576 APPswe transgenic mice. LaFerla's lab developed a triple transgenic model of AD (3xTg-AD) that expresses a mutant PS1M146V allele, the APPswe and TauP301L mutations. These transgenic mice develop both Aβ and NFT-like pathology within AD-relevant brain regions (Oddo, Caccamo et al. 2003), also Aβ accumulation precedes the development of tau pathology suggesting that Aβ could indeed promote and accelerate NFT formation. These animal AD models will be helpful to better understand the mechanisms by which Aβ induces tau hyperphosphorylation and NFT formation.

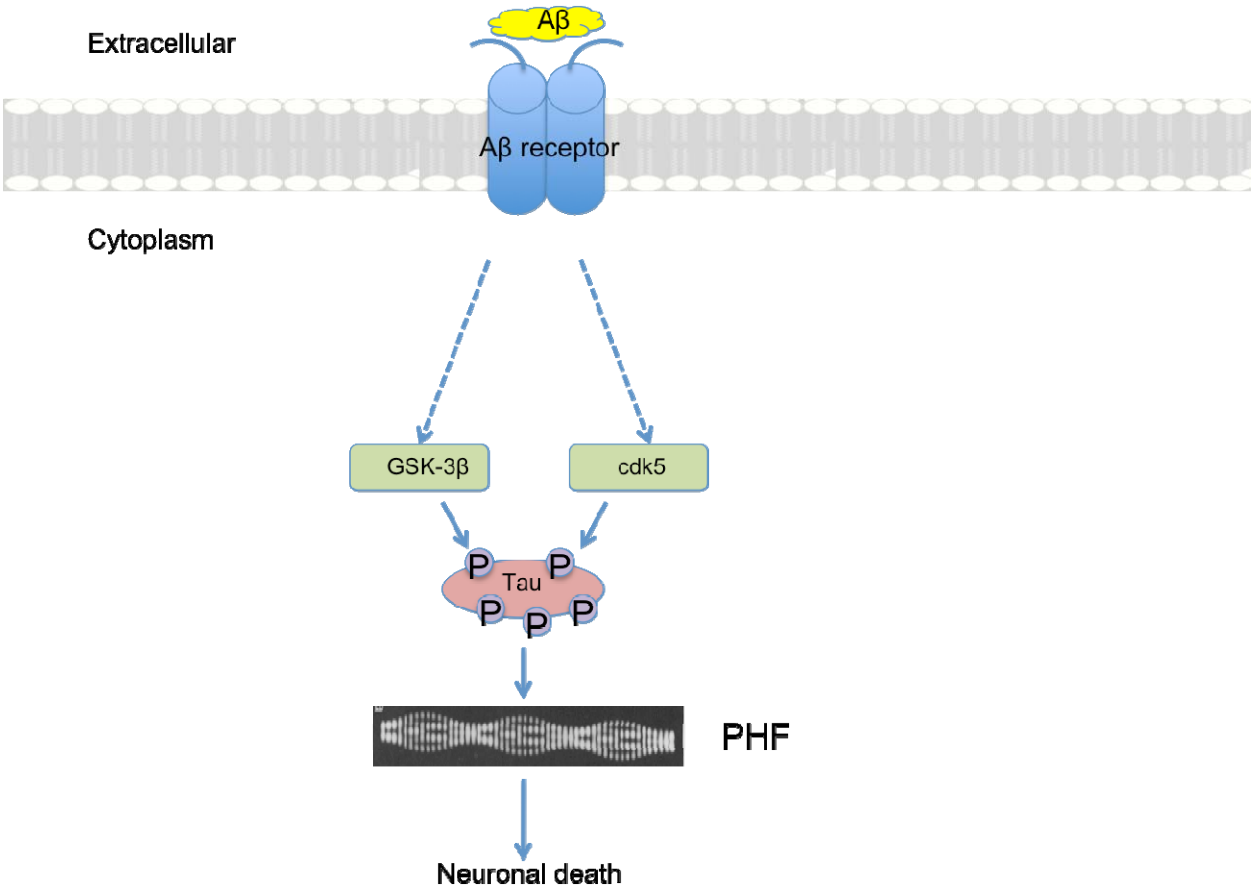


Figure 3 Aβ facilitates tau pathology by activating protein kinases

Extracellular Aβ binds with its receptors, such as integrin, and activates downstream signal pathways, leading to the activation of GSK-3β and cdk5. Those activated protein kinases phosphorylate tau, causing the formation of paired helical filaments (PHFs), and eventually leading to neuronal death.

AD brains exhibit profound activation of inflammation in response to the pathological accumulation of Aβ as well as neuronal and synaptic damage (Akiyama, Barger et al. 2000); (Wyss-Coray and Mucke 2002). Several proinflammatory cytokines and chemokines including IL-1β, IL-6, TNF-β, TGF-β and IL-8 have been reported to be elevated in the AD brain within pathologic regions (Griffin, Stanley et al. 1989; Akiyama, Barger et al. 2000). Also, a number of

activated microglia and astrocytes are observed in or around Aβ plaques in the AD brain (Duffy, Rapport et al. 1980; Griffin, Stanley et al. 1989; Sheng, Mrak et al. 1995). Moreover, *in vitro* experiments demonstrate that fibrillar Aβ can activate microglia, enhance the synthesis and release of proinflammatory cytokines, as well as activate the complement cascade, promoting microglial phagocytic activity (Rogers, Cooper et al. 1992; Jiang, Burdick et al. 1994; Chen, Frederickson et al. 1996). All these findings indicate that aggregated Aβ is sufficient to induce the inflammatory response (Galimberti, Baron et al. 1999; Tan, Town et al. 2000; Combs, Karlo et al. 2001). A large number of studies suggest that proinflammatory cytokines can accelerate tau pathology and NFT formation. For example, IL-1 exacerbates tau phosphorylation and NFT formation via p38-MAPK pathway in both cortical neurons and animal models (Sheng, Zhu et al. 2000; Sheng, Jones et al. 2001; Li, Liu et al. 2003). IL-6 induces tau hyperphosphorylation via enhancing CDK5 activity in rat hippocampal neurons (Quintanilla, Orellana et al. 2004). CDK5 activity also has been demonstrated to be required for lipopolysaccharide (LPS) injection induced brain inflammation and tau hyperphosphorylation in 3xTg-AD mice. Aβ-induced NO release from astrocytes has also been shown to contribute to tau phosphorylation within primary cultured neurons (Saez, Pehar et al. 2004). Therefore, increasing evidence suggests that brain inflammation may play a critical role in mediating the development of tau pathology in response to Aβ.

The ubiquitin proteasome system (UPS) plays an important role in a number of cellular functions by regulating the rapid turnover of proteins (Haglund and Dikic 2005). UPS dysfunction has been reported in many neurodegenerative diseases including Huntington's disease, ALS, Parkinson's disease, prion diseases, and AD. Recent evidence indicates that Aβ induces proteasomal dysfunction in both *in vitro* cell models and transgenic mouse models of

AD (Lopez Salon, Pasquini et al. 2003; Oh, Hong et al. 2005). Scanning transmission electron microscopy showed that Aβ physically interacts with the 20S proteasome within the barrel of proteasomes, suggesting Aβ may inhibit the proteasome directly (Gregori, Hainfeld et al. 1997; Kosik and Shimura 2005). Thus, accumulation of Aβ may inhibit proteasome activity to degrade its substrates such as tau. In AD, the UPS attempts to degrade misfolded and hyperphosphorylated tau (Kosik and Shimura 2005). It has been reported that phosphorylation of tau by GSK-3β and CDK5 triggers the interaction of tau and the chaperone protein Hsc70, which mediates tau degradation by UPS (Shimura, Schwartz et al. 2004). Therefore the UPS dysfunction may contribute to the buildup of tau aggregates. Also, PHFs co-immunoprecipitated with the proteasome in AD brains further supports the role of UPS in tau degradation (Keck, Nitsch et al. 2003). Increased levels of Aβ lead to proteasomal impairment (Keller, Hanni et al. 2000; Oh, Hong et al. 2005) and subsequently inhibit the degradation of misfolded tau, thereby providing an putative mechanism by which Aβ facilitates tau pathologies in AD.

Another cellular mechanism that may link Aβ and tau pathology in AD is axonal transport deficits. Previous studies demonstrated that the precursor protein of Aβ, APP, as well as the protein and mRNA of tau anterogradely move along axons by fast axonal transport (Aronov, Aranda et al. 2001; Aronov, Aranda et al. 2002; Utton, Noble et al. 2005). Impairment of axonal transport will cause altered distribution and production of Aβ and tau (Kamal, Almenar-Queralt et al. 2001; Lazarov, Lee et al. 2002; Stokin, Lillo et al. 2005). Interestingly, both Aβ and tau play a function to regulate axonal transport. Most recently, it has been indicated that Aβ itself can lead to diminished axonal transport also through the activation of GSK-3β (Pigino 2005). Taken together, these studies imply a complex relationship between APP, Aβ and

axonal transport and it remains unclear whether in AD transport deficits lead to increased Aβ generation or whether increased Aβ levels lead to transport deficits, or both.

Therefore, there are two possible hypotheses between Aβ and tau. One is that tau leads to increased Aβ production by impairing axonal transport. However, to date no evidence to show that tau pathology can modulate Aβ accumulation. Prior study demonstrates that overexpression of tau does not increase Aβ production *in vitro* (Goldsbury, Mocanu et al. 2006). Transgenic mice overexpressing wild type or mutant tau have axonal transport deficits (Ishihara, Hong et al. 1999; Zhang, Higuchi et al. 2004), but do not exhibit increased endogenous mouse Aβ. Also, FTDP-17 and other neurodegenerative diseases that exhibit tau accumulation do not show evidence of Aβ accumulation (Hardy, Duff et al. 1998), suggesting that tau-mediated transport deficits do not modulate Aβ. A second alternative hypothesis is that Aβ-induced transport deficits by GSK-3β activation could lead to mislocalization of tau. Most *in vivo* evidence supports the second hypothesis. For example, removal of accumulated Aβ by immunology leads to diminished tau pathology (Oddo, Caccamo et al. 2003; Oddo, Billings et al. 2004). More importantly, all genetic mutations that lead to enhanced Aβ42 production or aggregation also lead to NFT formation, consistent with the hypothesis that Aβ occurs upstream of tau in signaling pathways that contribute to AD. Currently, the majority of evidence suggests that Aβ facilitates NFT pathology via specific kinase activation, inflammatory response induction, proteasome inhibition or axonal transport dysregulation and subsequent mislocalization of tau. Among them, the most important way by which Aβ facilitates tau pathology is via activating protein kinase. GSK-3β and cdk5 have been well demonstrated to mediate this process. Interestingly, recent data indicates that a tyrosine kinase, c-Abl might be also involved in this process.

2.0 INTRODUCTION FOR C-ABL

2.1.1 Background of c-Abl

2.1.1.1 The structure and localization of c-Abl

The c-*abl* gene was originally identified as the cellular homologue of the oncogene of Abelson murine leukaemia virus and to encode a 150 KD non-receptor tyrosine kinase. c-Abl gene has been cloned from human, mouse, Drosophila, and nematode (Van Etten 1999). In mammals, c-Abl has two isoforms, type Ia and Ib for human c-Abl, and type I and type IV for murine c-Abl, generated by two alternative 59 exons with separate promoters. The only difference of these two isoforms is in their N-terminal sequences, where the type 1b/IV isoform of c-Abl contains a myristoylation site, while the 1a/I isoforms lack it (Hoffman-Falk, Einat et al. 1983; Goddard, Weiland et al. 1986) In vertebrates, there is only one homolog of c-Abl, called Arg (Abl-related gene). The Arg protein shares highly structural and sequence homology with c-Abl in the N-terminus (94% kinase domain, 90% SH2 & SH3 domain), but the C-terminal domain of Arg is quite different from c-Abl (29% overall, but 56% in the last 60 amino acids) (Kruh, Perego et al. 1990). Instead of three nuclear localization signals (NLSs) (Wen, Jackson et al. 1996; Wang, Miller et al. 2001) and three high-mobility group-like boxes (HLB) in c-Abl, in Arg, this region contains an additional F-actin-binding domain (Wang, Miller et al. 2001).

The functional domains of mammalian c-Abl have been well characterized. The N-terminus of c-Abl is homologous to Src-family members, containing the myristoylation site (only in human Ib and mouse IV c-Abl), the tyrosine kinase domain, as well the Src homology 2 (SH2) and 3 (SH3) domains (Songyang, Shoelson et al. 1994). Both SH2 and SH3 domains regulate c-Abl activity by mediating and determinating protein-protein interactions (Pawson 1994; Cohen, Ren et al. 1995). The SH3 domain of c-Abl mediates the interaction of c-Abl with various proteins, such as the Abl interactor protein (Abi) family of proteins (Dai and Pendergast 1995; Shi, Alin et al. 1995), ataxiatelangiectasia-mutated (ATM) (Baskaran, Wood et al. 1997; Shafman, Khanna et al. 1997), the protein tyrosine phosphatase SHPTP1 (Kharbanda, Bharti et al. 1996), the peroxiredoxin family member Pag/MSP (Van Etten, Jackson et al. 1989; Wen and Van Etten 1997) and regulatory factor X1 (RFX1) (Agami and Shaul 1998), via their proline rich regions containing the PxxP motif (Ren, Mayer et al. 1993). The SH2 domain mediates phosphotyrosine dependent protein-protein interactions(Songyang, Shoelson et al. 1993). Different from Src family members, c-Abl has a unique long C-terminus, which is crucial for c-Abl function (Schwartzberg, Stall et al. 1991; Tybulewicz, Crawford et al. 1991). Next to the catalytic domain is a proline-rich region that binds the SH3 domains of various adaptor proteins, including Crk, Grb2, and Nck (Feller, Knudsen et al. 1994; Ren, Ye et al. 1994). Also, DNA damage response kinase ATM (Baskaran, Wood et al. 1997), the tumor suppressors p53 (Goga, Liu et al. 1995) and Rb (Welch and Wang 1993), as well as RNA polymerase II carboxy terminal domain (Baskaran, Dahmus et al. 1993; Baskaran, Chiang et al. 1997) bind to c-Abl through its C-terminus. The C-terminal c-Abl contains three nuclear localization signals (NLSs) and one nuclear export signal (NES), a putative DNA-binding domain with three high mobility group-like boxes (HLB) that bind to A/T-rich DNA (Miao and Wang 1996; David-Cordonnier,

Hamdane et al. 1998) and an actin binding domain (McWhirter and Wang 1993; Van Etten, Jackson et al. 1994). These structural domains allow c-Abl to shuttle between the nucleus and cytoplasm, participating in many processes by direct protein-protein interactions.

In animal cells, c-Abl is ubiquitous but with different subcellular localization. In fibroblasts and other cell types, it locates predominantly in the nucleus but with a significant distribution in the cytoplasm that is associated mostly with filamentous actin (F-actin) and the plasma membrane (Van Etten, Jackson et al. 1989). While in primary haematopoietic cells and neurons, c-Abl is more cytoplasmic than nuclear. In contrast, all the transforming c-Abl variants, such as the oncogenic Bcr-Abl and v-Abl proteins are exclusively cytoplasmic, although they contain the three NLS (Van Etten, Jackson et al. 1989; McWhirter and Wang 1991; Vigneri and Wang 2001). This pattern of subcellular distribution indicates that c-Abl is involved in a number of cellular processes.

The cellular subcellular localization of c-Abl is tightly controlled by NLSs and NES. c-Abl has three NLSs, any one of them is sufficient to localize c-Abl to the nucleus, while they function differently in different cell types. The translocation of c-Abl to the cytoplasm is mediated by the NES at its C-terminus in Crm1 nuclear-export pathway, sensitive to leptomycin B7. In the cytoplasm, c-Abl is mainly associated with the F-actin cytoskeleton (McWhirter and Wang 1993; Van Etten, Jackson et al. 1994). A portion of myristoylated c-Abl is associated with the inner surface of the plasma membrane through its myristoyl group, which is required for membrane localization (Daley, Van Etten et al. 1992). The localization at multiple cellular compartments suggests that c-Abl may transduce signals between different cellular compartments in response to various physiological and pathological stimuli, and/or, plays distinct functions in different compartments.

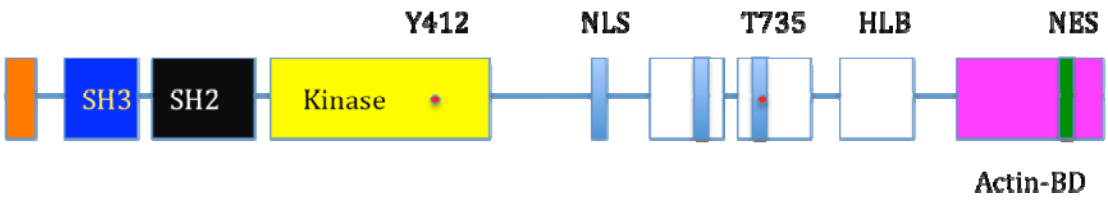


Figure 4 The functional domains of c-Abl.

The extreme N-terminus of the c-Abl protein contains a myristoylation site (orange). The Src homology-3 domain (SH3, dark blue), Src homology-2 domain (SH2, black) and the catalytic domain (Kinase, yellow) compose the remaining N-terminal half of c-Abl. In the C-terminal half there are three nuclear localization sequences (NLS, light blue), one nuclear export sequence (NES, green) and three high mobility group-like boxes (HLB, white). In addition, at the extreme C-terminus there is an actin-binding domain (Actin-BD, pink). The amino acid residue Y412 and T735 are indicated as red circles.

2.1.1.2 The activation and regulation of c-Abl

Inhibition of c-Abl

Mutated, oncogenic forms of c-Abl, such as v-Abl, whose expression leads to strongly increased tyrosine phosphorylation of c-Abl itself and other cell proteins, and also leads to transformation. In contrast, overexpression of wild-type c-Abl in mammalian cells does not induce either phosphotyrosine increase nor transformation, suggesting that the activity of c-Abl is tightly regulated (Franz, Berger et al. 1989; Jackson and Baltimore 1989). However, mechanisms regulating the activity of c-Abl's activity remain poorly understood, due to the complexity of its 3D structure and the diversity of its biological activities in the cell. Both intrinsic and extrinsic factors work together to affect c-Abl activity and ultimately determine

which substrates get phosphorylated and to what extent. Not a simple on-off switch, c-Abl is in a dynamic equilibrium of states, which is affected by many signaling inputs and regulated in many ways, including phosphorylation, intramolecular interaction, interaction with a variety of other proteins, and subcellular localization.

The c-Abl kinase is highly regulated and mostly inactive in cells (Muller, Slamon et al. 1982). Evidence suggests that the c-Abl SH3 domain plays a critical inhibitory role. Complete deletions or non-functional point mutations in the SH3 domain result in c-Abl kinase activation suggesting that this domain inhibits c-Abl kinase activity, either by an interaction with inhibitory proteins or by an intramolecular inhibitory mechanism (Welch and Wang 1993; Mayer and Baltimore 1994). However, out of a number of known SH3 binding proteins only Pag/MSP (Van Etten, Jackson et al. 1989) and amino acid permease 1 (Aap1) (Van Etten 1999) inhibit c-Abl kinase activity (Mayer and Baltimore 1994). In addition, Pag/MSP (Van Etten, Jackson et al. 1989) also inhibits c-Abl kinase activity independent of the SH3 domain, possibly by direct interaction with the kinase domain (Wen and Van Etten 1997). Furthermore, other SH3 binding proteins, such as Abi 1 and 2, the adaptor protein Crk (Feller, Ren et al. 1994), and the DNA-binding protein RFX1 (Agami and Shaul 1998), are phosphorylated by c-Abl (Shi, Alin et al. 1995) and even potentiate c-Abl kinase activity *in vitro*. Together these findings suggest that the SH3 domain inhibits c-Abl kinase activity likely by an intramolecular mechanism. Binding of the SH3 domain to the SH2-catalytic domain linker is considered to be important and necessary for the inhibitory closed conformation of c-Abl, since Superti-Furga and colleagues found that mutation of Pro-242 in this linker region induced an increased activity of c-Abl (Barila and Superti-Furga 1998). Evidence also suggests that the extreme N-terminus, including the

myristoyl group, contributes to retaining the kinase domain in a repressed state (Pluk, Dorey et al. 2002; Hantschel, Nagar et al. 2003; Nagar, Hantschel et al. 2003).

Inhibitor proteins that selectively bind to inactive c-Abl may further inhibit c-Abl activity, including Pag/Msp (Dai and Pendergast 1995), AAP1, tumor suppressor retinoblastoma protein (Rb) and F-actin (Pendergast, Muller et al. 1991; Shi, Alin et al. 1995; Zhu and Shore 1996; Prosperi, Ferbus et al. 1998). Some inhibitors are also substrates of c-Abl, indicating that the inhibition may be the result of competition with other substrates. On the contrary, Rb binds to the ATP binding lobe of c-Abl to inhibit catalytic activity without itself becoming phosphorylated (Welch and Wang 1993; Woodring, Hunter et al. 2001). F-actin can also inhibit c-Abl activity by directly interacting with F-actin-binding domain at the C-terminus of c-Abl (Woodring, Hunter et al. 2001). The activity of c-Abl is regulated by different mechanisms when localized in different cellular compartments. In the cytoplasmic and cytoskeletal compartments, the crystal structure of c-Abl indicated that the myristoyl group is buried in the C-lobe, which maintains c-Abl in an autoinhibited state (Nagar, Hantschel et al. 2003). However, the myristoyl group of the membrane-associated pool of c-Abl is inserted in the lipid bilayer and is not able to bind to the C-lobe. In this situation, inhibition of c-Abl may be mediated by PtdIns(4,5)P2 that binds directly to the catalytic domain of c-Abl and mimics the function of myristate, leading to a similar conformational change in the C-lobe (Plattner, Irvin et al. 2003).

Activation of c-Abl

There are over 500 protein kinases that have been identified and more that likely have not yet been identified. The activation mechanisms of these protein kinases include binding of another molecule, dissociation from an inhibitor, or phosphorylation/dephosphorylation by kinases/phosphatases. The phosphorylation of a residue on a mobile segment near the catalytic

cleft termed the “activation segment” is important for stimulating most kinases (Johnson, Noble et al. 1996). Commonly, the kinase structurally transitions from a “closed” to an “open” conformation to allow substrate binding and catalysis, followed by phosphorylation of the activation segment, which further stabilizes the open conformation (Hubbard, Mohammadi et al. 1998). The kinase exists in equilibrium between opened and closed states that normally favors the closed conformation. However, once phosphorylation of the activation segment occurs, the equilibrium shifts to favor the open conformation, resulting in full activation of the kinase. The X-ray crystal structure of the kinase domain of c-Abl indicates the activation of c-Abl is quite similar to this general model (Schindler, Bornmann et al. 2000). The c-Abl catalytic domain contains an activation segment that is found in all tyrosine kinases. A conserved tyrosine residue in the c-Abl catalytic domain (Y412) is phosphorylated in oncogenic forms of c-Abl, such as Bcr-Abl and v-Abl (Franz, Berger et al. 1989; Jackson and Baltimore 1989; Mayer and Baltimore 1994; Barila and Superti-Furga 1998) and associated with c-Abl activation (Brasher and Van Etten 2000; Dorey, Engen et al. 2001). Mutation of Y412 to phenylalanine causes a 90% reduction of c-Abl kinase activity. In its inactive state, the activation segment of c-Abl is non-phosphorylated and blocks the opening of the catalytic domain, preventing substrate binding.

This inactive conformation could be disrupted by auto or transphosphorylation at specific tyrosine residues in the activation loop of the catalytic domain and the SH2-catalytic domain linker (Brasher and Van Etten 2000), as well as by deletion of the SH2 or SH3 domains, or the myristoyl group (Hantschel, Nagar et al. 2003). The association of substrates with the SH3 or SH2 domain of c-Abl (e.g. binding of a phosphotyrosine ligand to the SH2 domain or a PXXP ligand to the SH3 domain) may increase the catalytic activity in situ to allow substrate

phosphorylation. As noted above, the SH3 domain contributes to c-Abl inhibition by intramolecular binding. Proline-rich ligands that engage the SH3 domain therefore could mediate c-Abl activation by interrupting this intramolecular binding, including c-Casitas B lineage lymphoma (c-Cbl) (Shishido, Akagi et al. 2000; Miyoshi-Akiyama, Aleman et al. 2001), RFX1 (Agami and Shaul 1998), ST5 (Majidi, Hubbs et al. 1998), and c-Jun (Barila, Mangano et al. 2000). For example, overexpression of c-Cbl efficiently, but c-Cbl mutant lacking the proline-rich region failed to induce c-Abl activation (Miyoshi-Akiyama, Aleman et al. 2001), suggesting that the proline-rich region is important for c-Abl kinase activation, probably by engaging with the SH3 domain, thus relieving inhibition and leading to kinase activation.

The activation loop of the catalytic domain can be phosphorylated by other kinases, such as Src (Plattner, Kadlec et al. 1999), or be autophosphorylated. A recent study showed that in the presence of ATP, unphosphorylated c-Abl underwent rapid autophosphorylation at Tyr412, in the activation segment of the kinase domain (Brasher and Van Etten 2000), with about 20-fold higher specific activity than the unphosphorylated form. However, the Y412F mutant does not prevent c-Abl autoactivation (Tanis, Veach et al. 2003), suggesting that phosphorylation of other tyrosine residues may contribute to autoactivation of c-Abl. Notably, phosphorylation of the activation loop tyrosine was concentration-dependent, suggesting that autophosphorylation was intermolecular (Cooper and MacAuley 1988; Moarefi, LaFevre-Bernt et al. 1997). That means phosphorylation of the activation loop tyrosine can be performed by a second c-Abl molecule, indicating the activation of c-Abl can be induced by increased local concentration of itself (Schlessinger 2000). Cellular processes that relocalize c-Abl to Focal adhesions (FAs) or to the plasma membrane may also simply activate c-Abl by increasing its local concentration.

Different from the SH2 domain in Src, which functions as a negative regulator, the SH2 domain in c-Abl almost exclusively enhances activity. The SH2 domain binds stably to tyrosine-phosphorylated proteins. This means that a newly phosphorylated substrate is not released, but is instead held close to the catalytic domain where it can be repeatedly or processively phosphorylated on multiple sites. The substrate can be stably phosphorylated even in the presence of high phosphatase activity. Consequently, the SH2 domain is important for efficient phosphorylation of substrates containing multiple potential phosphorylation sites (Duyster, Baskaran et al. 1995). For substrates containing a single phosphorylation site, however, the presence of the SH2 is not important (Duyster, Baskaran et al. 1995; Mayer, Hirai et al. 1995), supporting the notion that the SH2 does not regulate catalytic activity but instead regulates the ability to stably interact with and processively phosphorylate selected substrates. c-Abl may recruit other proteins containing SH2 domains to broaden the range of substrates that can be processively phosphorylated by c-Abl. For example, c-Abl binds to SH2/SH3 adaptors Crk, Grb2, and Nck through its proline-rich region just C-terminal to the catalytic domain (Feller, Knudsen et al. 1994; Ren, Ye et al. 1994), greatly increased the ability of c-Abl to processively phosphorylate other substrates like p130Cas, which specifically interacts with the SH2 domain of these adaptors (Mayer, Hirai et al. 1995).

In addition, c-Abl can be activated by other kinases including the DNA dependent protein kinase (DNA-PK) and ATM, which constitutively interact with c-Abl, but only phosphorylate c-Abl in response to ionizing radiation (Baskaran, Wood et al. 1997; Shafman, Khanna et al. 1997). ATM phosphorylates c-Abl at Ser-465 (Ren, Mayer et al. 1993), which is close to the activation loop and catalytic cleft on the large lobe of the kinase domain. Phosphorylation at this site may induce or stabilize the open conformation.

In addition to the functional domains in the N-terminus of c-Abl, such as SH3, SH2 and kinase domain as discussed above, the long unique C-terminus also is involved in c-Abl activity regulation by interacting with specific substrate proteins. For example, the phosphorylation of RNA polymerase II by c-Abl is dependent on a region close to the C-terminus of c-Abl (Baskaran, Dahmus et al. 1993). Deletion of C-terminus causes dysregulation of c-Abl activity.

In summary, the widely accepted model of c-Abl activity regulation is that c-Abl is in a dynamic equilibrium between the closed conformation (low activity) and the open conformation (high activity). This balance is tightly regulated by multiple intrinsic and extrinsic factors. The complexity in c-Abl kinase regulation may allow it to play a multitude of roles, to be regulated in a multitude of ways, depending on context and need, and to function as an integrator of multiple signals.

2.1.2 Function of c-Abl in dividing cells

c-Abl is involved in many cellular processes including cell cycle control, apoptosis, cytoskeletal regulation, growth factor signaling, cell growth and differentiation. Its function is tightly regulated by its subcellular localization. For example, nuclear c-Abl plays a role in transcription regulation to induce cell cycle arrest and apoptosis, particularly in response to DNA damage (Shaul 2000; Wang 2000). Cytoplasmic c-Abl is activated by growth factors and cell adhesion, localizing to dynamic regions of the cytoskeleton in actively spreading fibroblasts or the neurites of cortical neurons (Woodring, Litwack et al. 2002).

2.1.2.1 Nuclear c-Abl

Cell cycle arrest and apoptosis in response to DNA damage

Cell cycle progression is controlled by cell cycle checkpoints that generate a transient delay, cell cycle arrest, which acts to ensure proper completion of early events and genome integrity before progression. Furthmore, checkpoints allow DNA transactions to occur before progressing to the next phase of the cycle. Once the DNA is damaged, cells undergo cell cycle arrest to allow the damaged DNA to be repaired. If it is successful, DNA replication and cell growth occur, but if the repair fails, cells undergo apoptosis. Interestingly, c-Abl is involved in both cell cycle arrest and apoptosis in response to DNA damage.

Overexpression of wild-type c-Abl inhibits cell growth and leads to G1 arrest (Sawyers, McLaughlin et al. 1994; Goga, Liu et al. 1995). Cells expressing a mutant c-Abl kinase, however, are impaired in their ability to undergo G1 arrest in response to ionizing irradiation (IR) (Yuan, Huang et al. 1996; Yuan, Huang et al. 1996). In addition, expressing antisense c-Abl RNA in cells also has been shown to shorten G1/S transition (Daniel, Cai et al. 1995), suggesting that c-Abl may be involved in the G1/S checkpoint.

Apoptosis is an essential process in the development of multi-cellular organisms, in the maintenance of tissue homeostasis, in neurodegenerative diseases, and in response to stress. Several observations have implicated c-Abl in proapoptotic activity (Agami, Blandino et al. 1999; Yuan, Shioya et al. 1999). For example, overproduction of c-Abl tyrosine kinase in Saos-2 cells or NIH3T3 cells can activate apoptosis (Theis and Roemer 1998; Cong and Goff 1999). On the other hand, cells derived from c-Abl deficient mice or cells stably expressing the inactive c-Abl kinase mutant are resistant to IR or DNA damage induced apoptosis (Yuan, Huang et al. 1996), suggesting that the c-Abl is able to induce apoptosis and its kinase domain is required for

the pro-apoptotic activity. Consistent with this idea, c-Abl tyrosine kinase activity can be stimulated by DNA damage-inducers such as IR, cisplatin, mitomycin C, as well as topoisomerase inhibitors such as etoposide, doxorubicin and camptothecin (Kharbanda, Ren et al. 1995). A physiologic inducer of apoptosis, TNF, can also activate c-Abl tyrosine kinase (Liu, Baskaran et al. 1996).

The activation of nuclear c-Abl tyrosine kinase by IR requires the phosphorylation of c-Abl by ATM directly or indirectly through DNA-PK (Baskaran, Wood et al. 1997). Upon activation, c-Abl phosphorylates its nuclear substrates, including DNA-PK (Kharbanda, Pandey et al. 1997), Rad51 (Yuan, Mead et al. 1998), SHPTP1 (Kharbanda, Bharti et al. 1996) and the p85 subunit of phosphoinositide3-kinase (Yuan, Crane et al. 1997), negatively regulating their respective activities; or activates nuclear stress-activated kinase/jun N-terminal kinase (SAPK/JNK) (Kharbanda, Ren et al. 1995) and the related p38 kinase in response to IR. However, such IR-induced responses is normal in *abl*–/– primary murine embryo fibroblasts suggesting that c-Abl is not actually required. The role of c-Abl in IR-induced cell cycle arrest and DNA repair must be redundant, subtle, or both. Obviously, the precise role of c-Abl in these cellular responses needs to be further evaluated.

c-Abl might regulate cell cycle and apoptosis by binding to DNA directly, mediated by its three tandemly repeated DNA-binding domains at its C-terminus (Miao and Wang 1996). However, c-Abl has not been found to bind to a specific sequence, although it shows a weak preference for AT-rich oligonucleotides (Miao and Wang 1996). The functional significance of c-Abl DNA binding activity is not known. Although c-Abl has no intrinsic transcriptional activity, v-Abl and c-Abl can form a complex with transcription factors, such as CREB and E2F-1 (Birchenall-Roberts, Yoo et al. 1997), and modulate their transcriptional activity.

Current evidence suggests that p73 is a possible downstream effector of c-Abl induced apoptosis. p73 is member of the p53 tumor suppressor family, which includes p53, p63 and p73 (Levrero, De Laurenzi et al. 1999). Overexpression of either p53 or p73 can induce apoptosis (Jost, Marin et al. 1997; Levrero, De Laurenzi et al. 1999). p53 was considered to mediate c-Abl induced apoptosis because c-Abl interacts with and stabilizes p53. However, it is unlikely that p53 is involved in the c-Abl induced apoptosis. First, p53 can be induced following γ-irradiation, cisplatin treatment and UV radiation, but c-Abl cannot be activated by UV radiation. Second, c-Abl induces apoptosis in both p53-null cells and p53 positive cells (Yuan, Huang et al. 1997). In sharp contrast, p73, with similar transcriptional activation and DNA binding (Kaghad, Bonnet et al. 1997), can be induced by cisplatin, and this requires c-Abl (Gong, Costanzo et al. 1999) in wild type and p53-deficient cells. However, the induction of p73 by cisplatin is not observed in c-Abl-deficient cells. In contrast, the induction of p53 protein by cisplatin can occur without c-Abl (Gong, Costanzo et al. 1999). Furthermore, disruption of the c-Abl-p73 interaction results in a failure to induce apoptosis by IR (Agami, Blandino et al. 1999; Yuan, Shioya et al. 1999). Mutation of p73 at tyrosine 99 (Y99-F), the site that is phosphorylated by c-Abl, blocks the apoptotic response to IR (Yuan, Shioya et al. 1999). Thus, in response to DNA damage, c-Abl has an essential role in the regulation of p73 induced apoptosis, but it is not necessary for the activation of p53.

The possible mechanism by which p73 stimulates apoptosis is through transactivation of common downstream target genes shared with p53, such as p21 (Levrero, De Laurenzi et al. 1999). Consistent with this idea, a dominant negative variant of p73, delta-N-p73 protein, which lacks the N-terminal transactivation domain of p73, but contains an intact DNA binding domain of p73 (Yang, Walker et al. 2000), protects developing neurons from p53 mediated apoptosis

induced by the withdrawal of nerve growth factor (Pozniak, Radinovic et al. 2000). This result suggests that the delta-N-p73 may bind to the promoters of p53-activated genes and thus block p53 from activating apoptosis. The c-Abl kinase can directly bind to and phosphorylate the p73 protein, increasing the half-life of p73 (Gong, Costanzo et al. 1999). The SH3 domain of c-Abl appears to interact with a proline-rich region in p73 (Agami, Blandino et al. 1999), and this region is found in both the full-length and the delta-N-p73 proteins. Also the c-Abl phosphorylation site in p73, tyrosine 99, is located in both full-length and delta-N-p73. Therefore, the effect of c-Abl on apoptosis would then be determined by the expression pattern of the p73 gene. In cells that predominantly express delta-N-p73, such as the sympathetic neurons in neonatal brains, activation of the c-Abl kinase may enhance the anti-apoptotic function of delta-N-p73 and thus promote survival. In cells that predominantly express the full-length p73, such as colon cancer cells and mouse embryo fibroblasts, activation of the c-Abl kinase may induce the pro-apoptotic function of p73 and thus promote cell death (Gong, Costanzo et al. 1999; Levrero, De Laurenzi et al. 1999), indicating that the effect of c-Abl on apoptosis is highly dependent on the cell type.

The nuclear c-Abl activity is negatively regulated by interaction with Rb (Welch and Wang 1993). In the nucleus, a portion of c-Abl interacts with Rb in G1 phase of the cell cycle. The C-terminal pocket of Rb binds to the ATP-binding lobe of the c-Abl kinase domain, resulting in inhibition of c-Abl kinase activity. At the end of the G1 phase, Rb is phosphorylated by cyclin-D–cdk4/6 kinases and dissociates from c-Abl. Therefore c-Abl is allowed to be activated and stimulate the transcription of S-phase genes, probably via phosphorylation of the C-terminal domain (CTD) of RNA polymerase II. These observations suggest that c-Abl might have a growth-promoting role during S phase. However, fibroblasts derived from *abl*–/– mice do

not exhibit defects in S-phase progression, and no direct cellular target genes of c-Abl have been identified. *In vitro* studies demonstrated that induction of direct DNA damage activates c-Abl in cells that enter S phase, but failed to activate c-Abl in G0/G1 cells (Liu, Baskaran et al. 1996), indicating that c-Abl activation by DNA damage only occurs after cells enter S phase, when Rb is phosphorylated and inactivated. Taken together, the negative role of Rb in c-Abl activation indicates a hypothesis that the first response of a cell to DNA damage is cell cycle arrest, holding the cell at G0/G1 phase, inhibiting the synthesis of DNA. However, once the severity of DNA damage is over a particular threshold, cells lose their ability for growth arrest and enter the S phase. Plan B of protection again DNA damage is to release the inhibition of c-Abl from Rb and allow it to induce apoptosis, avoiding the spread of DNA damage. c-Abl is involved in both cell cycle arrest and apoptosis and Rb is the important switch to help a cell choose which way to go (Wang and Ki 2001).

2.1.2.2 Cytoplasmic c-Abl

c-Abl regulates the cytoskeleton

Less is known about specific functions of c-Abl in the cytoplasm. Among tyrosine kinases, c-Abl and its homolog Arg are unique in binding directly to F-actin. A large proportion of cytoplasmic c-Abl is associated with the F-actin cytoskeleton. c-Abl binds to both filamentous (F) and monomeric (G) actin through a conserved consensus actin-binding domain at the extreme C-terminus (McWhirter and Wang 1993), and together the two domains can mediate bundling of F-actin filaments. The kinase activity of c-Abl is increased by signals that stimulate F-actin rearrangement including extracellular matrix (ECM) proteins such as fibronectin (Lewis, Baskaran et al. 1996; Renshaw, Lewis et al. 2000; Woodring, Hunter et al. 2001). When

fibroblasts are plated onto a fibronectin matrix, c-Abl is recruited to FAs and c-Abl kinase activity increases transiently (Lewis, Baskaran et al. 1996). c-Abl can bundle F-actin *in vitro* and increases the number of F-actin microspikes and filopodia on spreading fibroblasts and neurons (Van Etten, Jackson et al. 1994). The kinase activity of c-Abl is required to modulate the F-actin cytoskeleton (Plattner, Kadlec et al. 1999; Zukerberg, Patrick et al. 2000; Kain, Gooch et al. 2003). Treatment of cells with c-Abl kinase specific inhibitor STI571 (Schindler, Bornmann et al. 2000) largely blocks this effect.

In vivo, the concentration of c-Abl within the cytoplasm is low, thus it is unlikely that c-Abl is a major modifier of the cellular F- and G-actin pools. However, it is possible that c-Abl affects the cytoskeleton locally. c-Abl has been detected in various F-actin structures, including FAs, pseudopodia, lamellipodia, filopodia, membrane ruffles, neuronal extensions and synapses. Previous studies indicate that c-Abl can regulate F-actin structures within the cellular cytoskeleton. The F-actin cytoskeleton is a fundamental component of all eukaryotic cells. The highly dynamic nature of the actin cytoskeleton is important for many essential cellular functions, including cell division, endocytosis, axon pathfinding and embryonic development. c-Abl regulates the assembly of F-actin polymers into different structures, depending on the extracellular signal. c-Abl has been shown to be involved in membrane ruffling, cell spreading, cell migration, and neurite extension in response to growth factor and extracellular matrix signals (Woodring, Hunter et al. 2003). Integrin receptors provide the adhesive connection between the F-actin cytoskeleton and the ECM (Boulter and Van Obberghen-Schilling 2006). c-Abl can activate, through binding and phosphorylation of the focal adhesion protein, paxillin (pax) (Lewis and Schwartz 1998). Since c-Abl contributes to several different F-actin dependent processes, its function must be determined by collaboration with other F-actin regulators or the

specific subcellular localization of multi-protein complexes containing c-Abl or c-Abl substrates. Further investigation is required to identify the key components in c-Abl cytoskeletal signaling pathways.

Interestingly, although c-Abl is activated by extracellular signals that regulate F-actin, it is also inhibited through binding with F-actin. Purified F-actin inhibits the activity of purified c-Abl *in vitro* (Woodring, Hunter et al. 2001). Cell attachment and spreading causes a dissociation of the c-Abl and F-actin, which is accompanied with increased c-Abl activity. Inhibition of c-Abl requires direct binding to F-actin, suggesting that F-actin may enhance the folding of c-Abl into the inactive conformation. In addition, consistent with the structural model for auto-inhibition of c-Abl (Nagar, Hantschel et al. 2003), the SH2 domain is also required for the inhibition of c-Abl by F-actin (Woodring, Hunter et al. 2001). Further structural analysis is necessary to determine the precise mechanism by which F-actin inhibits c-Abl. Taken together, the data suggest that there is a reciprocal relationship between c-Abl and F-actin. Although increased c-Abl activity can increase the movement of F-actin and thus enhance membrane ruffling and neurite extension, the association of c-Abl with F-actin is a negative feedback regulation, acting to decrease c-Abl activity. The reciprocal regulation of F-actin and c-Abl may be necessary for the rapid and dynamic regulation of c-Abl-dependent actin based processes.

In summary, c-Abl plays distinct functions in different cellular compartments. Activated nuclear c-Abl induces cell cycle arrest and apoptosis in response to DNA damage (Wang 2000; Puri, Bhakta et al. 2002). Cytoplasmic c-Abl primarily regulates F-actin. It is unclear if the cytoplasmic and the nuclear functions of c-Abl are coordinated. However, results from three recent studies support this model. Tyrosine phosphorylation of Crk by c-Abl disrupts the Cas-Crk complex. The loss of Cas-Crk coupling inhibits cell migration and promotes apoptosis

(Klemke, Leng et al. 1998; Cho and Klemke 2000; Kain and Klemke 2001). In addition, DNA damage cannot activate nuclear c-Abl tyrosine kinase activity in cells that are deprived of ECM signals (Woodring, Hunter et al. 2001), suggesting that the stable adhesion of fibroblasts to fibronectin is essential for DNA damage induced apoptosis by allowing the activation of c-Abl and p53. These cultured-cell-based results are intriguing but this hypothesis must be further assessed in animal and other model systems.

2.1.3 c-Abl in the nervous system

2.1.3.1 c-Abl in neurodevelopment and synaptic plasticity

The c-Abl tyrosine kinase is essential for proper embryonic development. c-Abl knockout mice are embryonic and neonatally lethal (Tybulewicz, Crawford et al. 1991). Surviving mice exhibit lymphopenia, thymic atrophy and/or osteoporosis, abnormal eyes, and defective spermatogenesis (Schwartzberg, Stall et al. 1991; Tybulewicz, Crawford et al. 1991; Li, Boast et al. 2000). These observations suggest that c-Abl is required for multiple cellular functions such as those described above. However, the specific role for c-Abl during development has remained unclear because of the short life span of the *abl*–/– mice. The c-Abl related gene (*arg)* was identified in 1986 (Kruh, King et al. 1986). The abl/arg double knockout mice show a more severe and rapid embryonic lethality, typically at stage E9-11 (Koleske, Gifford et al. 1998). However, unlike the *abl*–/– mice, *arg*–/– mice are born normal and do not exhibit lethality, but they exhibit behavioral phenotypes (Koleske, Gifford et al. 1998), suggesting that Arg must play redundant functions to sustain the development of c-Abl-deficient mice. However, these two proteins must play distinct functions, because c-Abl-deficient mice have developmental defects that are not observed with Arg-deficient mice (Koleske, Gifford et al. 1998).

Genetic studies of the Drosophila homolog of c-Abl, Drosophila Abl (D-Abl) suggest that D-Abl integrates the information from axon guidance and growth factor receptors and mediates to cytoskeleton, thus promoting cytoskeletal rearrangements in developing neurons and synapse formation. D-Abl is abundant and localized at axons in the developing nervous system (Gertler, Bennett et al. 1989). Mutations in D-Abl leads to defects in the nervous system, including aberrant midline crossing of longitudinal axons and defects in motor neuron axon guidance (Wills, Bateman et al. 1999; Wills, Emerson et al. 2002), indicating that D-Abl is required for the proper morphogenesis of neurons in the developing Drosophila nervous system. Vertebrate c-Abl and c-Abl related gene non-receptor tyrosine kinase (Arg) share the similar sequence with D-Abl, suggesting that c-Abl and Arg are similarly utilized for neuronal morphogenesis in vertebrates.

Genetic or biochemical interactions between c-Abl and cell surface receptors suggest that c-Abl regulates neurite growth by relaying signals from these receptors. These receptors include: roundabout, neurotrophin and ephrin, platelet-derived growth factor, drosophila leukocyte antigen-related protein tyrosine phosphatase, cadherins and integrins (Moresco and Koleske 2003). As previously described, integrin receptors can activate c-Abl kinase in cultured fibroblasts (Lewis, Baskaran et al. 1996). Several integrin receptors, such as alpha 5, are expressed in developing neurons to regulate adhesive interactions and neuronal migration (Bi, Lynch et al. 2001; Milner and Campbell 2002). c-Abl may mediate integrin signal induced neuronal cytoskeletal movement. In addition, c-Abl also interacts with neurotrophin and ephrin receptors, including the tyrosine kinase receptor A (TrkA), the receptor for NGF and the EphB2 receptor (Koch, Mancini et al. 2000; Yano, Cong et al. 2000; Yu, Zisch et al. 2001). However, it is unclear whether c-Abl is required to mediate the effects of these receptors on axon outgrowth

or guidance. Further studies are required to determine how c-Abl contributes to distinct biological responses controlled by various receptors. Once activated by cell surface receptors, Abl family kinases can influence cytoskeletal structure directly by organizing actin filaments, and indirectly by affecting other cytoskeletal regulators, such as, Ena/vasodilator-stimulated phosphoprotein (VASP) proteins; Abi-1 and Abi-2; and the 190 kD GTPase activating protein for Rho (p190RhoGAP). Previous studies demonstrated that Cdk5 is one of the mediators of c-Abl induced neurite outgrowth by forming a protein complex Cdk5-Cables-c-Abl. Cdk5 is a serine/threonine kinase that can be stimulated through phosphorylation at Y15 by c-Abl. Activated Cdk5 consequently phosphorylates specific substrates to regulate neuronal migration and neurite outgrowth (Zukerberg et al., 2000). This idea is also supported by the evidence that antisense RNA to Cables, a novel protein, interacting with Cdk5 in brain lysates, as well as expression of dominant negative Cdk5 each inhibit c-Abl induced embryonic neurite outgrowth.

In addition to their roles in neuronal development, more and more evidence suggests Abl family kinases regulate synaptic structure and function. c-Abl is expressed throughout the adult mouse brain, especially concentrated in synapse-rich regions (Koleske, Gifford et al. 1998; Moresco, Scheetz et al. 2003). c-Abl is confined to the active zone within presynaptic terminals and is prominent at the postsynaptic density in dendritic spines. Electrophysiological studies reveal that c-Abl knockout or inhibition by its specific inhibitor STI571 decreases paired-pulse facilitation (PPF), a transient form of presynaptic plasticity, in hippocampal slices, suggesting that c-Abl is required to modulate the efficiency of neurotransmitter release from the presynaptic terminal (Moresco, Scheetz et al. 2003). Genetic studies also suggest a functional relationship between D-Abl and the receptor tyrosine phosphatase Dlar in Drosophila (Wills, Bateman et al. 1999), whose mutations result in decreased numbers and enlarged active zones of synaptic

boutons. The functional antagonism between D-Abl and Dlar suggests that D-Abl might also be involved in proper active zone morphology and synaptic function. In addition, c-Abl regulates agrin-induced clustering of acetylcholine receptors on the postsynaptic membrane of the neuronal muscle junction by forming a complex with and phosphorylating the muscle-specific receptor tyrosine kinase (MuSK) (Finn, Feng et al. 2003).

Additional genetic studies suggest that c-Abl functions in axonal pathfinding and target recognition, possibly by mediating interactions between cell surface receptors and the cytoskeleton. Cell adhesion receptors are involved in the establishment, maintenance, and remodeling of contacts between presynaptic and postsynaptic components (Moresco and Koleske 2003). Mice lacking neural cell adhesion molecule (NCAM) exhibit reduced PPF at the neuromuscular junction (NMJ) (Rafuse, Polo-Parada et al. 2000; Polo-Parada, Bose et al. 2001) similar to that seen in the abl-/- hippocampus. All these data suggest that cell adhesion receptors could be the upstream signal for c-Abl mediated synaptic plasticity. Data presented below also supports this notion by providing evidence that interaction of c-Abl and cell adhesion proteins occur in human brain. However, the downstream pathway required for the synaptic function of c-Abl is not yet known. One c-Abl substrate, Ras interacting protein 1 (Rin1), may be a candidate, since it is localized to dendrites and modulates long-term synaptic plasticity (Dhaka, Costa et al. 2003). Further studies need to be addressed whether c-Abl is required for other synaptic functions, such as synapse shape, vesicle distribution, or active zone morphology.

2.1.3.2 c-Abl's function in neurodegeneration

c-Abl & AD

As described above, c-Abl functions in neurodevelopment, cell cycle arrest, and apoptosis in response to numerous stimuli. Interestingly, Aβ42 induces neuronal apoptosis and cell cycle activation also occurs in AD (Caltagarone, Jing et al. 2007). Therefore, studies to examine the potential role of c-Abl in neurodegeneration and AD are warranted.

This hypothesis is supported by several *in vitro* studies. Alvarez and colleagues showed that Aβ peptide fibrils induced an increase in c-Abl activity in rat hippocampal neurons as well as an increase in nuclear p73 protein levels and the p73-c-Abl complex. The inhibition of c-Abl activity by STI571 and the inhibition of c-Abl expression by RNAi rescued neurons from toxicity induced by Aβ fibrils (Alvarez, Sandoval et al. 2004). Moreover, consistent with this *in vitro* study, p73 is aberrantly distributed in the nuclei of hippocampal neurons in AD, but predominantly localized in the cytoplasm in normal brain (Wilson, Henry et al. 2004).

Other *in vitro* studies have linked c-Abl to the other major hallmark lesion of AD, tau pathology in NFTs. The abnormal hyperphosphorylation of tau in AD causes a dissociation of tau with microtubules and the formation of NFTs. The phosphorylation of tau on serine and threonine residues has been well studied, as most are located near or within the microtubule binding motifs of tau. However, the phosphorylation of tau at tyrosine residues has recently receiving more attention. Tyr-18 was reported as a site of phosphorylation by the Src-family kinase Fyn. Derkinderen et al identified phosphorylated Tyr-394 in PHF-tau from an Alzheimer brain, and in human fetal brain tau by mass spectrometry (Derkinderen, Scales et al. 2005). Mutational analysis determined that Tyr-394 is the major site of tyrosine phosphorylation in tau and c-Abl is responsible for the phosphorylation of tau at this residue, not Fyn, whose major site is Tyr-18 (Derkinderen, Scales et al. 2005). This data suggests that c-Abl could have a pathogenic role in tau pathology during AD.

Cdk5 has been considered a main kinase responsible for tau hyperphosphorylation in AD (Mandelkow 1999). Cdk5 can be activated by Aβ and elevated cdk5 activity has been detected in AD brain (Lee, Clark et al. 1999). However, the underlying mechanism of this induction is not yet clearly understood. c-Abl phosphorylates and activates cdk5 through the adaptor protein Cables during brain development (Zukerberg, Patrick et al. 2000), suggesting the potential involvement of c-Abl in cdk5 activation during AD. A recent study in Drosophila suggested the potential function of c-Abl in amyloid-initiated neurodegeneration via regulating cdk5, by showing that the suppression of c-Abl expression or kinase activity led to an approximate 50% reduction in the activation of cdk5 triggered by Aβ42 and also reduced neuronal cell death. The activated c-Abl interacted with cdk5 and enhanced cdk5 kinase activity through Y15 phosphorylation. Moreover, c-Abl also regulated the translocation of cdk5 from the cell membrane to a perinuclear localization, modulating its kinase substrates involved in cytoskeletal dynamics (Dhavan and Tsai 2001).

c-Abl may also enhance tau hyperphosphorylation indirectly by regulating the transcription of the serine/threonine kinase GSK-3β. c-Abl phosphorylates Fe65, a brain-enriched adaptor protein, at Y547 and enhances its nuclear translocation to regulate GSK-3β expression (Perkinton, Standen et al. 2004). The neuron-specific adaptor protein Fe65 binds with phosphorylated APP intracellular domain (AICD) at Thr-668 and induces nuclear translocation of AICD to regulate the transcription of its downstream target genes, including GSK-3β.

Given the many potential mechanisms by which c-Abl may contribute to regulating neurodegeneration, specific hypotheses have been generated regarding the functional role of c-Abl during AD.

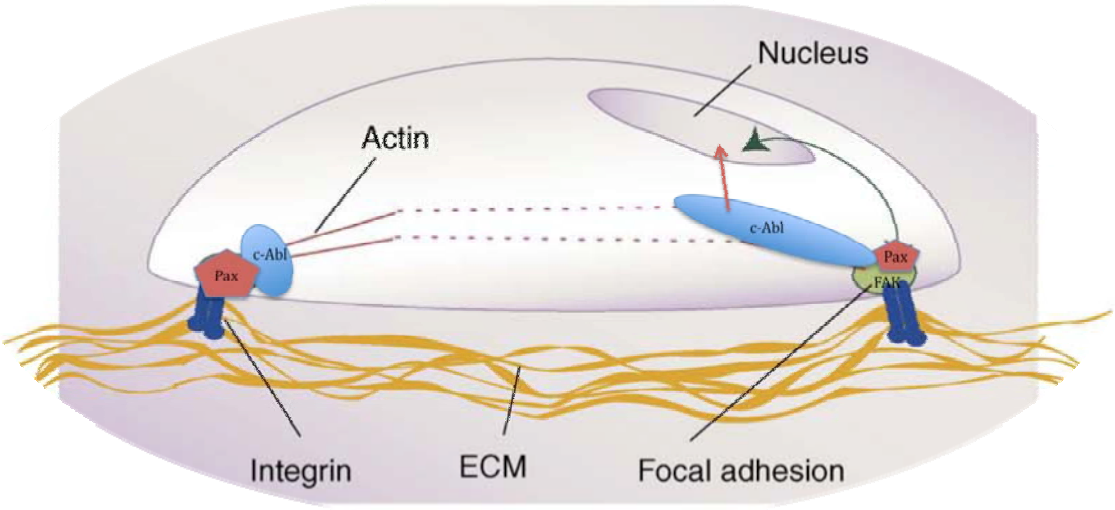


Figure 5 c-Abl & integrin signal pathway

In fibroblasts, once ECM interacts with integrin receptor, focal adhesion is stimulated to recruit the focal adhesion complex. Activated FAK phosphorylates paxillin. c-Abl can be activated by the integrin signaling pathway to phosphorylate paxillin. Activated c-Abl subsequently transmits the integrin signal to the cytoskeleton via interacting with actin, or enters the nucleus to regulate gene transcription.

3.0 THESIS GOALS

One mechanism by which Aβ facilitates tau pathology is through activation of specific protein serine/threonine kinases, such as cdk5 and GSK-3β. One tyrosine kinase, Fyn, has been shown to phosphorylate tau at residue Tyr18 and is associated with AD pathogenesis (Lee, Thangavel et al. 2004). However, a prior *in vitro* study showed that the major phosphorylation site of tau in COS-7 cells is Tyr394, and c-Abl is responsible for phosphorylation at this site (Derkinderen, Scales et al. 2005). Moreover, Aβ has been shown to bind to the integrin receptor on the membrane and transduce a signal from the extracellular space to the cell interior, regulating the cytoskeleton and/or gene transcription (Caltagarone, Jing et al. 2007). c-Abl can also be activated by integrin activation. **Therefore, we hypothesize that c-Abl is associated with Aβ facilitated tau phosphorylation via integrin binding and activation, which contributes to the generation of AD pathology through direct or indirect phosphorylation of tau.** We have begun to test this hypothesis by first examining the expression and distribution of c-Abl in the human hippocampus and by characterizing c-Abl interacting proteins in AD brain.

4.0 MATERIALS AND METHODS

4.1 SUBJECTS

Tissue samples were obtained from the University of Pittsburgh ADRC Tissue Bank after approval by "The Committee for Oversight of Research Involving the Dead" (CORID) at the University of Pittsburgh School of Medicine, Pittsburgh, PA. A blinded retrospective cross sectional study using the following postmortem brain tissue: 5 subjects with Alzheimer's disease (AD) Braak stage V-VI (late AD group), 5 subjects with no clinical history of dementia, but with AD pathology (early AD group, Braak stage II-IV), and 5 subjects with no clinical history of a neurodegenerative disease and no AD pathology (Aβ) and only rare tangles in the hippocampus (healthy control group, Braak stage 0-I). Braak stage is based on the distribution pattern of neurofibrillary tangles and neuropil threads, which correlates to AD progression and permits the differentiation of six stages. Stage 0: no discernible changes. Stage I-II (transentorhinal stages): either mild or severe alteration of the transentorhinal layer Pre-alpha. Stages III-IV (Limbic stages): conspicuous affection of layer Pre-alpha in both transentorhinal region and proper entorhinal cortex, as well as mild involvement of the first Ammon's horn sector. Stages V-VI (Isocortical stages): the destruction of virtually all isocortical association areas. A Bielschowsky stain was also performed on all cases. The diagnosis of AD was determined by the combined use of Consortium to Establish a Registry for AD (CERAD), the National Institute of Aging (NIA)

and the Reagan Institute (RI) criteria (Mirra, Heyman et al. 1991; Hyman and Trojanowski 1997). The University of Pittsburgh Brain bank currently collects few post-mortem tissue samples from AD subjects that have been extensively followed with cognitive assessment screens. Therefore we could not develop a research protocol using tissue samples from all subjects with pre-mortem cognitive assessments using definitions of mild cognitive impairment (MCI) criteria.

Patient demographics are listed in Table 2.

One Braak stage II case was assigned by the consulting neuropathologist to the healthy control group. This may occur in any research study, as some tau pathology can occur in elderly but cognitively intact individuals (68 years old in this case), and no amyloid pathology was evident in the subject. We also included an amyotrophic lateral sclerosis (ALS) case in the control group used for western blot analysis. This was due to limitations within the brain bank and a desire to obtain initial observations regarding the disease specificity of our findings. In fact, protein levels of c-Abl in this ALS case were no different than all other healthy controls used in this study.

Table 2 Clinical data of human subjects.

Clinical data of human subjects from non-neurologic disease controls, early AD (no dementia, with amyloid plaques and tangles) and neuropathologically defined AD (clinical history of dementia, amyloid plaques and tangles, no Lewy bodies). PMI, post mortem interval; F, female; M, male. Nd: non-dementia. HC: healthy control.

Table 2.1 Clinical data of subjects for western blot analysis.

CASE #	AGE	SEX	GROUP	PMI	BRAAK
1	61	M	HC (ALS)	15 hrs	0
2	50	M	HC	18 hrs	Nd
3	90	M	HC	4 hrs	Nd
4	74	M	HC	3 hrs	Nd
5	78	F	HC	6 hrs	Nd
6	85	M	Early AD	4 hrs	3
7	87	M	Early AD	5 hrs	4
8	86	F	Early AD	5 hrs	2
9	83	M	Early AD	12 hrs	3
10	85	F	Early AD	3 hrs	4
11	78	M	Late AD	7 hrs	5
12	82	M	Late AD	4 hrs	6
13	56	F	Late AD	3 hrs	5
14	79	F	Late AD	6 hrs	6
15	76	F	Late AD	9.5 hrs	6

Table 2.2 Clinical data of subjects for IHC study

CASE #	AGE	SEX	GROUP	CAUSE OF DEATH	PMI (hrs)	BRAAK
1	51	F	HC	Respiratory failure	5	0
2	51	M	HC	Pseudomonas aeruginosa pneumonia- right lung transplantation	6	0
3	57	M	HC		2	1
4	68	M	HC	Severe atherosclerosis of basilar artery	27	2
5	70	F	HC	Diffuse acute gram negative colitis	6	Nd
6	56	F	Early AD	Extensive acute myocardial infraction	19	2
7	66	M	Early AD	Multi-organ failure- micronodular cirrhosis	9	2
8	71	F	Early AD	Respiratory failure- bilateral pneumonia	15	2
9	72	M	Early AD	Probable cardiac arrhythmia	9	2
10	76	F	Early AD		13	3
11	57	F	Late AD		9	6
12	58	M	Late AD		7	6
13	69	F	Late AD		5	5
14	70	M	Late AD		3	6
15	71	F	Late AD		4.5	6

4.2 WESTERN BLOT ANALYSIS

Fresh frozen human hippocampi from control and AD subjects were homogenized in 20% w/v of cold Tris-Triton X buffer (50mM Tris at pH 7.4, 1% Triton X-100) with protease (Sigma) and phosphatase (Calbiochem) inhibitors. The homogenate was centrifuged at 5000g to get rid of the debris, then was centrifuged at 100,000g in ultracentrifuger at 4°C. The supernatant was saved containing soluble proteins, called the soluble fraction. The pellet contained the detergent insoluble proteins and organelles was resuspended in Tris-triton X buffer and called the insoluble fraction. The protein concentration of S2 and P2 fractions were quantitated using the BCA Protein Assay Kit (Pierce). We loaded 50 μg protein into each gel

lane and separated by SDS-PAGE and transferred to nitrocellulose membrane. Non-specific antigens were blocked with 5% dry milk in TBST for 1 hour at room temperature, and incubated at 4°C overnight with primary antibodies at the following concentrations: total c-Abl antibody (BD pharmingen) 1:600 dilution; c-Abl, p-Y412 antibody (Abcam) 1:1000 dilution; c-Abl, p-T735 antibody, (Cell signaling) 1:1000 dilution. Immunoblots were then incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Chemicon) and antigen visualized using ECL (Perkin Elmer Life Science) and exposed on Blue Basic Autorad Film (BioExpress) . Protein levels were quantified using NIH Image 1.63 and normalized by actin for both soluble fraction and insoluble fraction.

4.3 IMMUNOHISTOCHEMISTRY (IHC)

Tissue was immersion-fixed in 10% buffered formalin and embedded in paraffin. Human hippocampal tissue sections (8 μM) were heated to 60ºC for 20 minutes prior to deparaffinization with three consecutive 10 minutes incubations in 100% xylene. The tissue was then rehydrated and endogenous peroxidase activity blocked with 0.3% hydrogen peroxide for 30 minutes. To expose antigenic epitopes, tissue was steamed for 20 minutes in Target Retrieval Solution, High pH (Dako). To avoid the background due to endogenous biotin or biotin-binding proteins, lectins, or nonspecific binding substances present in the section, an avidin/biotin blocking kit was used according to company specifications (Vector Labs). Sections were incubated in Superblock (ScyTek Labs) for 10 minutes to block nonspecific antibody binding sites. Sections were next incubated with appropriate primary antibodies at 4°C overnight. For light microscopy, antibodies included anti-total c-Abl (Cell signaling) at 1:400 dilution, anti-c-Abl, p-T735 (Cell

signaling) at 1:100 dilution, anti-c-Abl, p-Y412 (Abcam) at 1:300 dilution, or anti-paired helical filaments (PHF) tau (clone AT8, Pierce) at 1:400 dilution. For each series of IHC staining, one no-primary antibody control slide was included to determine nonspecific immunoreactivity of the secondary antibody. A commercially available blocking peptide for c-Abl, p-T735 was also utilized. For the blocking peptide, brain tissue was immunolabeled with antibody containing a 3-fold volume (>100 molar excess) of specific blocking peptide as per manufacturer guidelines. Proteins bound to specific antibodies were visualized with Nova-Red chromagen (Vector Labs), dehydrated and mounted with permount (Fisher).

4.4 IMMUNOFLUORESCENT LASER SCANNING CONFOCAL MICROSCOPY (LSCM)

Immunofluorescent staining was carried out as stated above (section 4.3) except after primary antibody, slides were incubated with goat anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa Fluor-488 or –568 (invitrogen, 1:300 dilution) and mounted with Prolong Gold Antifade Reagent (Invitrogen). For double immunofluorescent staining of phospho-c-Abl and phospho-pax, tyramide signal amplification method was used as described before (Wang, Achim et al. 1999). Briefly, after blocking, the sections were incubated with rabbit anti-c-Abl, p-T735 antibody (1:5000, 4°C, overnight). At this concentration of anti-c-Abl, p-T735 antibody, the c-Abl, p-T735 antigen could not be detected by Alexa Fluor–568-conjugated goat anti-rabbit secondary antibody (1:200, (RT, 1 h) in the conventional IF method (in which the optimal dilution of anti-c-Abl, p-T735 antibody was 1:100), but was still detectable after TSA. The sections were rinsed in TBS and then incubated with biotinylated goat anti-rabbit IgG serum

(1:500, RT, 30 min). After being rinsed in TNT (0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) , the sections were incubated with TNB buffer (0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl, 0.5% Blocking Reagent) (RT, 30 min), excess TNB buffer was blotted, and then the sections were incubated with HRP-conjugated streptavidin (1:500 in TNB Buffer, RT, 30 min). The sections were rinsed in TNT and then incubated with TRITC-conjugated tyramide (1:100 in Amplification Diluent, RT, 10 min). After being rinsed in TNT and in TBS, the sections were incubated with rabbit anti-human phospho-pax antibody (1:100, RT, 2 h). The sections were rinsed in TBS and then incubated with Alexa Fluor-568-conjugated goat anti-rabbit secondary antibody (1:200, RT, 1 h). All the TSA reagents were provided in TSA^{TM} Fluorescence system (Perkin Elmer).

4.5 QUANTIFICATION OF IHC AND STATISTICAL ANALYSIS

The staining intensity of total c-Abl and phospho-c-Abl was determined by light microscopy with integrated software package (Simple PCI version 6.0). Images were taken from the same subfield of the CA3 and CA1 subfields as indicated in Figure 6A in each case and converted to 8-bit (gray and white). Preliminary data showed no significant difference of immunoreactivity of c-Abl between different areas within same subfield of hippocampus (CA1 or CA3). In each section, the subfield of interest (pyramidal layer) was manually (digitally) outlined as indicated in Figure 6B, and its area was measured. A threshold was set and only the objects with a staining level above this threshold were measured (Fig. 6B). Staining intensity was measured in the fraction of subfield of region of interest (ROI) area occupied by the threshold

object area (object area/ROI area) within the selected areas determined by the drawing tool. The object area/ROI area values were statistically compared with ANOVA (GraphPad Prism 4.0a). When $P < 0.05$,results were considered statistically significant and post-hoc test was performed to compare each pair of groups.

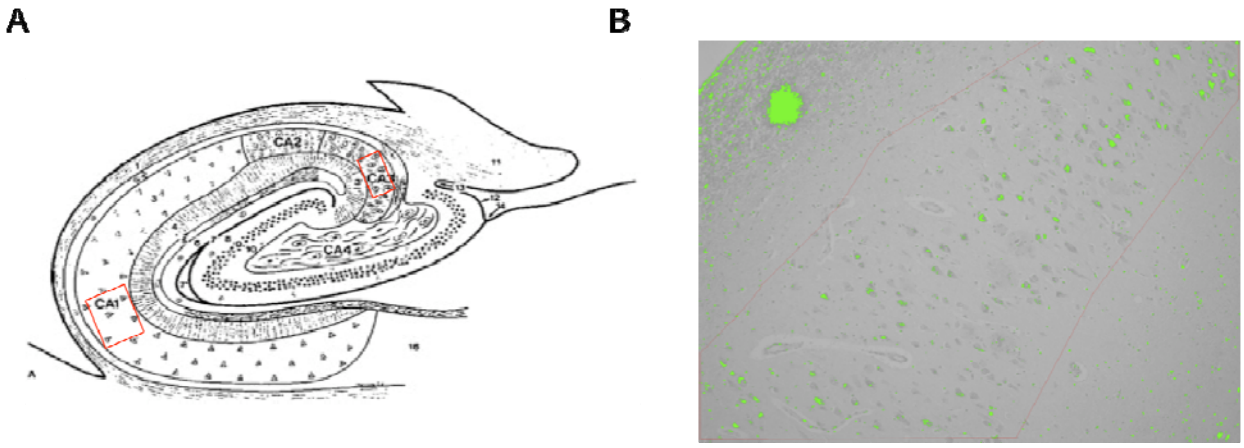


Figure 6 A schematic figure to show the method for quantify the IHC results.

A schematic figure to show the method for the IHC quantification. A. The area in CA1 and CA3 where the image (20X magnification) was taken was indicated by red frame. B. An example to show how the software works. Briefly, in each section, the subfield of interest (pyramidal layer) was manually (digitally) outlined as indicated in Figure 6B, and its area was measured. A threshold was set and only the objects with a staining level above this threshold were measured (Fig. 6B). Staining intensity was measured in the fraction of subfield of region of interest (ROI) area occupied by the threshold object area (object area/ROI area) within the selected areas outlined by the drawing tool.

4.6 CO-IMMUNOPRECIPITATION (CO-IP)

All Fresh frozen hippocampal tissue were homogenized in RIPA-DOC buffer with a same weight/volume ratio (1:5) (50mM Tris at pH 7.4, 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease and phosphatase inhibitor cocktails) in glass Telfon homogenizer (Ghanevati and Miller, 2005). We used 200µl total homogenate for each IP. Immunoprecipitation studies were carried out using the MultiMACS Protein A/G Kit from Miltenyi Biotec, according to the manufacturer's protocols. Samples were separated by SDS-PAGE, transferred to nitrocellulose membrane (Biorad), and incubated with appropriate primary antibodies at 4°C overnight, antibodies included anti-total c-Abl (BD Pharmingen, 1:600 dilution), anti-c-Abl, p-Y412 (Abcam, 1:2000), anti-c-Abl, p-T735 (Cell Signal, 1:2000), anti-total pax (BioSource, 1:2000), anti-pax, p-Y31 (Epitomics, 1:1000), anti-pax, p-Y118 (BioSource, 1:1000), anti-14-3-3 (Abcam, 1:1000), anti-actin (Chemicon, 1:100,000), or anti-PHF-tau (clone AT8, Pierce, 1:800 dilution). Blots were then incubated with appropriate HRP-conjugated secondary antibodies and visualized using ECL detection kit (Perkin Elmer Life Science), and exposed on Blue Basic Autorad Film (BioExpress). Separate blots was used to detected different phosphorylated forms of c-Abl or pax.

4.7 PRIMARY CORTICAL NEURON CELL CULTURE

The primary cortical neurons were derived from Sprague–Dawley rats at embryonic day 17. Cortical cells were plated in polylysine and laminin coated plates at $1.2X10^6$ in plating media

for 5 hours. Then, the cells were maintained in neurobasal medium (Gibco Invitrogen Corporation. Carlsbad, CA), supplemented with B27 (Gibco Invitrogen Corporation), plus 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen Corporation) for 8 days before cell treatments.

4.8 Aβ FIBRIL PREPARATION, CELL TREATMENT AND CYTOTOXICITY ASSAYS

Cortical cells were treated with 10μM Aβ42 fibrils. To obtain the Aβ fibrils, Aβ42 peptide powder, purchased from Bachem, was dissolved in aqueous solution (10^{-5} M) at a concentration of 1mg/ml. Aβ42 solution was incubated in a 37°C water bath for 24 hours to aggregate before use. The fibril formation was confirmed by thioflavin S staining. The peptides were then diluted in serum-free DMEM to final concentration of 10μM. The concentration of the fibrils was not measured. Cortical cells were treated with prepared Aβ42 fibrils at a final concentration of 10μM. At indicated times after treatment, cell viability was measured by propidium iodide (PI) uptake and Hoechst 33342 staining. PI uptake -- Cell death can be quantitatively measured by adding PI (5μg/mL in PBS, pH 7.2, 5 minutes incubation) and by calculating the percentage of PI+ stained cells in random fields. Hoechst 33342 staining – cells were fixed in 100% precooled ETOH for 15 minutes, permeabilized for 5 minutes with 0.1% Triton X-100, treated with 0.2 mM Hoechst 33342 dye (Sigma) for 5 minutes, and coverslipped. The number of Hoechst+ nucleus was used to calculate the total number of cells within the same field. Cell viability was counted as the ratio of PI+ to Hoechst+ cells. 3 fields for each sample at

each time point were chosen randomly. The cell viability at this time point is determined by the mean of cell viability within these 3 fields.

4.9 CELLULAR FRACTIONATION AND WESTERN BLOT ANALYSIS

The NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) was used to obtain the nuclear and cytoplasmic fractions of primary cultured neurons. The procedure was followed per manufacturer's instructions. Briefly, isolate cells by centrifugation at 500 x *g* for 2-3 minutes. Get rid of the supernatant. Add ice-cold CER I to the cell pellet. Incubate on ice for 10 minutes. Add ice-cold CER II and incubate on ice for 1 minute and then vortex for 5 seconds vigorously. Centrifuge the tube for 5 minutes at maximum speed in a microcentrifuge (~16,000 x *g*). Supernatant was saved as cytoplasmic extract fraction. Resuspend the insoluble (pellet) fraction, which contains nuclei, in ice-cold NER. Vortex vigorously for 15 seconds. Return the sample to ice and continue vortexing for 15 seconds every 10 minutes, for a total of 40 minutes. Centrifuge the tube at maximum speed (~16,000 x *g*) in a microcentrifuge for 10 minutes. The supernatant was saved as nuclear extract fraction. The protein concentration of cellular fractions was quantitated using the BCA Protein Assay Kit (Pierce). We loaded 20 μg protein into each gel lane and separated protein by SDS-PAGE, then transferred it to nitrocellulose membrane. Non-specific antigens were blocked with 5% dry milk in TBST buffer for 1 hour at room temperature, and incubated at 4°C overnight with primary antibodies at the following concentrations: total c-Abl antibody (BD pharmingen) 1:600 dilution; Lamin B antibody (Santa Cruz) 1:100 dilution; β-tubulin antibody (Sigma) 1:100,000 dilution; actin antibody (Chemicon) 1:200,000 dilution. Immunoblots were then incubated with appropriate horseradish peroxidase-

conjugated secondary antibody (Chemicon) and antigen visualized using ECL (Perkin Elmer Life Science) and exposed on Blue Basic Autorad Film (BioExpress). Protein levels were quantified using NIH Image 1.63 and normalized by tubulin for the cytoplasmic fraction or Lamin B staining for the nuclear fraction.

4.10 *IN VITRO* KINASE ASSAY

Neurons ($1.2X10^6$) per well were treated with 10μM Aβ **fibrils**. The cells were then washed in ice-cold PBS and lysed in immunoprecipitation buffer (RIPA buffer; 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40) including phosphatase inhibitors cocktail (Calbiochem) and a protease inhibitor cocktail (Sigma). The lysates were incubated overnight at 4°C with anti-total c-Abl antibody (polyclonal, Cell Signal) and 3 h with protein A beads at 4°C. The c-Abl immunoprecipitates were first washed with RIPA buffer (three times) and then with kinase buffer (20 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, phosphatase inhibitors, protease inhibitors) (Alvarez et al., 1999). The kinase reaction was performed in the same buffer with 25μM ATP + 10μCi [γ-32P] ATP (Amersham Pharmacia Biotech) and specific substrate peptide (**EAIYAAPFAKKK,** New England Biolabs). After 30 min at 30°C, the reaction mixtures were stopped on phosphocellulose p81 paper (Whatman, Pleasanton, CA) and washed three times with 0.75% v/v phosphoric acid. Put the washed p81 papers into scintillation vials and measure the radioactivity by scintillation counting. Instead of c-Abl precipitated from cell lysate, a pure c-Abl kinase protein (New England Biolabs) or no c-Abl buffer was incubated with other components of the reaction as positive or negative control. Duplicate was done for each time point and the whole experiment was repeated three times.

4.11 STATISTICAL ANALYSIS

For IHC and immunoblot studies using tissues from human subjects, statistical analysis was performed by Kruskal-Wallis test (non-parametric one way ANOVA) across all subject groups, followed by post-hoc test to compare each pair of groups. The difference considered significant when $p < 0.05$ for all experiments, expect for correlation analysis, the difference considered significant when $p < 0.1$. Such borderline setting ($p<0.1$) for correlation has been used for other published papers considering the relative big variation between human subjects (Burri, Djonov et al. 2003; Tseng 2005).

For immunoblot and kinase assay using primary neurons, student t-test was performed to compare treated group and untreated group. The difference considered significant when $p<0.05$ (indicated by *).

5.0 ALTERATION OF C-ABL EXPRESSION AND DISTRIBUTION IN HUMAN HIPPOCAMPUS

5.1 INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia in the elderly and neuropathologically characterized by accumulation of extracellular neuritic plaques and intracellular and extracellular neurofibrillary tangles (NFT) within specific brain regions (Morishima-Kawashima and Ihara 2002). In AD, memory loss and cognitive impairment are accompanied by the degeneration of select neuronal populations in the hippocampus and cerebral cortex (Morrison and Hof 1997). Alvarez et al (2004) reported that c-Abl was activated and induced cell death by forming a complex with p73 upon treatment of primary hippocampal neurons with Aβ fibrils. The neuronal cell death induced by Aβ fibrils was prevented by pharmacologic inhibition of c-Abl (Alvarez, Sandoval et al. 2004). Prior study has also suggested a role for c-Abl in NFT formation by direct phosphorylation of tau (Derkinderen, Scales et al. 2005). Moreover, c-Abl may also induce tau phosphorylation indirectly through activation of downstream targets, cdk5 and GSK-3β, which both have been shown to phosphorylate tau (Klafki, Staufenbiel et al. 2006). However the localization and distribution of c-Abl in AD brain is unknown.

Therefore we examined the expression and distribution of c-Abl in the hippocampus of AD and age matched control subjects. We found that the immunoreactivity of non-phosphorylated total c-Abl was decreased in AD hippocampus compared to healthy control. However, its activated form, phosphorylated on tyrosine amino acid residue 412 (c-Abl, p-Y412) localized in neuronal cell bodies in healthy controls, was sequestered in granulovacuolar degeneration bodies (GVD) in hippocampal pyramidal neurons of late-stage AD subjects. Another phosphorylated form of c-Abl, T735, co-localized with additional hallmarks of AD, including amyloid plaques, tangles and GVDs. These data support the hypothesis that c-Abl is associated with AD pathology.

5.2 RESULTS

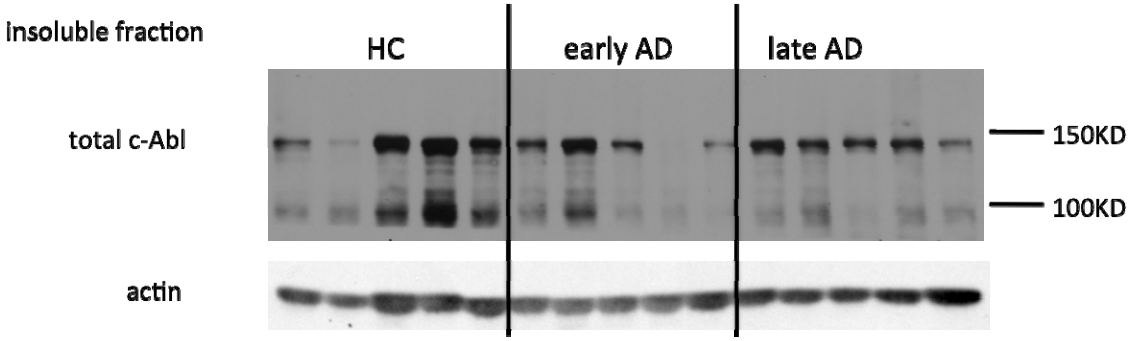
5.2.1 Expression and distribution of c-Abl in human hippocampus

5.2.1.1 c-Abl expression in hippocampus by western blot analysis

We first examined the protein levels of total c-Abl in healthy control, early- and late-stage AD subjects by immunoblot (see Materials and Methods for classification parameters). We used mouse monoclonal anti-c-Abl antibody, clone 8E9 to detect total c-Abl in tissue lysate. The 8E9 clone has been reported to react with an epitope in the tyrosine kinase domain of murine abl proteins (Wang et al, 1988). Total c-Abl was present predominantly in the insoluble fraction (Fig. 7A). Unfortunately, we did not detect any significant difference between healthy control and AD subjects within insoluble fractions (Fig. 7B). Its level in the soluble fraction was too low

to be detected (data not shown). This may be caused by the low sensitivity of this clone 8E9 antibody.

A.



B.

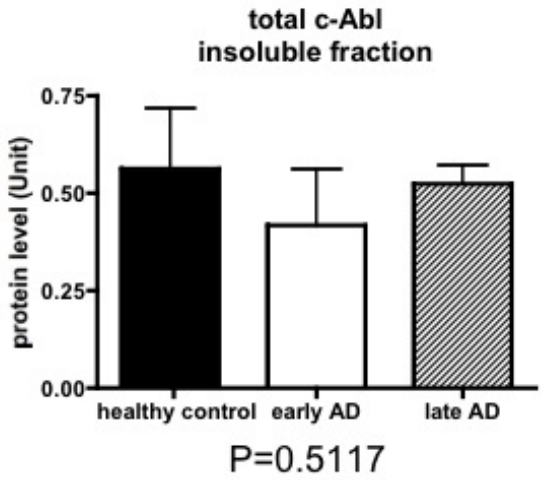


Figure 7 Western blot analysis for total c-Abl in human hippocampus.

Fresh frozen hippocampus from non-neurologic disease control and neuropathologically confirmed early- and late-stage AD cases were homogenized as described in Methods to general soluble and insoluble fractions. A) Equal amounts of protein from each case were loaded in gel lanes and immunolabeled using antibody specific to total c-Abl. The blot was also probed with anti-actin antibody as loading control. Lane 1-5 from control subjects, lanes 6-10 from early-stage AD subjects and lane 11-15 from late-stage AD subjects. B) Quantitative measurements of total c-Abl protein level in control and AD subjects, normalized by loading control (actin). Protein level was shown as mean±SE, n=5 for each group. The highest protein level among all

*15 cases was set to 1. Black column: healthy control, White column: early AD, grey column: late AD. Statistical analysis was performed by Kruskal-Wallis test (non-parametric one way ANOVA) across all subject groups (ANOVA P value was indicated in the figure), followed by post-hoc test to compare each pair of groups (marked by * when $p<0.05$ between the indicated groups). No significant difference across these three groups.*

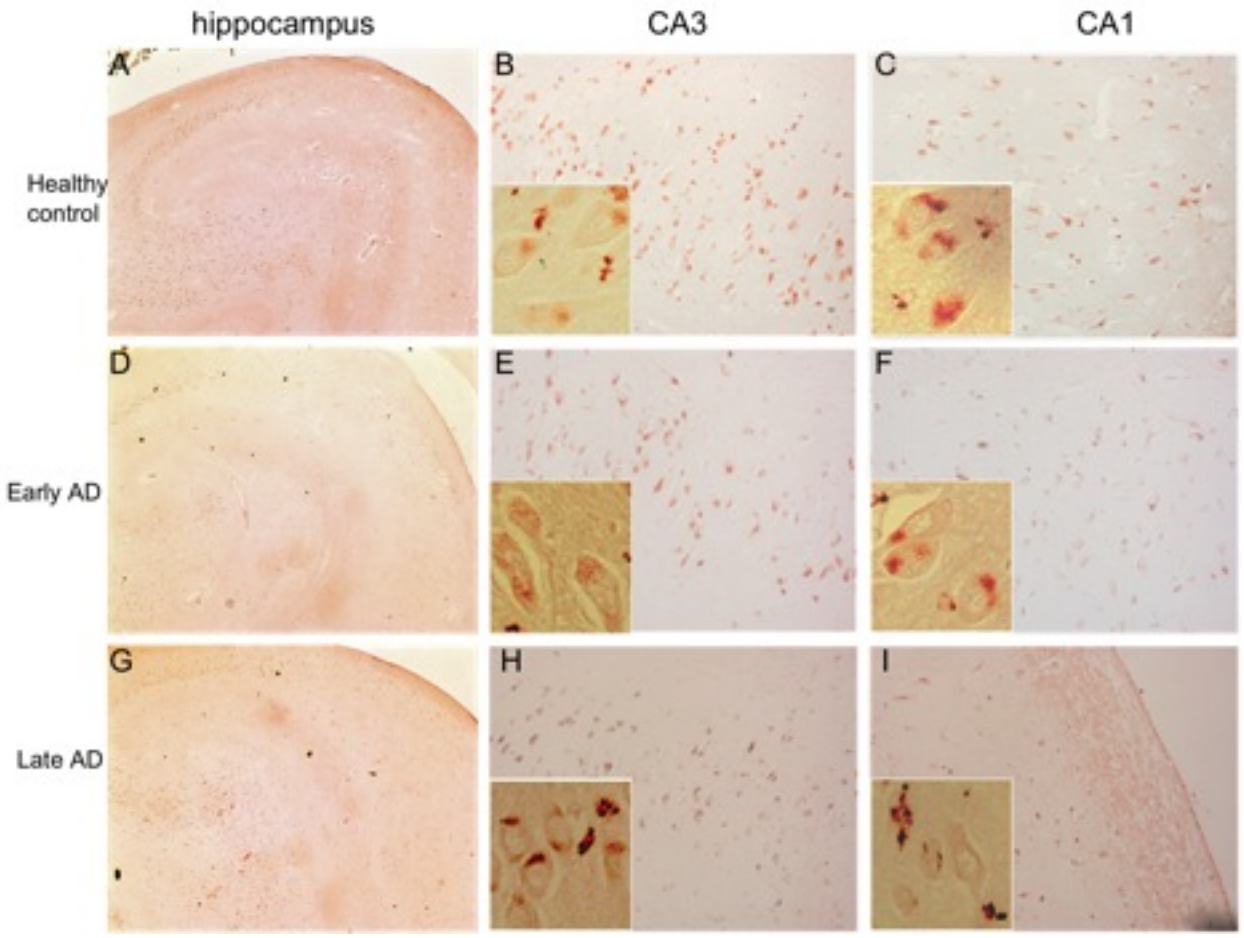
5.2.1.2 c-Abl distribution in hippocampus by IHC

For c-Abl immunohistochemistry we used an antibody different from the antibody we used for western blot analysis. This antibody recognizes a sequence close to the C-terminus of human c-Abl, whose binding is not directly impacted by the phosphorylation status of c-Abl. We detected c-Abl in the cytoplasm of pyramidal neurons throughout the hippocampus in all cases (Fig. 8A). No staining was observed in Dentate granule neurons (Fig. 8A). In healthy control and early-stage AD subjects, c-Abl was typically observed in punctate structures in the cell body of neurons (Fig. 8A). We also observed some granular staining outside the cell bodies of pyramidal neurons (Fig. 8A). Since c-Abl immunoreactivity was mainly observed in neuronal cell bodies and neuropil in both grey and white matter (Fig. 8A, panel I), the granular staining most likely were neuronal processes. In late stage AD, the staining pattern exhibited little difference in CA4 and CA3/CA2 compared to normal control or early AD. However, we observed a loss of diffuse cytoplasmic c-Abl immunoreactivity in CA1 neurons with increased granular staining, suggesting protein sequestration or transportation of c-Abl from cell body to neuronal processes (Fig. 8A, panel I). We failed to detect c-Abl in amyloid plaques or cytoskeletal pathology in any AD case.

The staining intensity of total c-Abl was determined by light microscopy with integrated software package (Simple PCI version 6.0, MicroBrightField Inc.), taking images of pyramidal

neurons from the same region of the CA3 and CA1 subfields in each case. Quantification demonstrated that the overall intensity of c-Abl immunoreactivity was lowest in hippocampi of early-stage AD comparing to non-demented healthy control and late-stage AD subjects. Within CA1 neurons, c-Abl immunoreactivity increased moderately in late AD, but was still much lower than in that in control subjects (Fig. 8B). The c-Abl immunoreactivity exhibited no significant difference between all three groups, healthy control, early-stage AD and late-stage AD groups (Fig. 8B). However, in CA3, c-Abl exhibited a significant difference across all the three groups (Fig. 8B).

A.



B.

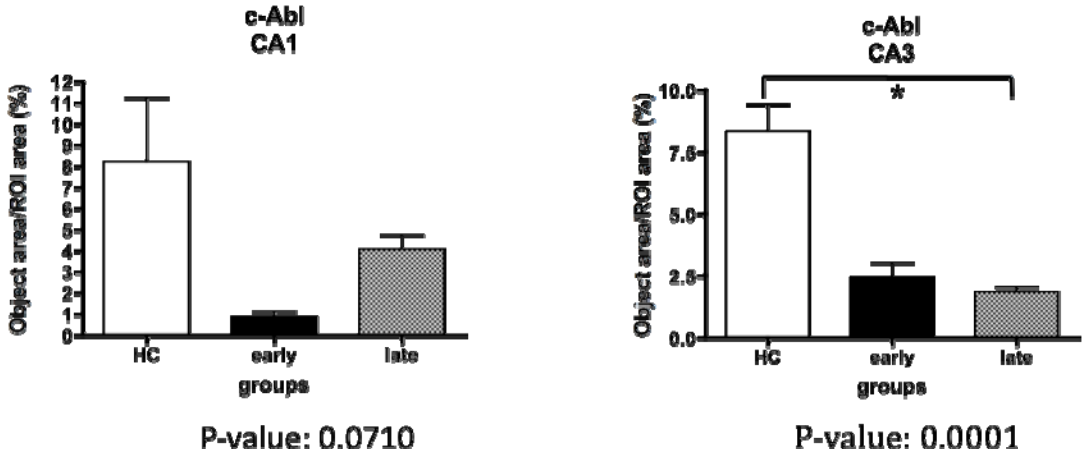


Figure 8 The subcellular distribution of total c-Abl in control, early- and late AD hippocampus.

A. *The expression of total c-Abl in human hippocampus. Whole hippocampus (left column), CA3 (middle column) and CA1 (Right column). Healthy controls (panel A-C), early stage AD (panel D-F), and late stage AD (panel G-I). Magnification for left column is 20X, middle and right columns is 200X, and inset magnification is 400X.*

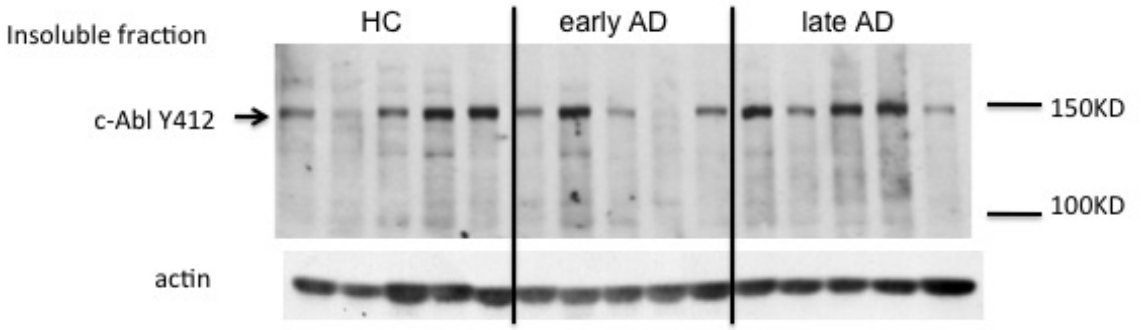
B. *Quantification result of total c-Abl immunoreactivity was shown by bar charts with the mean and standard error of each group. White bars denote the healthy control group, and the black and grey bars denote the early-stage AD and late-stage AD respectively. Statistical analysis was performed by ANOVA with comparisons across all three subjects groups, with $P < 0.05$ considered statistically significant. ANOVA P value was indicated in the figure. Immunoreactivity was shown as mean±SE, n=5 for each group. Post-hoc test was used to compare each pair of groups, showing a significant difference between healthy control and late AD. *, $p<0.05$ between the indicated groups.*

5.2.2 Expression and distribution of c-Abl, p-Y412 in human hippocampus

5.2.2.1 c-Abl, p-Y412 expression in hippocampus by western blot analysis

We first examined the protein levels of c-Abl, p-Y412 in the same healthy control, early- and late-stage AD subjects as we used before by immunoblot (see Materials and Methods for classification parameters) using an antibody produced against synthesized phospho-peptide derived from human c-Abl around the phosphorylation site of tyrosine 412 (D-T-Y^P-T-A), which detects endogenous levels of c-Abl only when phosphorylated at tyrosine 412. C-Abl, p-Y412 was detected in the insoluble fractions and showed significant differences across three sample groups (Fig. 9A, B). The protein level of c-Abl, p-Y412 in the insoluble fraction was significantly decreased during early AD compared to healthy control, and it showed a trend for increased c-Abl, p-Y412 in late-stage AD although the difference was not statistically significant (Fig. 9B). A total of 15 cases were used for this analysis. The signal of c-Abl, p-Y412 in the soluble fraction was too low for reliable detection.

A.



B.

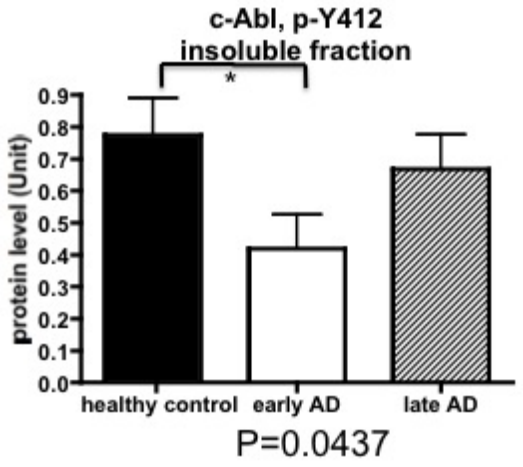


Figure 9 Western blot analysis for c-Abl, p-Y412 in human hippocampus.

Fresh frozen hippocampus from non-neurologic disease control and neuropathologically confirmed early- and late-stage AD cases were homogenized as described in Methods to general soluble and insoluble fractions. A) Equal amounts of protein from each case were loaded in gel lanes and immunolabeled using antibodies specific to c-Abl, p-Y412. The blot was also probed with anti-actin antibody as a loading control. Lane 1-5 from control subjects, lanes 6-10 from early-stage AD subjects and lane 11-15 from late-stage AD subjects. B) Quantitative measurements of c-Abl, p-Y412 protein level in control and AD subjects, normalized by loading

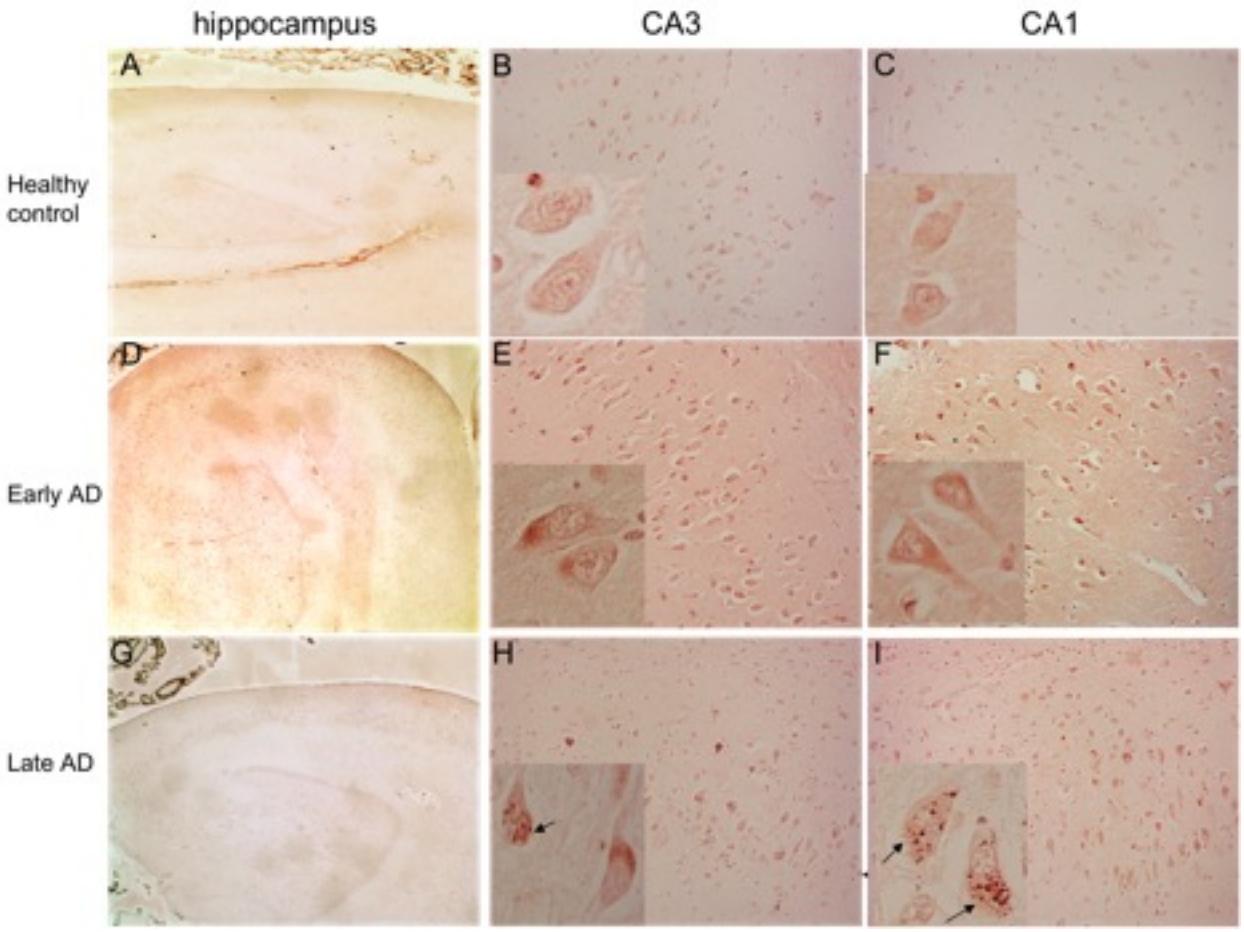
*control (actin). Protein level was shown as mean±SE, n=5 for each group. The highest protein level among all 15 cases was set to 1 for different fractions respectively. Statistical analysis was performed by Kruskal-Wallis test (non-parametric one way ANOVA) across all subject groups (ANOVA P value was indicated in the figure and the difference considered significant when P < 0.05.), followed by post-hoc test to compare each pair of groups (marked by * when p<0.05 between the indicated groups.*

5.2.2.2 c-Abl, p-Y412 distribution in hippocampus by IHC

We next examined the distribution of c-Abl, p-Y412, the phosphorylated form of c-Abl indicating c-Abl kinase activation, in the hippocampus by immunohistochemistry (IHC) using the same antibody. In healthy control and early AD cases, c-Abl, p-Y412 was predominantly distributed in neuronal cell bodies (Fig. 10A, panel A-F). The overall staining intensity was increased in the CA1 subfield in early-AD (Fig. 10A, panel F). Interestingly, we observed granular staining of c-Abl, p-Y412 within neuronal cell bodies surrounded by clear vacuoles (indicated by arrows in Fig. 10A, panel I). The structures share morphological characters with GVDs that are most often detected in hippocampal pyramidal neurons in AD (Tomlinson and Kitchener 1972; Okamoto, Hirai et al. 1991). c-Abl, p-Y412 was detected within GVD bodies throughout the CA4 to CA1 subfields in late-stage AD (Fig. 10A, panel H, I). In CA1, c-Abl, p-Y412 was predominantly localized in GVDs (Fig. 10A, panel I). All the c-Abl, p-Y412 positive neurons are GVD positive. In CA3, the percentage of GVD positive neuron varies greatly between subjects (52.56±20.66, mean±SE, n=5). No amyloid plaque or neurofibrillary tangle staining was observed using this anti-c-Abl, p-Y412 antibody. Low levels of nuclear immunoreactivity were observed throughout the hippocampus in all cases.

The staining intensity of phospho-c-Abl-Y412 isoforms was determined using a light microscope with integrated software package (Simple PCI version 6.0, Hamamatsu Corporation) by taking images of pyramidal neurons from the same region of the CA3 and CA1 subfields in each case. Contrary to our findings with total c-Abl, analysis showed that the overall immunoreactivity of c-Abl, p-Y412 in early-stage AD is higher than that in control and late-stage AD subjects, and there is a significant difference in CA1 between all three groups, suggesting this activated form of c-Abl accumulates during the early-stage of AD and more related to CA1 subfield (Fig. 10B). The apparent reduction of c-Abl, p-Y412 in late-stage AD may be due to its sequestration into GVDs.

A.


hippocampus
CA3
CA1
Healthy control
Early AD
Late AD
A
B
C
D
E
F
G
H
I

B.

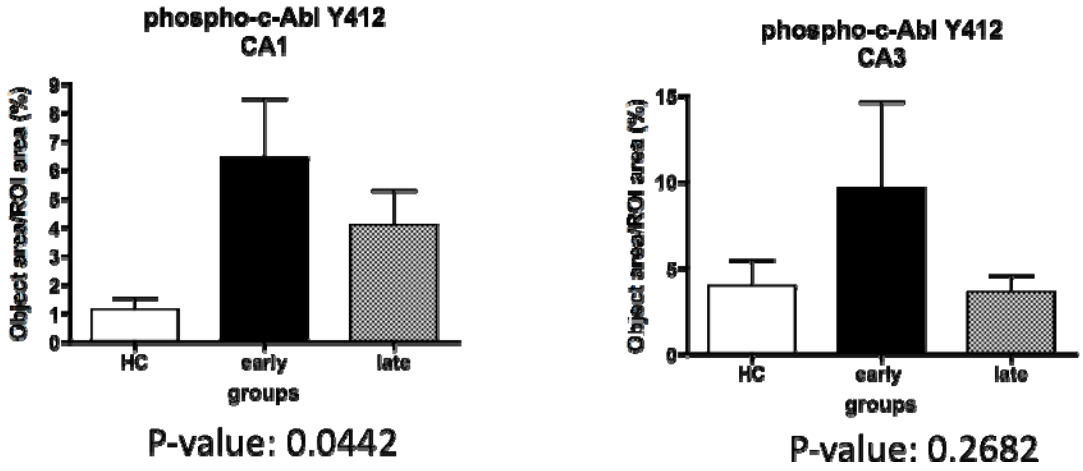


Figure 10 The distribution of c-Abl Y412 in control, early AD and late AD hippocampus.

A. *Immunohistochemistry of c-Abl Y412 in human hippocampus. Whole hippocampus (left column), CA3 (middle column) and CA1 (Right column). Healthy controls (panel A-C), early stage AD (panel D-F), and late stage AD (panel G-I). Magnification for left column is 20X, middle and right columns is 200X, and inset magnification is 400X. C-Abl, p-Y412 was observed in both a granular staining and GVDs in neuronal cell bodies as indicated by arrows (insets in H and I).*

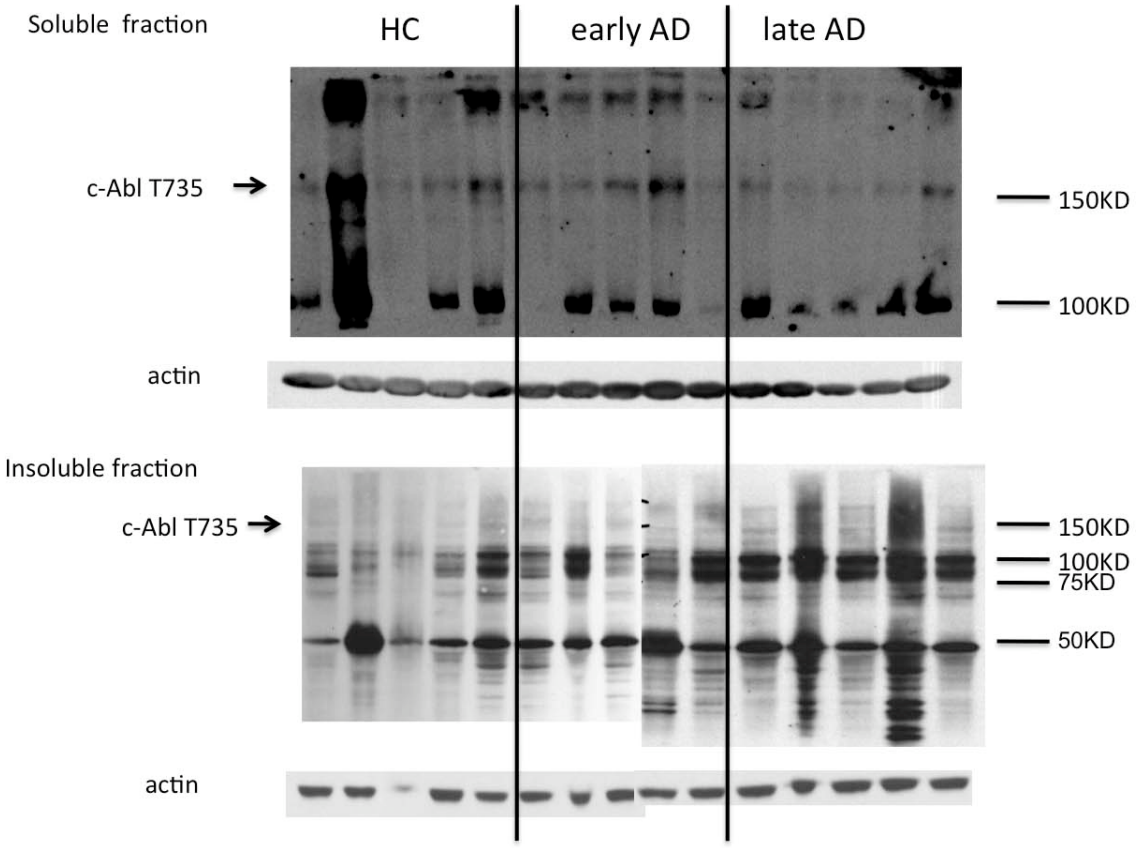
B. *Quantification result of c-Abl, p-Y412 immunoreactivity was shown by bar charts with the mean and standard error of each group. White bars denote the healthy control group, and the black and grey bars denote the early-stage AD and late-stage AD respectively. Statistical analysis was performed by ANOVA with comparisons across all three subjects groups, with $P < 0.05$ considered statistically significant. ANOVA P value was indicated in the figure. Immunoreactivity was shown as mean±SE, n=5 for each group. Post-hoc test showed no significant difference ($p<0.05$) between any two groups.*

5.2.3 Expression and distribution of c-Abl, p-T735 in human hippocampus

5.2.3.1 c-Abl, p-T735 expression in hippocampus by western blot analysis

We next examined the protein levels of c-Abl, p-T735 in the same healthy control, early- and late-stage AD subjects as we used before by immunoblot (see Materials and Methods for classification parameters). The antibody we used for phospho-c-Abl Thr735 detects endogenous levels of c-Abl only when phosphorylated at threonine 735. C-Abl, p-T735 was detected in soluble fraction (Fig. 11A, top panel). We did not detect any significant difference between healthy control and AD subjects in the soluble fraction (Fig. 11B). The bands corresponding to c-Abl, p-T735 were too weak to be quantified in the insoluble fraction (Fig. 11A, bottom panel). A total of 15 cases were used for this analysis.

A.



B.

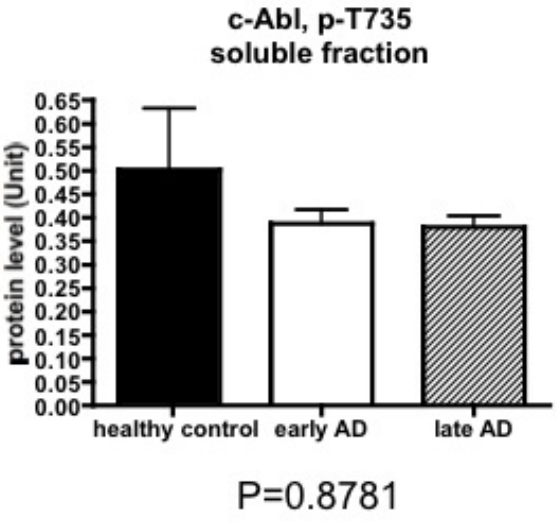


Figure 11 Western blot analysis for c-Abl, p-T735 in human hippocampus.

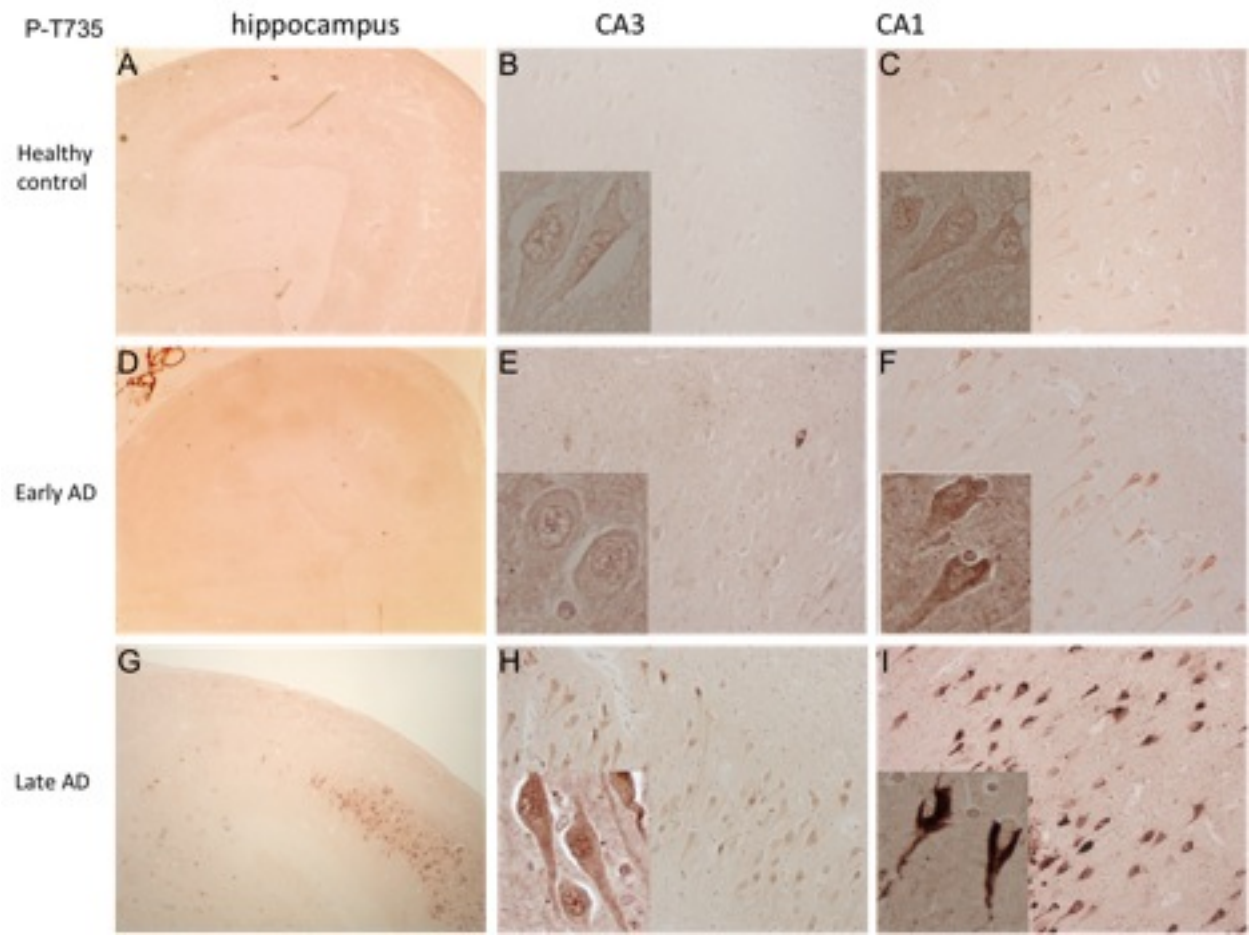
Fresh frozen hippocampus from non-neurologic disease control and neuropathologically confirmed early- and late-stage AD cases were homogenized as described in Methods to generate soluble and insoluble fractions. A) Equal amounts of protein from each case were loaded in 8% SDS-PAGE gel for soluble fraction, loaded in 4-12% SDS-PAGE gel (invitrogen) for insoluble fraction. The blot was probed with antibodies specific to c-Abl, p-T735, and then reprobed with anti-actin antibody as loading control. Lane 1-5 from control subjects, lanes 6-10 from early-stage AD subjects and lane 11-15 from late-stage AD subjects. B) Quantitative measurements of c-Abl, p-T735 protein level in control and AD subjects, normalized by loading control (actin). Statistical analysis was performed by ANOVA across all subject groups, and the difference considered significant when $P < 0.05$. ANOVA P value was indicated in the figure. Protein level was shown as mean±SE, n=5 for each group. The highest protein level among all 15 cases was set to 1.

5.2.3.2 c-Abl, p-T735 distribution in hippocampus by IHC

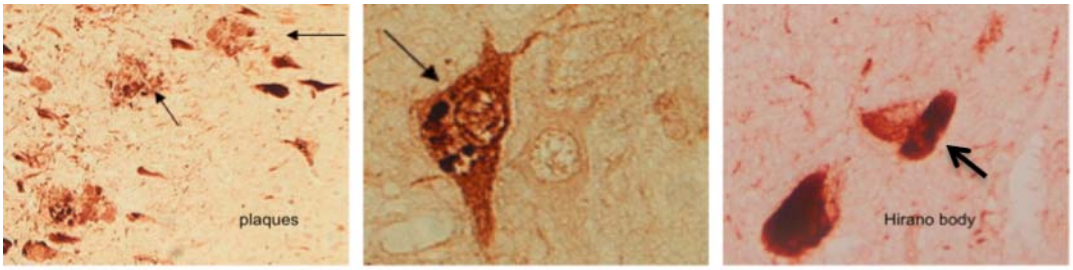
Phosphorylation of c-Abl on threonine residue 735 (T735) is required for binding to 14-3-3 protein and regulates the nuclear translocation of c-Abl (Yoshida, Yamaguchi et al. 2005). Therefore, we determined the presence and distribution of c-Abl, p-T735 in the hippocampus of AD and control subjects by immunohistochemistry using the same antibody we used for western blot analysis. In healthy control, c-Abl, p-T735 showed very weak immunoreactivity in hippocampal pyramidal neuron cell bodies (Fig. 12A, panel A-C). In early-stage AD, c-Abl, p-T735 showed increased cytoplasmic immunoreactivity in CA1 (Fig. 12A, panel D-F). However, in late-stage AD, c-Abl, p-T735 displayed increased cytoplasmic immunostaining in CA1 pyramidal neurons compared to healthy control and early-stage AD (Fig. 12A, panel I). Notably, the immunoreactivity of c-Abl, p-T735 in CA3, CA4 and dentate gyrus (DG) of late-stage AD hippocampus was reduced to the levels observed in control subjects (Fig. 12A, panel G), indicating that c-Abl, p-T735 may contribute to AD pathology specifically in the CA1 subfield. In the CA1, subiculum and entorhinal cortex, amyloid plaques and NFTs were detected by this antibody (Fig. 12A, panel I, inset; Fig. 12B, left panel). In addition, GVDs were also immunostained by anti-c-Abl, p-T735 antibody in CA1 and cerebral cortex (Fig. 12B, middle panel). Interesting, we also observed Hirano body immunoreactivity in late-stage AD subjects, indicating that c-Abl T735 is sequestered in Hirano bodies during AD (Fig. 12B, right panel). We also observed nuclear immunoreactivity in hippocampal neurons of all cases (represented in Fig. 12A, panel A-H). Approximal 80% neurons in CA3 exhibited a slight nuclear staining. C-Abl, p-T735 immunoreactivity was completely eliminated by preincubation of the antibody with blocking peptide (Fig. 12C).

The staining intensity of phospho-c-Abl-T735 was determined using a light microscope with integrated software package (Simple PCI version 6.0, Hamamatsu Corporation) by taking images of pyramidal neurons from the same region of the CA3 and CA1 subfields in each case. For c-Abl, p-T735, immunoreactivity in the CA3 exhibited no statistically significant difference between different groups. However, in CA1 the immunoreactivity of c-Abl, p-T735 increased moderately and significantly in early-stage AD comparing to control subjects. It was also increased in last-stage AD dramatically and the difference between all three groups is statistically significant (Fig. 12D).

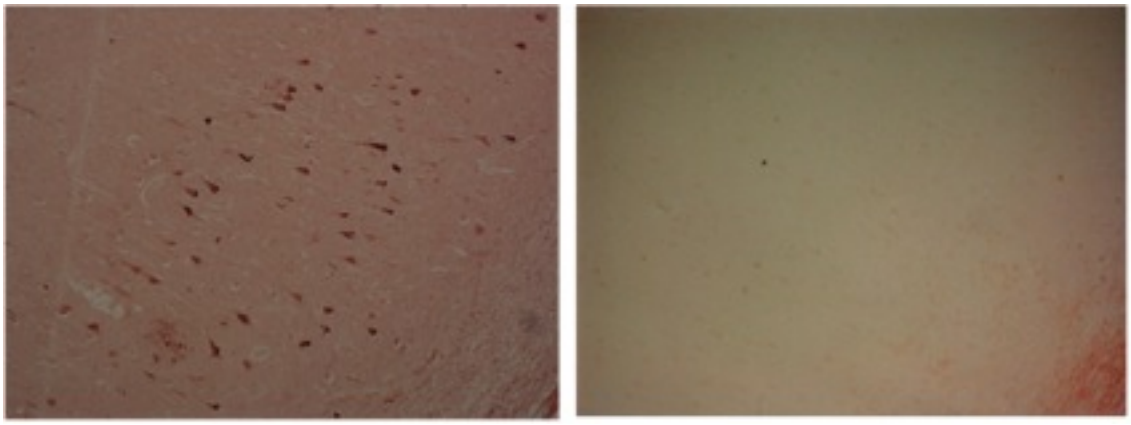
A.


P-T735
hippocampus
CA3
CA1
A
B
C
Healthy control
D
E
F
Early AD
G
H
I
Late AD

B.


plaques
Hirano body

C.



D.

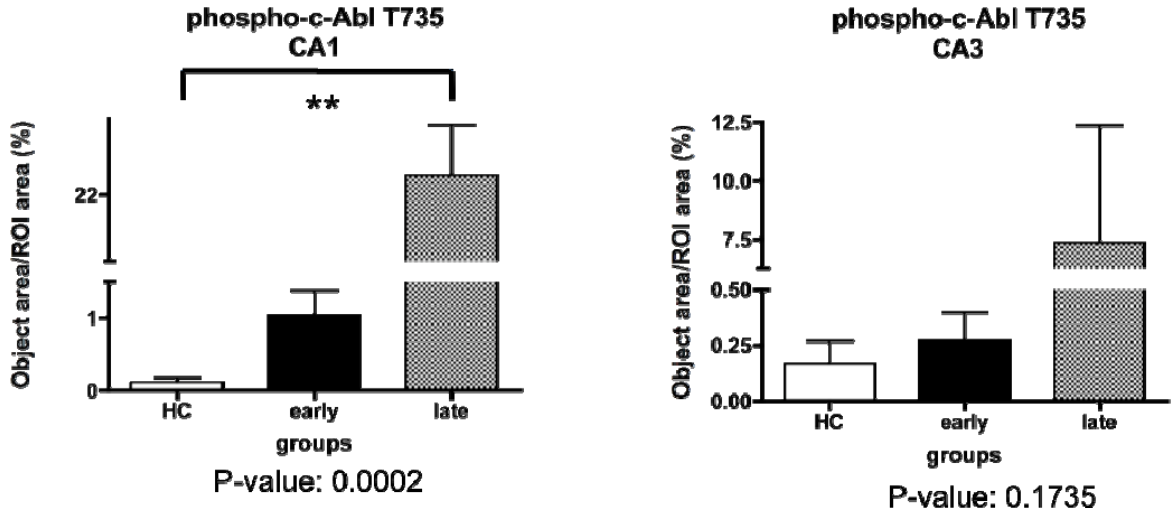


Figure 12 The distribution of c-Abl, p-T735 in control, early AD and late AD hippocampus.

A. Immunohistochemistry of c-Abl T735 in human hippocampus. Whole hippocampus (left column), CA3 (middle column) and CA1 (Right column). Healthy controls (panel A-C), early stage AD (D-F), and late stage AD (G-I). Magnification for left column is 20X, middle and right columns is 200X, and inset magnification is 400X.

B. c-Abl, p-T735 localized to neuropathologic hallmarks of AD. A) c-Abl, p-T735 localized to plaques and NFTs (magnification: 200X). B) c-Abl, p-T735 localized to GVDs (magnification: 400X). C) c-Abl, p-T735 localized to Hirano bodies (magnification: 400X).

C. A commercially available blocking peptide for phospho- c-Abl T735 was utilized. Left panel: AD hippocampus slide incubated with anti-c-Abl, p-T735 antibody alone; Right panel: brain tissue was immunolabeled with antibody containing a 3-fold volume (>100 molar excess) of specific blocking peptide as per manufacturer guidelines. All the staining in left panel was blocked and disappeared in the right panel, indicating that the antibody is specific to its epitope and the staining is not non-specific induced by secondary antibody.

*D. Quantification result of c-Abl, p-T735 immunoreactivity was shown by bar charts with the mean and standard error of each group. White bars denote the healthy control group, and the black and grey bars denote the early-stage AD and late-stage AD respectively. Statistical analysis was performed by ANOVA with comparisons across all three subjects groups, with $P < 0.05$ considered statistically significant. ANOVA P value was indicated in the figure. Immunoreactivity was shown as mean±SE, n=5 for each group. Post-hoc test was used to compare each pair of groups, showing a significant difference between healthy control and late AD. **, $p<0.01$ between the indicated two groups.*

5.2.3.3 Co-localization of c-Abl, p-T735 with AD pathology

To further examine and confirm the co-localization of c-Abl, p-T735 with AD pathology, we performed confocal microscopy for c-Abl, p-T735 with amyloid plaques, labeled by anti-Aβ antibody, or with AT8 labeled tau pathology, including NFTs and GVDs. We observed that in CA1 pyramidal neurons c-Abl, p-T735 co-localized with phospho-tau in NFTs (panel A-C) and

GVDs (panel D-F) within pyramidal neurons, but not localized in phospho-tau positive dystrophic neurites (Fig. 13, panel A-C). Within CA1 and CA3 of AD hippocampus, all the p-T735 c-Abl positive neurons are phospho-tau positive, and all the phospho-tau positive neurons with a staining pattern like GVD are p-T735 c-Abl positive. However, there are approximal 10-20% phospho-tau positive neurons exhibiting a diffuse staining pattern, which are p-T735 c-Abl negative. C-Abl, p-T735 was also observed within amyloid plaques, although it was not co-localized with amyloid, suggesting c-Abl, p-T735 containing neurites were sequestered into and surrounding amyloid plaques during AD (Fig. 13, panel G-I). All the amyloid plaques we observed within AD hippocampus contain p-T735 c-Abl positive punctate immunoreactivity.

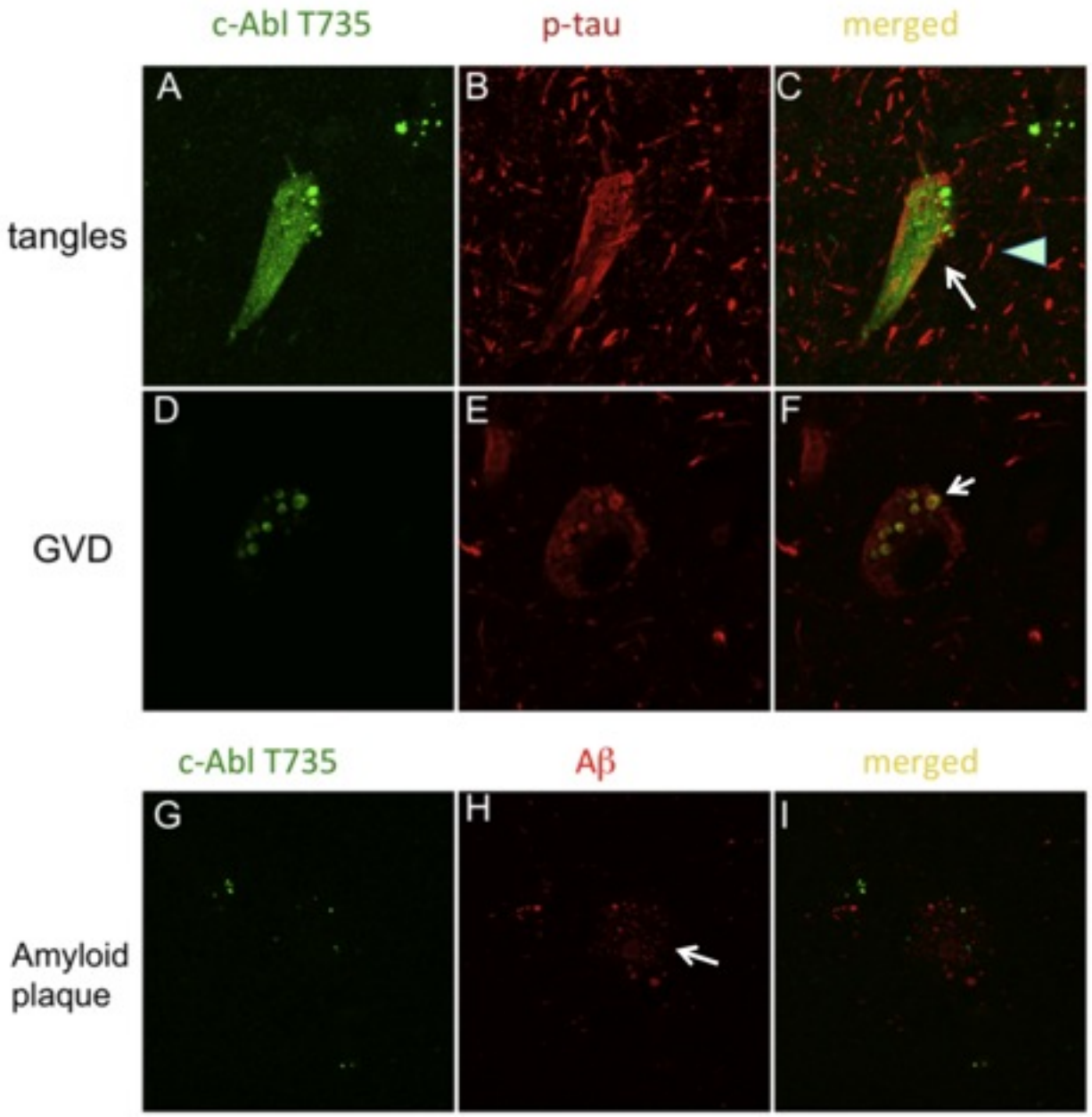


Figure 13 Confocal microscopy of c-Abl, p-T735 with tau and β-amyloid in late stage AD.

Panel A-F: *Double-label confocal microscopy using antibodies to c-Abl T735 and phospho-tau (AT8). (Magnification: 600X.) c-Abl T735 co-localizes with NFTs (indicated by arrow) but not p-tau positive dystrophic neurites (indicated by arrowhead) (panels A-C). Phosphorylated tau and c-Abl T735 co-localize to GVDs (indicated by arrow, panels D-F). (green, panel A & D: c-Abl T735, red, panel B & E: AT8, yellow, panel C & F: merged).* ***Panel G-I:*** *Double-label*

confocal microscopy for c-Abl T735 and beta-amyloid. Punctate c-Abl T735 immunoreactivity is contained within amyloid plaques, although c-Abl T735 is not co-localized with Aβ . (green, panel G: c-Abl T735, red, panel H: Aβ, yellow, panel I: merged. Amyloid plaque indicated by arrow).

5.3 CONCLUSION AND DISCUSSION

In summary, the highest level of total c-Abl immunoreactivity occurs in healthy control hippocampus. c-Abl, p-Y412 exhibited a most intensive staining during early-stage of AD. However, another phosphorylated form of c-Abl, c-Abl, p-T735, showed increased immunoreactivity specifically in the CA1 subfield of late stage AD. Since the phosphorylation of c-Abl might modulate its function, these results suggest the functional status of c-Abl is altered throughout different stages of AD disease progression. In addition, phospho-c-Abl co-localizes with phospho-tau within AD pathology.

c-Abl is a non-receptor tyrosine kinase that shuttles between the cytoplasm and nucleus and functions in multiple physiological and pathological processes (Woodring, Hunter et al. 2003). In the cytoplasm, c-Abl regulates the cytoskeleton through interactions with actin, whereas in the nucleus it can induce apoptosis in response to DNA damage (Woodring, Hunter et al. 2003). Cultured neurons were rescued from Aβ induced toxicity via inhibiting c-Abl activity by a specific c-Abl inhibitor, STI571, or by c-Abl RNAi (Alvarez, Sandoval et al. 2004). We observed increased levels of c-Abl, p-Y412 in early stage AD, with increased c-Abl, p-T735 in late stage AD (Fig. 10 and 12). Activation of c-Abl during early AD may induce downstream phosphorylation of proteins such as GSK-3β, and collectively contribute to tau phosphorylation

and NFT formation. Further studies are required to test this hypothesis. The increased levels of c-Abl, p-T735 during late AD may represent additional c-Abl phosphorylation that occurs upon c-Abl accumulation in NFTs and GVDs. However the relationship between phosphorylation at Thr735 and the kinase activity of c-Abl is not clear yet.

Although our immunohistochemistry results demonstrated that c-Abl was predominantly in the cytoplasm of pyramidal neurons, we did observe a nuclear immunostaining of c-Abl, p-Y412 and T735 within some hippocampal neurons in AD. This result is consistent with prior studies that showed a portion of c-Abl colocalized with nuclear DNA in mouse neuroepithelial cells, while the majority of c-Abl was located in the cytoplasm (Koleske, Gifford et al. 1998). Our results do not exclude the possibility of the involvement of nuclear c-Abl in the pathogenesis of AD, as c-Abl may participate in nuclear events contributing to apoptosis during AD (Caltagarone, Jing et al. 2007). Alternatively, c-Abl may contribute to APP/Fe65-mediated gene transcription by direct interaction with Fe65 (Perkinton, Standen et al. 2004). In addition, activated c-Abl may only transiently enter the nucleus, thereby making its detection in post-mortem tissues much more difficult. Our data suggest a preferential sequestration of c-Abl, p-Y412 into GVD.

GVD is considered a protective mechanism during early stage of apoptosis via degrading mutant or toxic proteins (Levine and Yuan 2005). Sequestering c-Abl in GVDs may represent a neuroprotective response as has been suggested for other proteins located in GVDs (Mott and Hulette 2005).

The rabbit polyclonal anti-total c-Abl antibody we used for IHC is against a sequence close to the carboxy-terminus of human c-Abl. This antibody should recognize c-Abl independent of its phosphorylation status, though no evidence is currently available to confirm or

refute this assumption. In this study we observed an immunostaining pattern distinct from that seen using antibodies to phosphorylated forms of c-Abl, indicating that c-Abl phosphorylation may induce protein conformational changes that impede binding of c-Abl C-terminal antibody. Therefore, it is possible that this antibody only recognizes non-phosphorylated form of c-Abl when used for IHC. We failed to immunolabel neuropathologic hallmarks of AD with the c-Abl antibody to non-phosphorylated protein, suggesting phosphorylation of c-Abl may be required for its re-distribution to pathologic hallmarks of AD. A punctate staining pattern of total c-Abl in AD hippocampus was observed using an antibody against a sequence within C-terminal of c-Abl. Unfortunately, we were unable to use the anti-C-terminal c-Abl antibody in confocal microscopy experiments and therefore could not further characterize the c-Abl positive granules using this antibody to non-phosphorylated c-Abl.

We also observed c-Abl, p-Y412 in GVDs in the CA1 subfield of late AD (Fig. 10A, panel I). GVDs, a special type of autophagosome, are considered a marker for AD (Mott and Hulette 2005). Prior studies have shown that the GVD granules are immunoreactive with antibodies to phosphorylated tau (Bondareff, Wischik et al. 1991). A recent *in vitro* study demonstrated that c-Abl phosphorylated tau at Tyr394 and this specific phosphorylated form of tau was identified in human brain by mass spectrometry (Derkinderen, Scales et al. 2005). Thus, we propose that c-Abl activation, as denoted by phosphorylation at Y412, occurs during early AD and may contribute to tau phosphorylation and subsequent formation of GVDs during AD. This hypothesis is further pursued in Chapter 7.

We also examined the protein level of total and phosphorylated c-Abl in human hippocampus by performing western blot analysis. Different from the results of IHC, the protein levels of total c-Abl and c-Abl, p-T735 did not show a statistically significant difference between

groups (Fig. 7B & 11B). However, we noticed the antibodies against those two proteins always recognize another band at 100kD (Fig. 7A & 11A, bottom panel). Interestingly, the changes of intensity of this 100kD bands between groups seem similar to the IHC results, showing the highest intensity in healthy control group for total c-Abl and the highest intensity in late AD group for c-Abl, p-T735. Future studies using mass spectrometry based amino acid sequencing must be performed to identify the protein.

In summary, this study represents the first examination of the expression and distribution of c-Abl in human AD brain, and indicates the functional status and subcellular distribution of c-Abl is altered during AD progression. Further functional studies of c-Abl and downstream signaling proteins are currently underway and may provide novel therapeutic targets for AD and other neurodegenerative diseases.

6.0 C-ABL IS ASSOCIATED WITH TAU PHOSPHORYLATION IN AD

6.1 INTRODUCTION

Based on the IHC results in Chapter 5, we hypothesize that c-Abl may contribute to tau phosphorylation during AD. To test this hypothesis, we next examined the correlation, the co-localization and the interaction of phospho-c-Abl with p-tau. In addition, we investigated one possible mechanism which may link c-Abl's function to tau phosphorylation in AD.

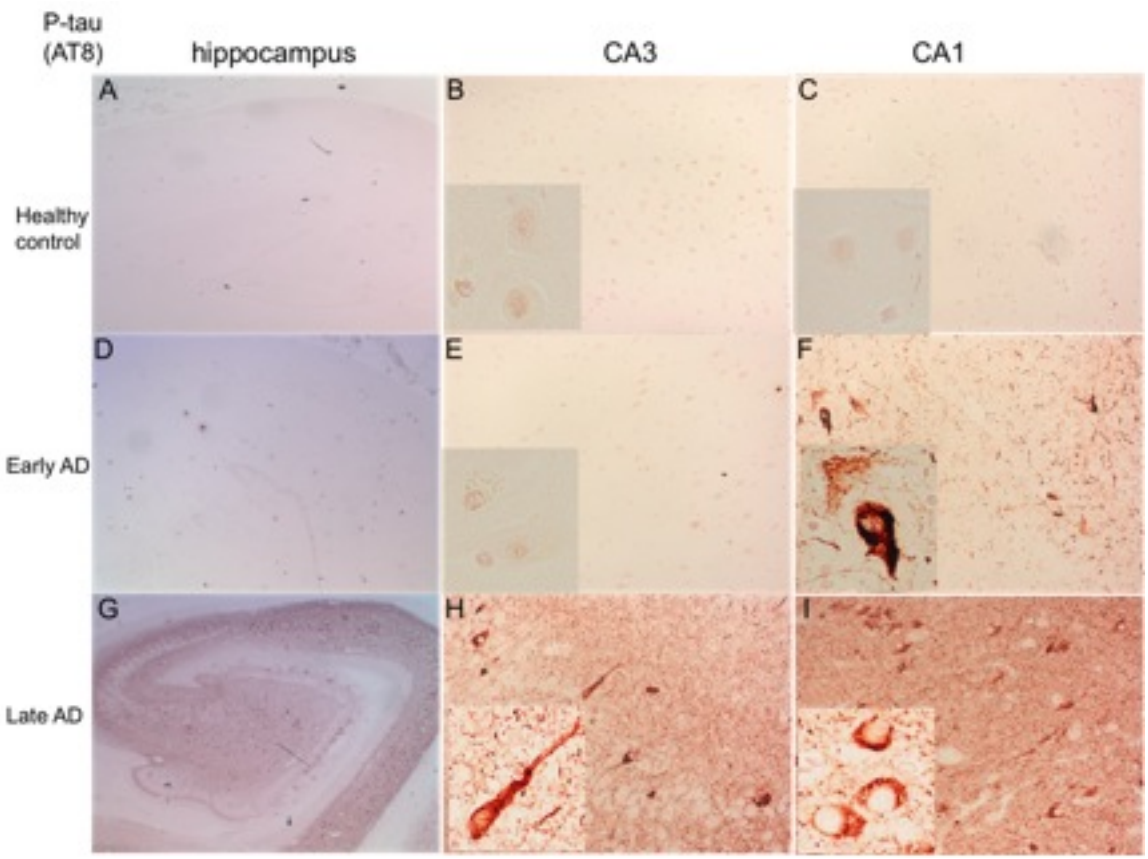
6.2 RESULTS

6.2.1 Immunoreactivity of phospho-c-Abl is correlated with phospho-tau

To determine if the immunoreactivity of phospho-c-Abl has a correlation with p-tau, we performed IHC using anti-p-tau antibody (AT8) with consecutive tissue slides from the same subjects we used for IHC study for c-Abl (Fig. 14A). We quantified the immunoreactivity of tau in CA1 and CA3 subfields of hippocampus of each case using the same method as described in the Materials and Methods and Chapter 5 (Fig. 14B). Our results showed a much stronger immunoreactivity of AT8 in CA1 compared to CA3 in AD subjects. This is consistent with other previous published data (Kitamura, Sugimori et al. 2005), in which the authors tested several

antibodies against different isoforms of p-tau and found AT8-positive NFT was graded as CA1 = entorhinal cortex = subiculum > CA2 = CA3 = CA4.

A.



B.

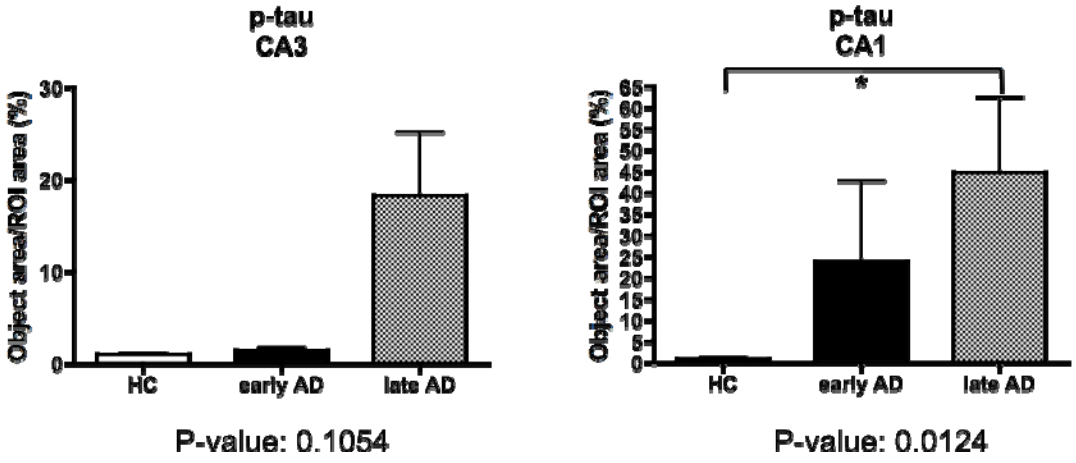


Figure 14 The distribution of p-tau in control, early AD and late AD hippocampus.

A. Immunohistochemistry of p-tau in human hippocampus. Whole hippocampus: left column, CA3 (middle column) and CA1 (right column). Healthy controls (panel A-C), early stage AD (panel D-F), and late stage AD (panel G-I). Magnification for left column is 20X, middle and right column is 200X, and inset magnification is 400X.

*B. Quantification result of p-tau immunoreactivity was shown by bar charts with the mean and standard error of each group. White bars denote the healthy control group, and the black and grey bars denote the early-stage AD and late-stage AD respectively. Statistical analysis was performed by ANOVA with comparisons across all three subjects groups, with $P < 0.05$ considered statistically significant. Post-hoc test was used to compare each pair of groups, showing a significant difference between healthy control and late AD. **, $p<0.01$ between the indicated two groups. Immunoreactivity was shown as mean±SE, n=5 for each group.*

The immunoreactivity of phospho-c-Abl was used as the value of X-axis, and the immunoreactivity of p-tau of the same subject was used as the value of Y-axis. Therefore, we

plotted a total of 15 data points representing the immunoreactivity of phospho-c-Abl and phospho-tau for each subjects. Then, we performed linear regression to test the correlation between phospho-c-Abl and phospho-tau.

We noted a positive correlation between c-Abl, p-Y412 and phospho-tau immunostaining within the CA1 across the subject groups (r=0.2667, p=0.0586), suggesting that as the immunoreactivity of c-Abl, p-Y412 is increased, there was a corresponding increase of tau phosphorylation. No significant correlation was detected between these two proteins in the CA3 subfield (r=0.0422, p=0.4813) (Fig. 15). In addition, phosphorylated c-Abl T735 also showed a positive correlation with phospho-tau in both the CA1 subfields (r=0.4893, p=0.0054) and CA3 subfields (r=0.6677, p=0.0002) (Fig. 15).

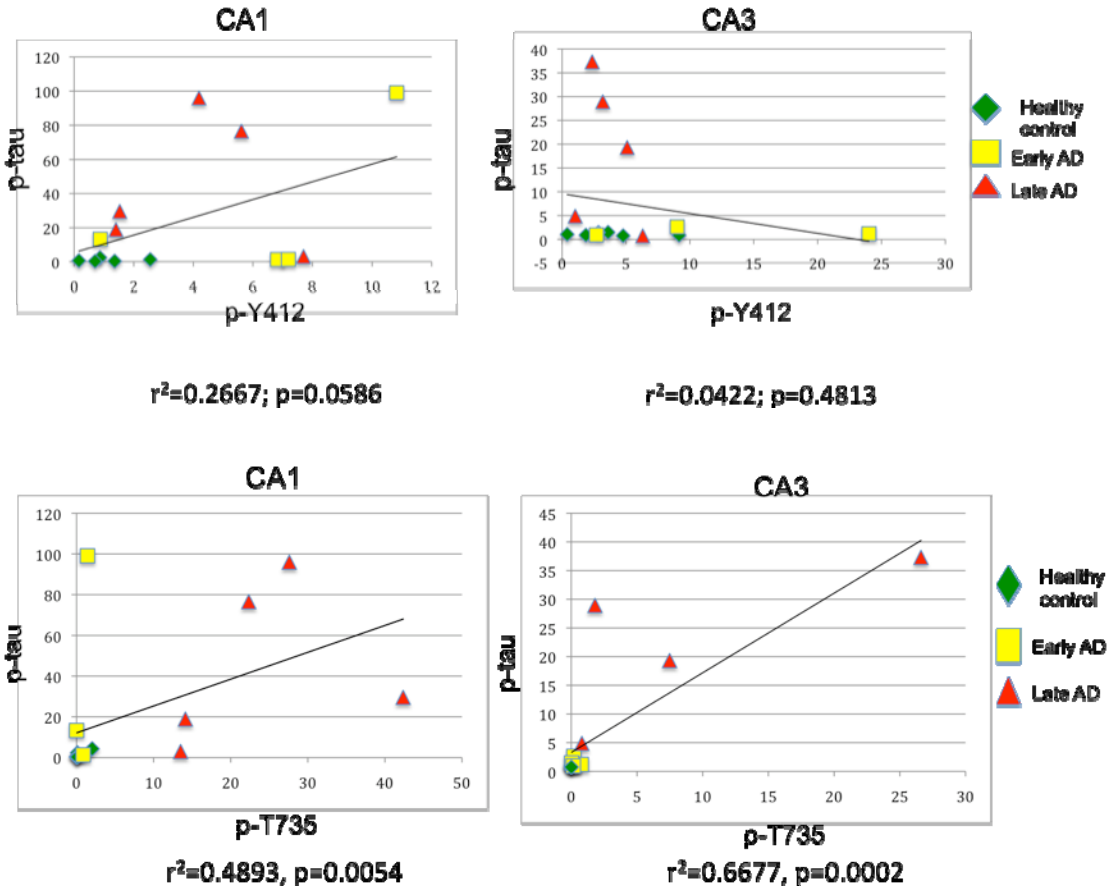


Figure 15 Correlation of phospho-c-Abl and p-tau.

Top panel showed the correlation of c-Abl, p-Y412 and p-tau in CA1 and CA3 subfields of human hippocampus as indicated. Bottom panel showed the correlation of c-Abl, p-T735 and p-tau in CA1 and CA3 subfields of human hippocampus as indicated. The corresponding value of r^2 *and p was shown below the linear regression curve. Statistical significance considered when* $p < 0.1$.

6.2.2 Co-localization of phospho-c-Abl with phospho-tau in AD hippocampus

We examined the co-localization of phospho-c-Abl isoforms with phospho-tau during AD by double-label laser confocal microscopy. The results demonstrated that c-Abl, p-Y412 co-localized with phosphorylated tau in GVDs within NFTs bearing neurons and co-localized within the tau-positive granules within amyloid plaques in late AD hippocampus (Fig. 16). We used the same antibody as we used for IHC to perform immunofluorescence staining. Unfortunately, this antibody did not work well for immunofluorescence. We have to use TSA method (as described in materials and methods) to amplify the signal. Even though, only very few neurons in AD hippocampus showed as p-Y412 c-Abl positive. We observed a co-localization of p-Y412 c-Abl and phospho-tau within all these p-Y412 c-Abl positive neurons, although almost of phospho-tau positive neurons are p-Y412 c-Abl negative. As described in Chapter 5, c-Abl, p-T735 displayed co-localization with hyperphosphorylated tau in both GVD and NFT structures (Fig. 13, panel A-F). Again, as we described before, within CA1 and CA3 of AD hippocampus, all the p-T735 c-Abl positive neurons are phospho-tau positive, and all the phospho-tau positive neuron with a staining pattern like GVD are p-T735 c-Abl positive. However, there are approximal 10-20% phospho-tau positive neurons exhibiting a diffuse staining pattern p-T735 c-Abl negative. Punctate c-Abl, p-T735 immunostaining was also observed within amyloid plaques (Fig. 13, panel G-I), although it was not co-localized with amyloid. All the amyloid plaques we observed within AD hippocampus contain p-T735 c-Abl positive punctate immunoreactivity.

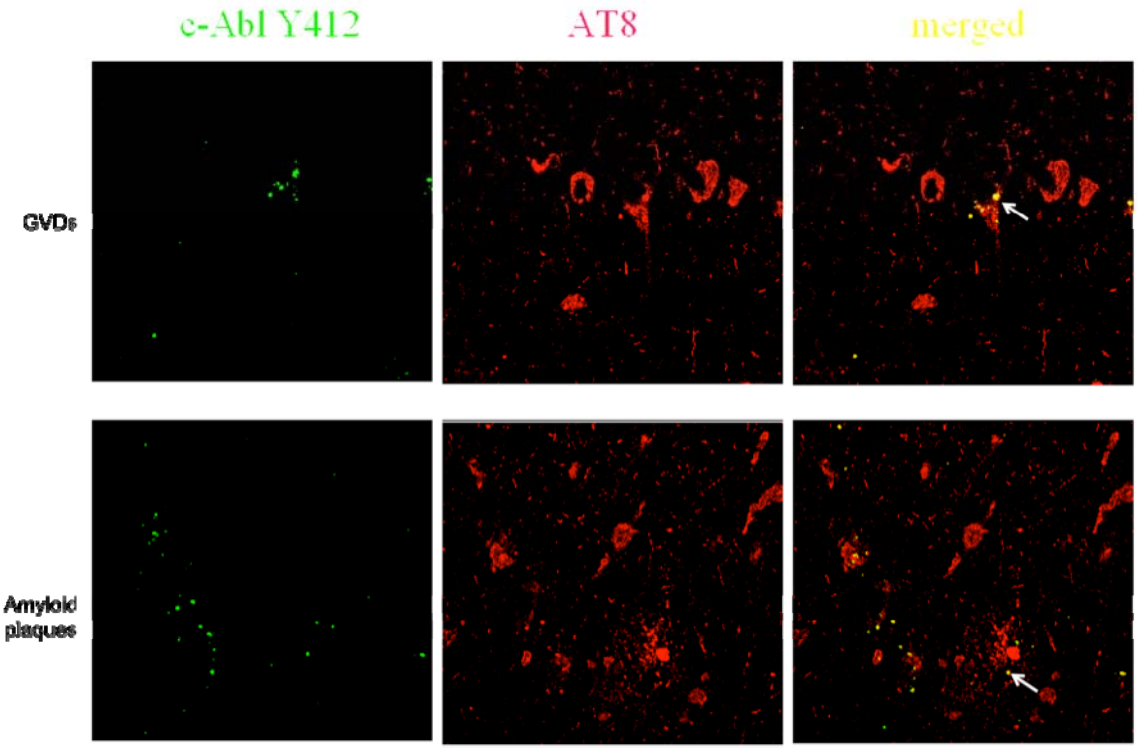


Figure 16 Confocal microscopy of c-Abl, p-Y412 and phospho-tau in CA1 late stage AD.

Top panel: c-Abl Y412 co-localized with phospho-tau (labeled by AT8) in GVDs within tangle-bearing pyramidal neurons (arrow in merged image). (Magnification: 400X.) Bottom panel: c-Abl Y412 co-localized with phospho-tau (labeled by AT8) in punctate structures within AT8 positive amyloid plaques (arrow in merged image). (green: c-Abl Y412, red: p-tau, yellow: merged. Magnification: 400X.)

6.2.3 Interaction of c-Abl and phospho-tau in AD hippocampus

6.2.3.1 Co-IP method establishment

Co-IP is one of the most common techniques for the analysis of the protein-protein interactions. Traditionally, protein-G or protein-A sepharose beads that bind most antibodies are used to precipitate the complex of the protein of interest and its specific antibody, which is added

into the cell lysate prior to the precipitation. In this traditional method, the non- binding protein is typically removed by multiple wash steps. However loss of beads and inefficient removal of non-specific proteins often occurs using this technique. MACS® Technology (Miltenyi Biotec) provides a convenient and easy way to precipitate proteins efficiently and specifically using μMACS Protein A/G MicroBeads which does not need centrifugation steps. μMACS™ Protein A and μMACS Protein G MicroBeads are colloidal, super-paramagnetic MicroBeads, which are conjugated to Protein A or Protein G, respectively. Instead, the protein complex from cell lysates is passed over a separation column placed in the magnetic field of a MACS Separator. The protein-antibody-beads complex is retained within the column while other proteins are efficiently washed away. Moreover, the size of the MicroBeads is extremely small comparing to agarose beads, only 50 nm in diameter, allowing a more effective protein-protein interaction.

We compared the magnetic beads with more traditional agarose beads for precipitating our proteins of interest. We tested the interaction of c-Abl and paxillin using agarose and magnetic beads, since these two proteins have been shown to be associated following integrin activation in fibroblast (Lewis and Schwartz 1998). Figure 17, top panel shows that c-Abl only can be precipitated by paxillin when magnetic beads are used. The results indicated the magnetic bead method is superior for detecting proteins interacting with c-Abl under our experimental system. In addition, the results also demonstrated that more interacting proteins can be detected in total lysate of human hippocampus tissue, instead of the separated soluble fraction, suggesting the interaction of c-Abl and at least paxillin tested in this study may occur solely in the insoluble fraction. Similarly, pax was also precipitated by itself at the highest level when magnetic beads and total homogenate used (Fig. 17, bottom panel). However, such a comparison was the result

of one experiment. This experiment must be repeated at least three times to confirm this initial observation.

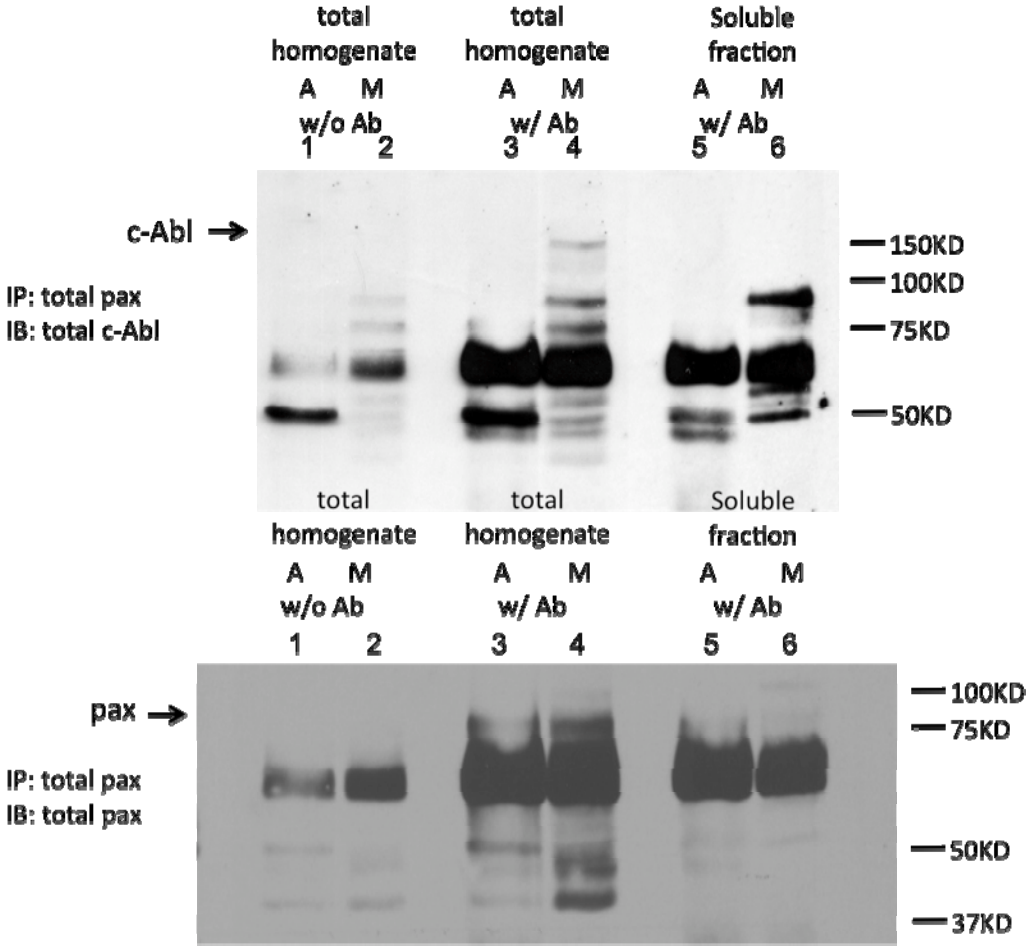


Figure 17 Direct comparison of agarose and magnetic beads (n=1).

Co-immunoprecipitation was performed using hippocampal tissue extracts from late AD subjects. Proteins were immunoprecipitated with anti-total pax antibody from hippocampal tissue homogenates and precipitated proteins separated by SDS-PAGE. The resulting blot was probed with anti-total c-Abl antibody (top panel) and reprobed with anti-total pax antibody (bottom panel). From left to right, lane 1 and 2 are total homogenate of AD hippocampus without antibody as a negative control; lane 3 and 4 are total homogenate of AD hippocampus with antibody as indicated; lane 5 and 6 are soluble fraction of AD hippocampus with antibody

as indicated. Lane 1, 3 and 5 denoted the co-IP using agarose beads (marked as "A"). Lane 2, 4 and 6 denoted the co-IP using magnetic beads (marked as "M"). No IgG is shown in this panel since different species antibodies used for immunoprecipitation and immunoblot (IP: rabbit, IB: mouse).

6.2.3.2 Interaction of c-Abl and phospho-tau in AD hippocampus

To further characterize direct protein interactions with c-Abl, we immunoprecipitated c-Abl from late AD and control hippocampus and detected co-precipitation of phosphorylated tau in AD but not control brain (Fig. 18, top panel). We also performed the reverse IP to confirm the interaction between total c-Abl and p-tau. We tried repeating these findings using 2 additional AD and 2 additional control subjects, but the results showed equivalent levels of c-Abl in the control and AD IP lanes. This lack of reproducibility is perhaps due to homogenization of the whole tissue. Possibly better results would be obtained by microdissecting out only the specific hippocampal regions of interest (CA1 or CA3), or by using laser capture microdissection to remove only pyramidal neurons from a specific region of the hippocampus.

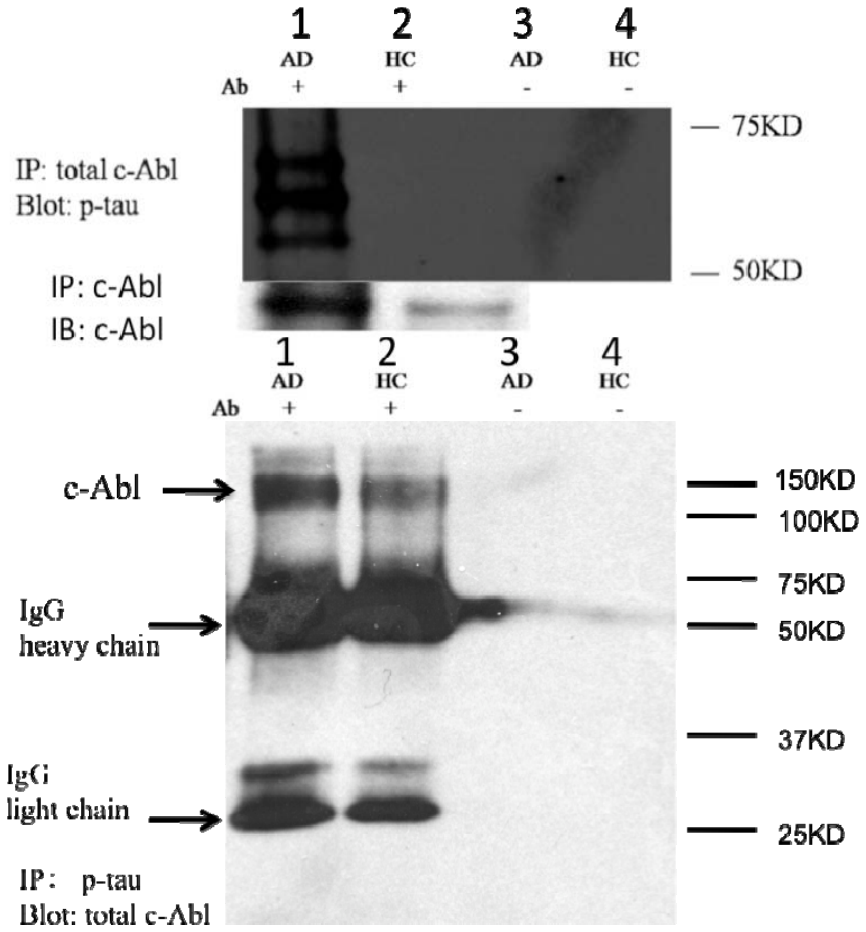


Figure 18 Total c-Abl interacts with phospho-tau in AD brain.

Co-immunoprecipitation was performed using tissue extracts from healthy controls or late AD subjects. ***Top panel:*** *Proteins were immunoprecipitated with anti-total c-Abl antibody from hippocampal tissue homogenates and precipitated proteins were separated by SDS-PAGE. The resulting blot was probed with anti-phospho-tau antibody (AT8). Lane 1: AD subject, lane 2: healthy control subject, lane 3 and lane 4: No antibody negative controls (no antibody used for immunoprecipitation). N=1. The blot was also probed with antibodies against total c-Abl to demonstrate the precipitation was successful and to normalize the amount of p-tau precipitated by total c-Abl.* ***Bottom panel****: Proteins were immunoprecipitated with AT8 antibody from hippocampal tissue homogenates and precipitated proteins were separated by SDS-PAGE. The*

resulting blot was probed with anti-total c-Abl antibody. Lane order as same as the top panel.

In addition, we also showed an association between phosphorylated tau and both c-Abl, p-Y412 and T735 in AD hippocampi (Fig. 19, top panel). Additional repetitions of this experiment are needed to confirm this result. p-Tau was also precipitated by total c-Abl while with a weaker band compared to figure 18, top panel, which may be caused by a decrease in the total amount of tissue used for co-IP this time. We also performed the reverse IP (IP: p-tau, IB: c-Abl, p-Y412 or c-Abl, p-T735). The reverse experiment demonstrated that phospho-c-Abl was also precipitated by p-tau in both healthy control and AD subjects (Fig. 19, bottom panel). An input should be added for the reserve IP experiment.

Tau protein is present in all neurons and therefore in all cases, and phospho-dephosphorylation reactions are constantly occurring in all cells. Phosphorylated tau displays a series of bands between 55 – 74 kDa, with additional higher molecular weight multimers in the absence of strong detergents and denaturing conditions (6M urea). We observed additional AT8 immunoreactive bands in this healthy control subject (HC) than typically expected (Fig. 19, top panel input HC lane). We currently do not know why this case exhibits the additional immunoreactive species, as healthy control subjects typically exhibit fewer and weak immunoreactive bands by immunoblot. An IHC examination of this case may help to determine the presence of excessive p-tau in this case and if additional glial cells are immunopositive in this case, since the tissue lysates will contain proteins from all cells in the tissue. This additional data may also help explain why c-Abl was immunoprecipitated by p-tau in this healthy control subject (Fig. 18, bottom panel). An immunoprecipitation control (IP: AT8, IB: AT8) should be performed to demonstrate the level of p-tau immunoprecipitated in AD and this HC

subject. Therefore additional experiments using more human subjects are required to confirm or refute these initial findings.

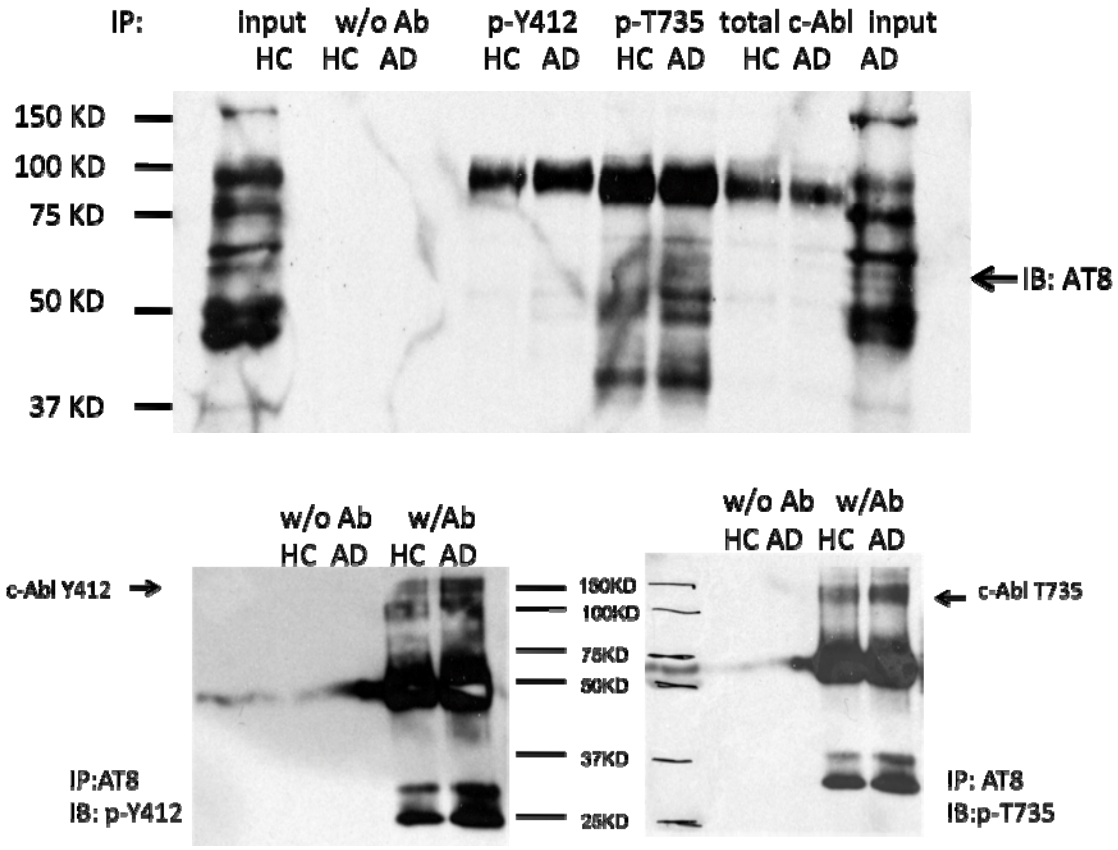


Figure 19 Phospho-c-Abl interact with p-tau in human hippocampus (n=1).

Co-immunoprecipitation was performed using 100 ul of a 20% hippocampal tissue homogenate from healthy controls or late AD subjects. ***Top panel:*** *Proteins were immunoprecipitated with anti-c-Abl, p-Y412 or T735 antibody, or total c-Abl antibody as indicated from hippocampal tissue homogenates and precipitated proteins separated by SDS-PAGE. The resulting blot was probed with anti-phospho-tau antibody (AT8). In each panel, left lane is healthy control subject. Right lane is AD subject. No IgG is shown in this panel since different species antibodies used for immunoprecipitation and immunoblot.* ***Bottom panel****: proteins were immunoprecipitated*

from hippocampal tissue homogenates using anti-phospho-tau antibody and the resulting blot probed with anti-c-Abl, p-Y412 or p-T735 antibody. In each panel, first two lanes are no antibody negative controls, right two lanes are AT8 precipitation lanes with healthy control subject and AD subject as indicated.

6.2.4 c-Abl is associated with the integrin signaling pathway possibly via paxillin

In tissue, the extracellular matrix (ECM) regulates many aspects of cellular function including growth, proliferation, survival, differentiation, morphology, migration, and death (Howe, Aplin et al. 1998; Jin, Peel et al. 2004). The ECM signals through cell surface integrins, which mediate both cell/ECM and cell/cell adhesions, although they do not contain intrinsic enzymatic activities (Lambert, Stevens et al. 1994; Howe, Aplin et al. 1998; Zhao, Reiske et al. 1998; Williamson, Scales et al. 2002). Instead, they associate with numerous intracellular effectors, cell adhesion molecules (CAMs). Activated integrins induce CAM activation by tyrosine phosphorylation during the initial stages of cell adhesion (de Curtis and Malanchini 1997; Zhao, Pestell et al. 2001; Kasahara, Koguchi et al. 2002; Brown and Turner 2004). These CAMs assemble into immature less dense peripherally located focal adhesion (FA) complexes and mature into more dense centrally located FA complexes associated with actin stress fibers (Calalb, Polte et al. 1995; Kasahara, Koguchi et al. 2002). More than 50 CAMs are localized to FAs coupling to the actin cytoskeleton and regulating the structural components of the FAs to efficiently organize multiple signaling pathways. Structural CAMs include actin, α-tubulin, hydrogen peroxide inducible clone 5 (hic-5), paxillin, Crk associated substrate (p130cas), talin, tensin, vinculin, and zyxin. Signaling CAMs include FAK, Fyn, phosphoinositide-3 (PI-3) kinase, c-Abl, Crk, Csk, Grb-2, Nck, and PYK2 (Lambert, Stevens et al. 1994; Zhao, Reiske et

al. 1998; Jin, Peel et al. 2004). Many of these signaling CAMs are tyrosine kinases known to be upstream of serine/threonine kinases including members of the mitogen activated protein kinase (MAPK) pathway, cdk5, and GSK-3β (Lambert, Stevens et al. 1994; Howe, Aplin et al. 1998; Kasahara, Koguchi et al. 2002; Cordes and Meineke 2004; Jin, Peel et al. 2004). In addition, protein tyrosine phosphatases (PTP-1D, PTP-PEST, and PTP-1B) have been shown to dephosphorylate CAMs upon cell detachment and regulate FA turnover (Lambert, Stevens et al. 1994; Howe, Aplin et al. 1998; Giancotti and Ruoslahti 1999; Jin, Peel et al. 2004). CAMs localized to FAs have been shown to be involved in bidirectional signaling including the compartmentalization of integrin activated downstream signaling molecules regulating "outside-in-signaling" and the localization and affinity of integrin receptors to regulate "inside-out signaling" (Howe, Aplin et al. 1998; Zhao, Reiske et al. 1998; Cordes and Meineke 2004; Jin, Peel et al. 2004; Verdier and Penke 2004). Ultimately, cell adhesion signaling through integrins and FAs can impact the viability of the cell.

Typically, specific ECM molecules bind to integrin cell surface receptors and activate downstream FA cell adhesion molecules (CAMs) involved in the regulation of anchorage-dependent cell survival signals (Giancotti and Ruoslahti 1999; Aplin 2003; Cordes and Meineke 2004; Verdier and Penke 2004). Integrins and numerous FA CAMs are expressed in all cells throughout the brain and numerous studies indicate a role for integrin signaling in neurite outgrowth during differentiation and in response to the toxic effects associated with neurodegeneration (Leventhal and Feldman 1996; de Curtis and Malanchini 1997; Nishiya, Tachibana et al. 2001; Brown and Turner 2004; Jin, Peel et al. 2004). Integrins have been shown to bind Aβ and activate FAs through integrin clustering, CAM mobilization, and/or cooperatively with growth factor signaling through cell surface growth factor receptors (Sonoda,

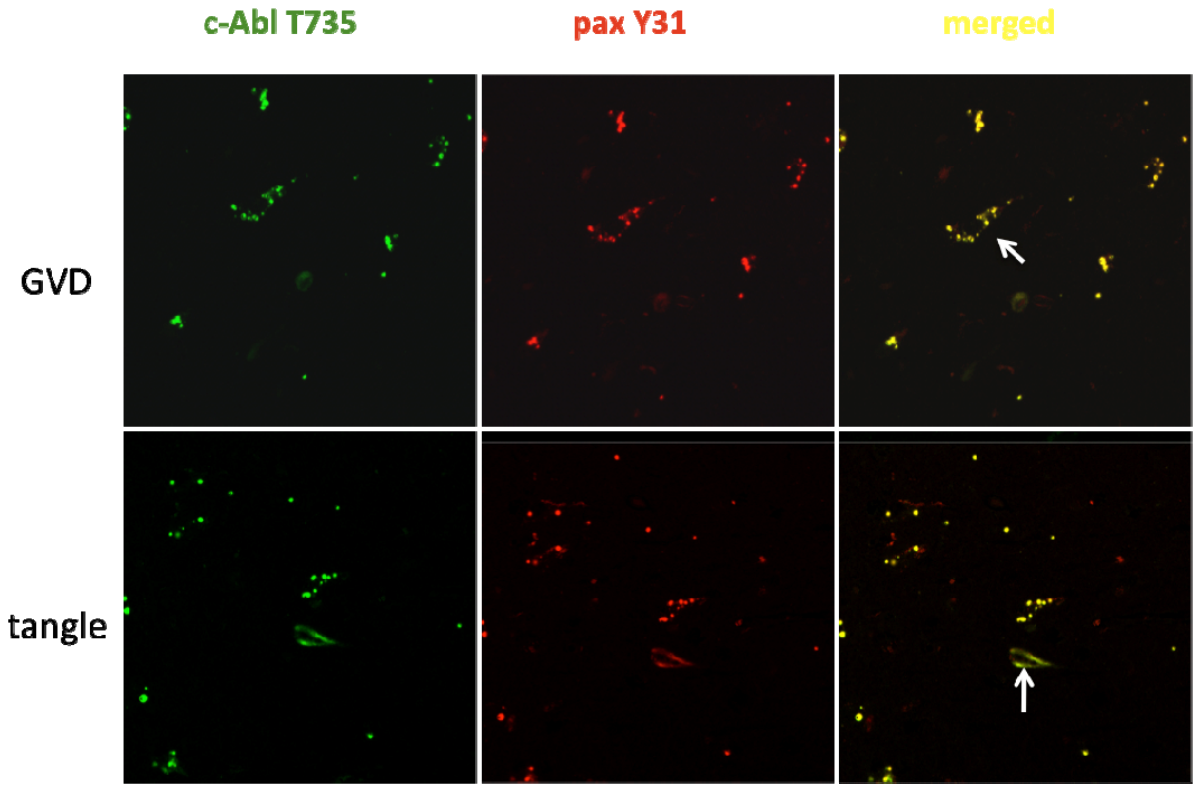
Watanabe et al. 1999; Nishiya, Tachibana et al. 2001; Gilmore 2005; Reddig and Juliano 2005). Specifically, if fibrillar Aβ is added to cultured cells, both FAK and paxillin are rapidly phosphorylated leading to downstream signaling events that can regulate cell viability (Sonoda, Watanabe et al. 1999; Nishiya, Tachibana et al. 2001; Reddig and Juliano 2005). Neuronal viability and synaptic loss during the course of AD and potentially other neurodegenerative disorders may be mediated through FA signaling. As mentioned above, c-Abl can interact with FA proteins (see Chapter 2) and therefore we investigated direct interactions between c-Abl and FA proteins in control and AD brains.

Paxillin is an adaptor protein in focal adhesions (Mitra, Hanson et al. 2005). Paxillin interacts and serves as a substrate of adhesion-integrin activated c-Abl (Lewis and Schwartz 1998). It recruits both structural and signaling molecules to focal adhesions and transducing signals from adhesions and growth factors to regulate cell migration and gene expression (Brown and Turner 2004). Grace and Busciglio reported that fibrillar Aβ induced neuronal dystrophy is mediated by activation of focal adhesion proteins and required paxillin (Grace and Busciglio 2003).

Paxillin contains a proline-rich region in its amino terminal, which may permit binding to c-Abl. Near this proline-rich region there are two known paxillin tyrosine phosphorylation sites, Y31 and Y118. Phosphorylation at these two tyrosines by FAK and Src generates docking sites for SH2 domain containing proteins and facilitates signal transduction (Brown and Turner 2004). Interestingly, by performing confocal microscopy, we found that phospho-paxillin Y31 co-localized with c-Abl, p-T735 at both NFTs and GVDs, while phospho-paxillin Y118 only co-localized with c-Abl, p-T735 at GVDs but not NFTs, supporting our hypothesis that c-Abl and paxillin interact and indicating the binding preference between different phosphorylated forms of

c-Abl and paxillin due to their localizations at different AD pathologies (Fig. 20). Since both antibodies for c-Abl, p-T735 and phospho-pax are rabbit, we had to use TSA signal amplification method to perform this double staining (refer to Methods and Materials). The antibody for c-Abl, p-T735 was first incubated with the tissue slide and amplified by TSA, thus the signal of c-Abl, p-T735 is much stronger than phospho-pax. However, such co-localization was observed in all phospho-pax positive tangles or GVDs. Unfortunately, the anti-c-Abl, p-Y412 and anti-phospho-paxillin antibodies were the same isotype and double-label confocal microscopy was not successful using this antibody combination.

A.



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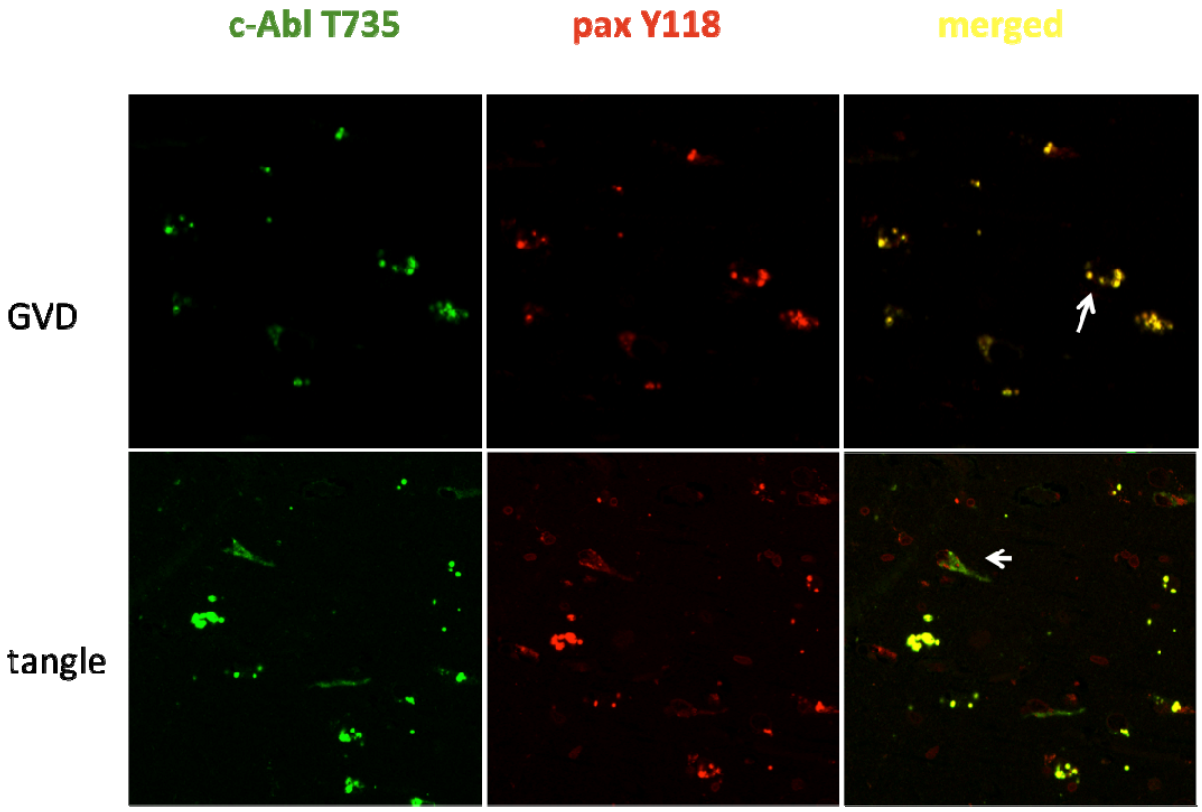


Figure 20 Co-localization of c-Abl, p-T735 and phospho-pax in CA1 of AD hippocampus.

A. C-Abl, p-T735 co-localized with pax, p-Y31 in both GVDs (top panel) and tangles (bottom panel). Magnification: 400X. B. C-Abl, p-T735 co-localized with pax, p-Y118 in GVDs (top panel), but not in tangles (bottom panel) (green: c-Abl, p-T735, red: pax, p-Y31 or Y118, yellow: merged). Magnification: 400X.

We determined the interactions of paxillin and c-Abl in the hippocampus of control and AD subjects by co-immunoprecipitation (Fig. 21). The results were inconclusive. Repetitions of this experiment and reverse IP need to be performed to obtain reliable and reproducible results from the IP experiments.

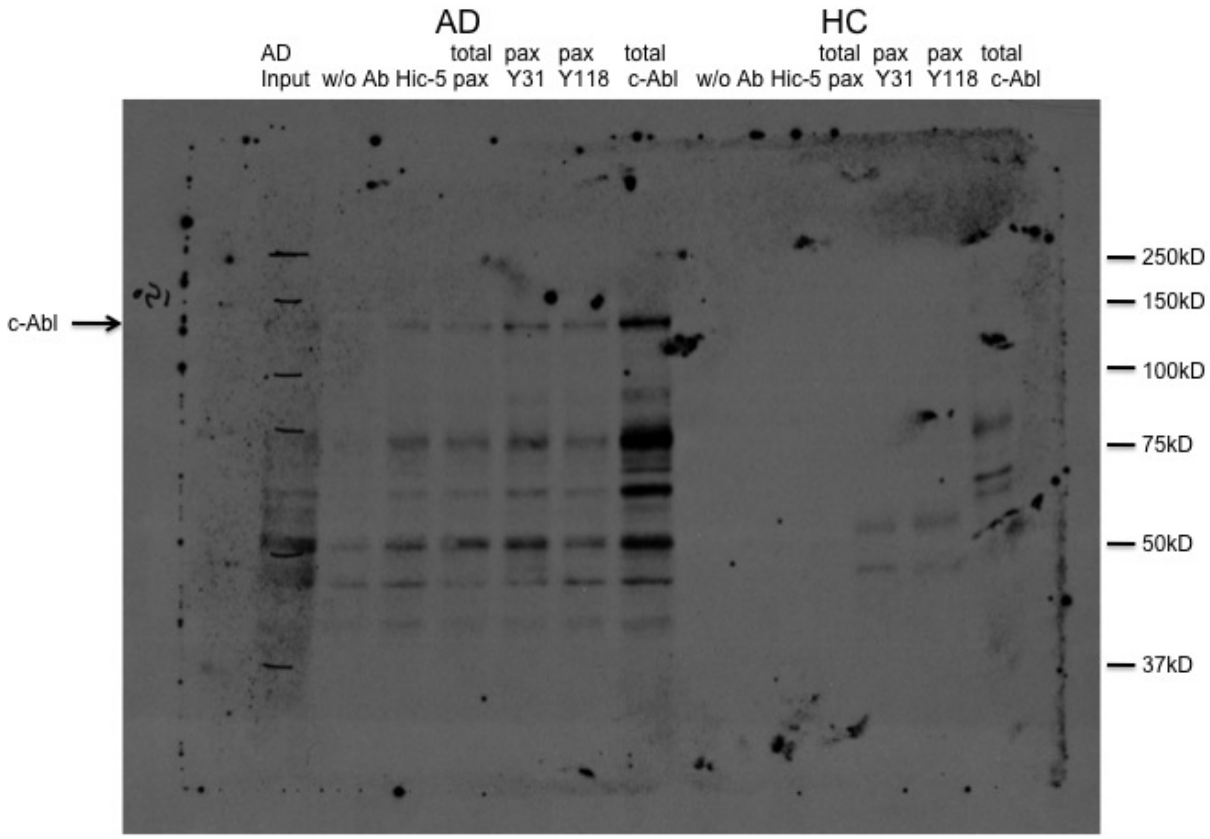


Figure 21 Interaction of total c-Abl with total and phospho-pax.

Co-immunoprecipitation was performed using hippocampus tissue extracts from healthy controls (HC) or AD patients. Proteins were immunoprecipitated without antibody as negative control (w/o Ab) or with anti-Hic-5, total pax, pax p-Y31, pax p-Y118 or total c-Abl antibody (rabbit) as indicated from hippocampal tissue homogenates and precipitated proteins separated by SDS-PAGE. The resulting blot was probed with anti-total c-Abl antibody (mouse). First 7 lanes are AD subject. Right 6 lanes are HC subjects.

6.3 CONCLUSION & DISCUSSION

Previous in vitro study showed that c-Abl phosphorylates tau at tyr 394, a phosphorylation site of tau presents in PHF purified from AD (Derkinderen, Scales et al. 2005). In our study, we performed correlation analysis, confocal microscopy and co-IP using human hippocampus to show the association of c-Abl and p-tau in vivo, although the co-IP experiments needed to be repeated for confirmation.

c-Abl is likely to mediate interactions between cell surface receptors and the cytoskeleton in the synapse, however, it is unclear which cell surface receptors recruit Abl family kinases in the synapse. Cell adhesion receptors play roles in the establishment, maintenance, and remodeling of contacts between presynaptic and postsynaptic components. Furthermore, activation of FAK/paxillin by Aβ can induce tau phosphorylation, cell cycle activation, and the loss of cell adhesions can lead to subsequent cell death, suggesting focal adhesion may activate c-Abl and contribute to AD pathogenesis. However, there is no evidence to support the interaction of NCAM and c-Abl either *in vitro* or *in vivo*. Our study is the first to show the interaction of c-Abl with one of NCAM, paxillin in human hippocampus, providing evidence to link c-Abl with the pathogenesis of AD. The hypothesized model is shown in Fig 22. Upon Aβ binding to integrin receptors on the membrane, the integrin signaling pathway is activated and c-Abl is recruited and activated by interacting directly with paxillin, which can bind to the integrin receptor. Activated c-Abl phosphorylates tau directly at tyr 394 or indirectly by enhancing other serine/threonine kinases, such as GSK-3β and cdk5, eventually causing tau hyperphosphorylation and neuronal cell death during AD. Further experiments are required to validate these findings and the potential role of c-Abl in tau phosphorylation during AD.

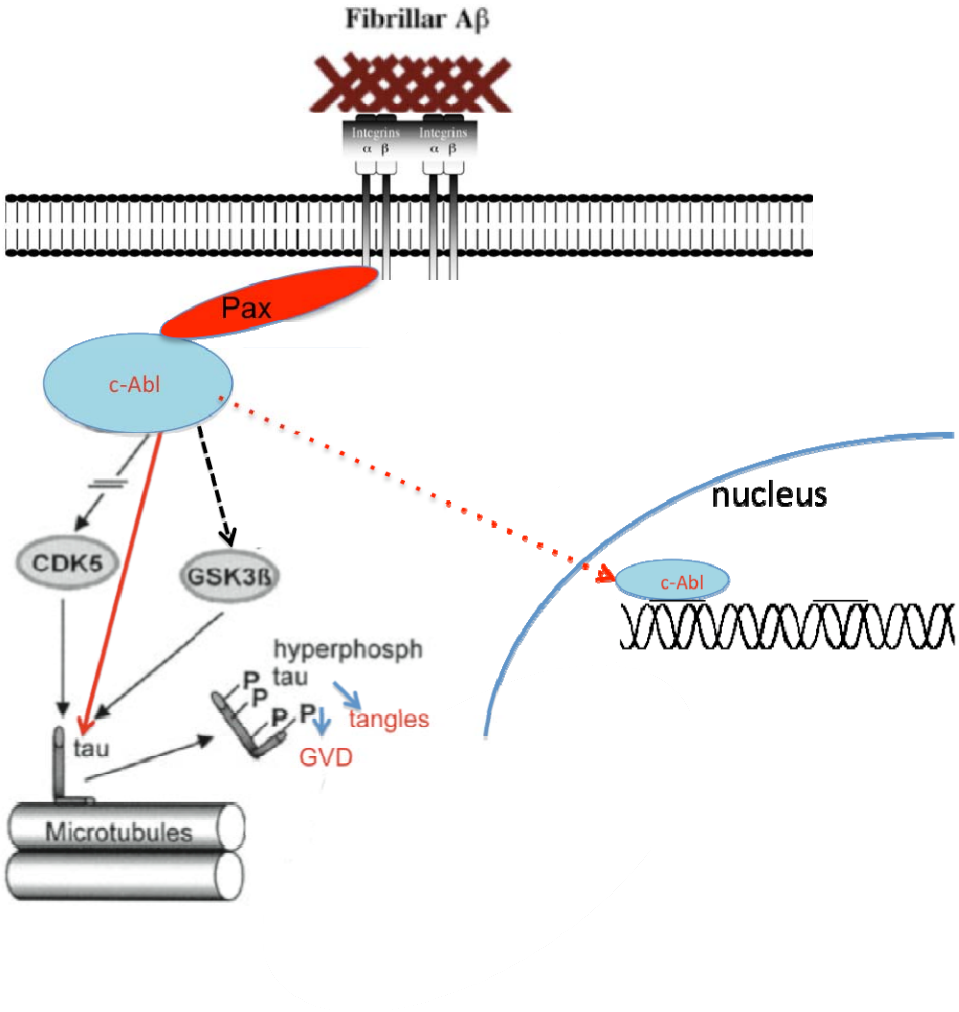


Figure 22 A model of c-Abl's signaling pathway contributing to AD pathogenesis.

7.0 INDUCTION OF C-ABL BY ABETA IN PRIMARY NEURONS

7.1 INTRODUCTION

Alvarez and colleagues reported that c-Abl activation contributes to Aβ40 induced neuronal cell death (Alvarez, Sandoval et al. 2004). Aβ40 fibrils induced c-Abl activity in rat hippocampal neurons, likely through increasing nuclear p73 protein levels and increasing formation of the p73-c-Abl complex. The neuronal cell death induced by Aβ fibrils was prevented by either the inhibition of c-Abl via its specific inhibitor, STI571, or RNAi, which reduced c-Abl protein. These results indicate that c-Abl may be a therapeutic target for the treatment of AD.

To validate this result, we performed the following cell culture experiments. Primary cortical neurons were treated with Aβ42 fibrils, instead of Aβ40 that was used in the Alvarez study. Aβ peptide length varies from 39 to 43 amino acids. Aβ40 represents approximately 90% of the total secreted Aβ (Vigo-Pelfrey, Lee et al. 1993). However, Aβ42 is more relevant to the pathogenesis of AD than Aβ40. First, familial-AD-linked mutations of APP and presenilins lead to increased production of Aβ42 (Suzuki, Cheung et al. 1994; Borchelt, Thinakaran et al. 1996). Second, Aβ42 is the isoform most increased during AD, although Aβ40 is the most abundant (Canevari, Abramov et al. 2004). Studies demonstrated that the elevated Aβ42/Aβ40 ratio, not simply the increase in the Aβ42 level, is a major determining factor for the early onset of AD

(Younkin 1995). Thirdly, Aβ42 aggregates more rapidly than Aβ40 (Snyder, Ladror et al. 1994) and is essential for parenchymal and vascular amyloid deposition in mice (McGowan, Pickford et al. 2005) A recent study suggested that the different 2 amino acid at end of Aβ causes an increased rigidity of the Aβ42 C-terminus that may therefore serve as an internal seed for aggregation (Yan and Wang 2006). Finally, Aβ42 exhibits greater neurotoxicity than Aβ40 (Dore, Kar et al. 1997; Zou, Gong et al. 2002). Aβ42, not Aβ40, upregulates the key proapoptotic protein "bax" (Paradis, Douillard et al. 1996). Interestingly, Zou and colleagues reported that Aβ40 protects neurons from damage induced by Aβ42 in culture and in rat brain, suggesting a mechanism by which increased Aβ42/Aβ40 ratio accelerates the development of AD (Zou, Kim et al. 2003).

The protein level and kinase activity of c-Abl was determined in a series of time points following Aβ42 treatment in primary cortical neurons to confirm a role for Aβ42 in c-Abl activation.

7.2 RESULTS

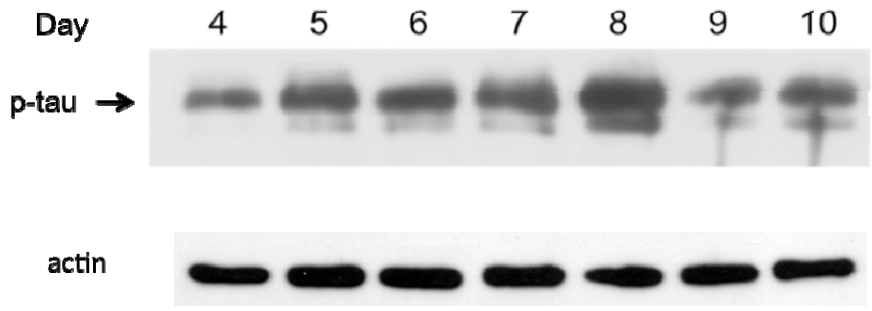
7.2.1 c-Abl is induced by Aβ in primary neurons

7.2.1.1 Cell culture model

Primary cortical neurons were obtained from fetal pups of pregnant female Sprague Dawley Rat at embryonic day E17. The cells were plated at a density of $1.2X10^6$ cell/ml in media containing fetal bovine serum. After 5h, the medium was changed to B27 medium that promotes neuronal growth. To determine the optimal time of Aβ administration to induce altered protein

phosphorylation, we examined protein levels of phospho-tau in untreated primary cortical neurons at a series of time points. Previous studies suggested that Aβ toxicity in neuronal cell death is dependent on the presence of phospho-tau, with peak toxicity when peak levels of phospho-tau are noted (Liu, Perry et al. 2004). Western blot analysis showed that the level of phospho-tau protein was highest at day 8 in our primary cortical neurons (Fig. 23). Therefore, we treated the neurons with 10μM Aβ42 fibrils at day 8 for all subsequent experiments. Aβ42 was dissolved in water of final concentration at 1mg/ml and incubated at 37°C for 24h before administration to the neuron culture. In our study, we confirmed the fibril formation by thioflavin S staining. Aβ42 was added to primary cultured neurons at 10μM. Cell viability was determined by PI and Hoechst staining at 48 h post-treatment. In the control group of vehicle treatment, the cell viability was approximately 70±3.3% of total cells. Cell viability decreased to 35± 6.0% of total cells in Aβ treatment group (mean ± SEM, n=3, $p<0.05$). The experiment was repeated 3 times.

A.



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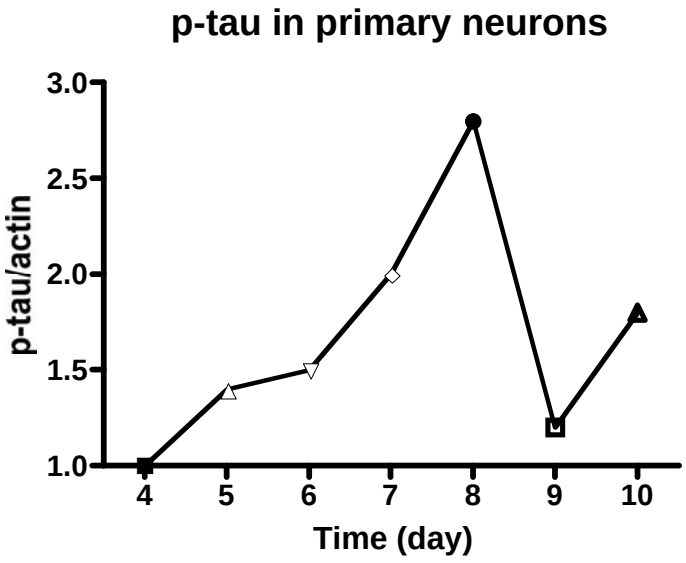


Figure 23 Induction of p-tau levels in primary neurons.

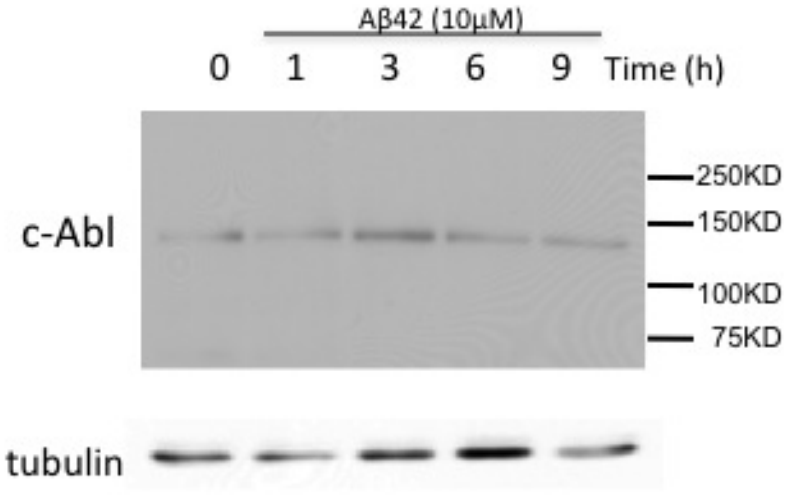
A. Whole cell lysates were collected at culture day 4,5,6,7,8,9 and 10. Equal amount of protein was loaded per lane. The phospho-tau (p-tau) was detected by AT8 antibody.

B. The phosphorylation of tau was increased in the primary culture following neuronal cells maturation. The protein level of p-tau was normalized to actin. The p-tau protein level at day 4 was set to 1.

7.2.1.2 c-Abl protein level is induced by Aβ

Whole cell lysates were prepared following 0h, 1h, 3h, 6h and 9h of Aβ treatment. The results showed that total c-Abl protein level was induced by Aβ42 fibrils and reached a peak level at 3h, then returned to near basal level by 9h post-treatment (Fig. 24).

A.



B.

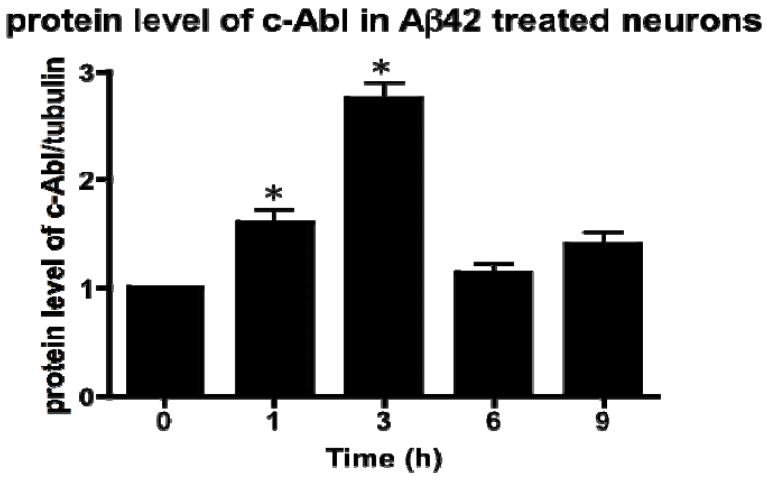


Figure 24 Protein level of c-Abl in Aβ42 treated neurons.

A. *Primary neurons were treated with 10μM Aβ42. Whole cell lysates were collected at 0,1,3,6 and 9h post-treatment. Equal amounts of protein were loaded per lane. The total c-Abl protein level was normalized by tubulin.*

B. *Quantification of the results from 3 independent experiments, revealing an increase in total c-Abl protein level following 10μM Aβ42 treatment in primary cortical neurons. The c-Abl protein level at 0h was set to 1, and shown as mean ±SE. (n=3). The protein level of c-Abl at 1h and 3h was significant different from untreated control ($p<0.05$).*

7.2.1.3 Kinase activity of c-Abl is induced by Aβ

We next examined the functional activity of c-Abl after exposure to Aβ42 fibrils. Whole cell lysates were collected at the same time points as above after Aβ42 treatment. c-Abl was immunoprecipitated by an anti-c-Abl specific polyclonal antibody (Cell Signaling). The immunoprecipitated proteins were incubated with a peptide containing c-Abl substrate sequence (**EAIYAAPFAKKK,** New England Biolabs) and γ^{32}P-ATP. After 30 min at 30°C, the reaction mixtures were stopped on p81 paper and unbound γ^{32}P-ATP was washed away. The kinase activity of c-Abl was denoted by the amount of radioactivity measured by scintillation counting. Instead of c-Abl precipitated from cell lysate, a pure c-Abl kinase protein (New England Biolabs) or no c-Abl buffer was incubated with other components of the reaction as positive or negative control. Each time point was run in duplicate and the whole experiment was repeated three times. The kinase activity of c-Abl was induced by Aβ42 in a similar time frame as the change of total c-Abl protein level (Fig. 25).

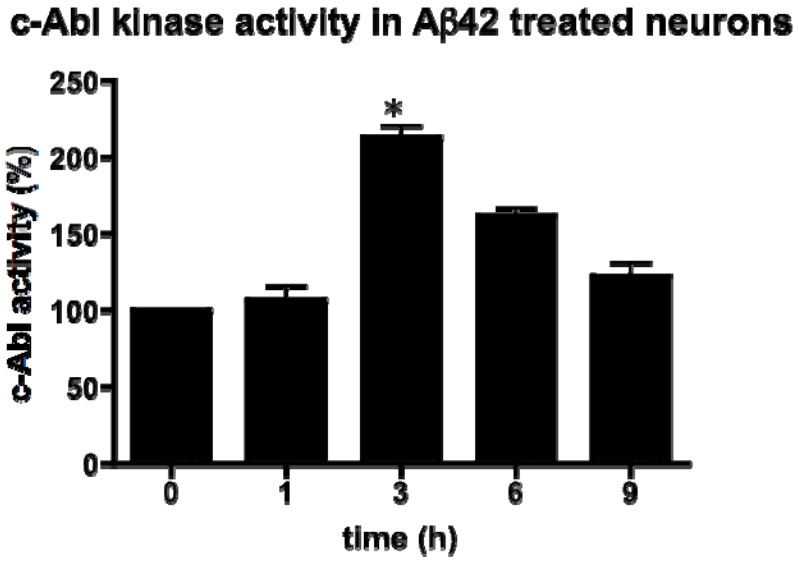


Figure 25 c-Abl kinase activity was induced by A42.

The primary neurons were treated with 10μM Aβ42. Whole cell lysates were collected at 0,1,3,6 and 9h post-treatment. The c-Abl kinase activity was measured as described above. Quantification from 3 independent experiments revealed an increase in c-Abl kinase activity following 10μM Aβ42 treatment in primary cortical neurons. Kinase activity was normalized to the level at 0h that was set to 100%.). The kinase activity of c-Abl at 6h was significant different from untreated control ($p<0.05$, $n=3$).

7.3 CONCLUSION & DISCUSSION

Our data demonstrated c-Abl is induced by Aβ fibrils in primary cultured neurons. We observed an increase of both c-Abl protein level and kinase activity following Aβ42 treatment.

Below we note limitations to the current experimental design and future experiments that address each limitation:

First, in order to determine the time to expose the cells to Aβ, we tested the p-tau level in untreated primary cortical neurons at a series of time points, from day 4 to day 10. A peak level of p-tau was shown at day 8, therefore we decided to treat cells with Aβ at day 8, since a prior study showed that Aβ toxicity is dependent on p-tau induction (Liu, Perry et al. 2004). However, this experiment only was performed once. Repetitions are necessary to confirm the results. In addition, it would be better if we can also examine the protein level of total tau, and the level of p-tau should be normalized by total tau, thus excluding the possibility that the increased level of p-tau is caused by the increased level of total tau.

Second, in our study, we just confirmed the formation of Aβ fibrils by thioflavin S staining, which binds to β-sheet structure. Other methods, such as EM and Congo Red, should also be utilized to further define the structure of Aβ aggregation (fibril, oligomer or mixture). We incubate the Aβ peptide solution in a water bath for a relatively long time, since we diluted the peptide solution to 1mg/ml. The speed of Aβ aggregation is highly dependent on the initial concentration of the peptide solution. For further experiments, different methods should be tested to get a better aggregation of Aβ, probably oligomer and fibril mixture with the maximum toxicity. In addition, to make sure the concentration of Aβ fibril added to each sample is consistent, quantification for Aβ fibril concentration should be performed by densitometric measurements using Congo Red.

Third, to determine the period to treat the cells with Aβ, we just compared the cell viability at 24h and 48h post-treatment. More time points should be tested. Untreated control cells are also needed at each time point.

Last, PI staining is testing cell membrane permeability, indicating necrosis or late stage of apoptosis. Another method to detect apoptosis is by testing DNA fragmentation (TUNEL or

nucleus morphology test) which should be used combined with PI to determine the cell viability following Aβ treatment.

The results we showed in this chapter are preliminary data to determine the function of c-Abl in Aβ toxicity. In the future, specific c-Abl inhibitor, RNAi for c-Abl knockdown, or c-Abl knockout transgenic mice should be utilized to further confirm the role of c-Abl in Aβ toxicity. That will provide in vitro data to support our hypothesis that c-Abl mediate Aβ facilitated tau pathology during AD.

8.0 SUMMARY & CONCLUSIONS

c-Abl is a ubiquitous and conserved protein, which is expressed in almost all tissue types and has been cloned from nematode to human. c-Abl is associated with many essential physiological processes as well as contributing to disease pathogenesis, such as chronic myeloid leukemia (CML). In the nervous system, c-Abl also plays an important role in neurite outgrowth and neuronal cell death during development. However, its distribution in the adult human brain and function in neurodegeneration has not been examined. Recent *in vitro* studies suggest that c-Abl is involved in both Aβ induced cell death and tau phosphorylation. However, the expression of c-Abl in AD brain has not been examined. Our study for the first time systemically examined the expression and distribution of c-Abl using human hippocampus from both healthy control and various stage AD subjects.

First, we found that the activation status of c-Abl is altered during AD progression. Second, we demonstrated the association of c-Abl and tau phosphorylation in AD by multiple ways, including immunohistochemistry and correlation analysis, confocal microscopy and co-IP assays to demonstrate direct interactions between c-Abl and phospho-tau. Finally, we provided preliminary evidence to show possible interaction of c-Abl with paxillin, indicating c-Abl may mediate Aβ facilitated tau pathology via integrin signaling (more repetitions of experiments needed). Our study supports continued investigations to understand the function of c-Abl during AD, which may be a potential therapeutic target for AD. Noteworthy, we also localized activated

c-Abl to a specific pathological inclusion, GVDs, during late stage AD. This finding may help to understand the formation and function of GVDs in AD.

9.0 DISCUSSION & FUTURE DIRECTIONS

Aβ and tau pathology are the two main hallmarks of AD and many prior studies indicate the functional relationship between these two pathologic alterations of AD. Previous *in vitro* studies indicated that c-Abl can be activated by Aβ and contribute to tau phosphorylation (Alvarez, Sandoval et al. 2004; Derkinderen, Scales et al. 2005). Our *in vitro* data demonstrates c-Abl is induced by Aβ fibrils in primary cultured neurons. We observed an increase of both c-Abl protein level and kinase activity following Aβ42 treatment. Therefore, we hypothesized that c-Abl links Aβ toxicity and tau pathology, thus contributing to AD pathogenesis. However few prior studies have examined the expression and distribution of c-Abl in adult human brain. Thus, studying c-Abl using AD brain is both relevant and timely towards further understanding its function in AD.

To test our hypothesis, we first examined the expression and distribution of c-Abl and specific phospho-c-Abl isoforms in human hippocampus from both healthy control and AD subjects. Total c-Abl was located predominantly in neuronal cell bodies (Fig. 8). The activated form and c-Abl, phospho-Y412 exhibited a punctate staining in neuronal cell body and the highest immunoreactivity during early AD (Fig. 10). Phosphorylation of c-Abl is dependent on its local concentration, as activation can be increased high local concentration within the cell (Smith and Mayer 2002). These data suggested activation of c-Abl during early stages of AD. However, in late AD, c-Abl, p-Y412 was predominantly localized at GVDs, a special type of

autophagosome, which are considered a marker for AD (Anderton 1997). Prior studies have shown that the GVD granules are immunoreactive with antibodies to phosphorylated tau (Bondareff, Wischik et al. 1991). Our study demonstrates that c-Abl is a new protein component of GVDs, an important finding from this work. A recent *in vitro* study demonstrated that c-Abl phosphorylated tau at Tyr394 and this specific phosphorylated form of tau was identified in human brain by mass spectrometry (Derkinderen, Scales et al. 2005). Thus, we propose that c-Abl activation, as denoted by phosphorylation at Y412, occurs during early AD and contributes to tau phosphorylation. Phosphorylated c-Abl and tau are then sequestered into GVDs during AD. It is not possible that the tau phosphorylation occurs after their sequestration into GVDs, because only phosphorylated tau has been detected in GVDs (Bondareff, Wischik et al. 1991). This hypothesis is further supported by our observed correlation between levels of c-Abl, p-Y412 and phospho-tau in the CA1 subfield of AD brain (Fig. 15), Furthermore, our confocal microscopy results demonstrated that c-Abl, p-Y412 and phospho-tau co-localized to GVDs within hippocampal pyramidal neurons (Fig. 16). Our co-immunoprecipitation data also demonstrate a direct protein interaction between tau and c-Abl which might be mediated by the proline-riched subfield of tau and the SH3 domain of c-Abl (Lee 2005). However, prior study excludes the possibility of tau tyrosine phosphorylation by c-Abl because of the failure of interaction between tau and SH3 domain of c-Abl, a potential binding area in c-Abl for tau. Contrary, SH3 domain of Fyn and Src binds to tau, leading people to focus on the role of Fyn and Src in tau tyrosine phosphorylation. The failure of interaction between tau and a recombinant SH3 domain of c-Abl cannot exclude the possibility of tau and c-Abl interaction. One possibility is that the interaction between these two proteins requires other domains in c-Abl. Second, c-Abl might be associated with tau through other proteins. In addition, tau binds to the SH3 domain of

Src and upregulates its kinase activity. It would be interesting to determine if binding with tau also enhance the kinase activity of c-Abl.

9.1 C-ABL & AD PATHOLOGY

Echarri et al reported that activated c-Abl is degraded by the ubiquitin-proteasome dependent pathway (Echarri and Pendergast 2001). Immunohistochemical examinations demonstrated that the majority of centrally located granules within GVDs were positive for ubiquitin. Therefore sequestration of activated c-Abl to GVDs may be a cellular response to target the protein for degradation. Western blot analysis showed that the protein level of c-Abl, p-Y412 in the insoluble fraction, which is the cellular part that contains membrane structures including GVDs, has a significant decline in early AD compared to healthy control, and a trend of increase in late-stage AD subjects, possibly because c-Abl, p-Y412 is sequestered into GVDs in late AD.

Kinases such as c-Jun and JNK have also been detected in GVD granules within hippocampal pyramidal neurons during AD, which has been reported to contribute to tau phosphorylation in AD (Zhu, Raina et al. 2001; Zhu, Lee et al. 2002; Lagalwar, Berry et al. 2007). Interestingly, a functional relationship between these two kinases and c-Abl has been reported, with positive feedback mechanisms between c-Abl and c-Jun (Barila, Mangano et al. 2000). GSK-3, a substrate of c-Abl, also has been located in GVDs in AD brain (Ishizawa, Sahara et al. 2003). Therefore, c-Abl may contribute to tau pathology by directly phosphorylating tau or indirectly via regulating other serine/threonine kinases, which contribute to tau pathology in AD (Okazawa and Estus, 2002; Hooper, Killick et al. 2008). The association

between c-Abl and mitogen-activated protein kinases will be further examined in future studies. β-catenin also has been detected in GVDs (Ghanevati and Miller 2005), which is involved in Aβ induced synaptic dystrophy via the Wnt signal pathway (Morgan, Colombres et al. 2004). Its phosphorylation by c-Abl has been shown to regulate its nuclear translocation (He 2006) and modulate N-cadherin-mediated cell adhesion (Rhee, Mahfooz et al. 2002).

As mentioned above, phosphorylation of c-Abl at threonine 735 is required for the interaction of c-Abl with 14-3-3 and retention of c-Abl in the cytoplasm of cells. Strong cytoplasmic immunoreactivity of c-Abl, p-T735 in AD compared to normal brain suggests that c-Abl might be sequestered in the cytoplasm by 14-3-3 and in cytoplasmic aggregates, such as tangles, GVDs and Hirano bodies (Fig. 12). The binding of phosphorylated proteins with 14-3-3 may be refractory to dephosphorylation by phosphatases, which might explain why c-Abl, p-T735 exhibits a much higher expression in AD comparing total and activated c-Abl, Y412. Moreover, increased immunoreactivity of c-Abl, p-T735 was specific in the CA1 of late AD hippocampus, where the neuronal loss is most severe in AD, but this was not evident in other hippocampal subfields. Interestingly, our results demonstrated that only c-Abl, p-T735 displayed immunoreactivity in p-tau labeled NFTs. This data suggests association of c-Abl, p-T735 with phosphorylated tau. Additional studies are required to examine the functional significance of T735 phosphorylation and cytoplasmic accumulation of c-Abl during AD. We also observed c-Abl, p-T735 in Hirano bodies, suggesting c-Abl accumulation in both tau and actin based cytoskeletal abnormalities during AD. Maselli et al reported that expressing CT fragment (amino acids 124-295) of actin induced formation of Hirano body in slime mold Dictyostelium cell (Maselli, Davis et al. 2002). Utilizing this cultured cell model could help us determine the potential function of c-Abl in the formation of Hirano body.

It is not surprising that we failed to detect total c-Abl (in this study we considered it as the unphosphorylated form) in amyloid plaques or cytoskeletal pathology in any AD case. This result is consistent with our hypothesis that phosphorylation of c-Abl is essential for its association with AD pathology.

9.2 C-ABL & INTEGRIN

c-Abl is likely to mediate interactions between cell surface receptors and the cytoskeleton in the synapse, however, it is unclear which cell surface receptors recruit Abl family kinases in the synapse. Cell adhesion receptors play roles in the establishment, maintenance, and remodeling of contacts between presynaptic and postsynaptic components. Noteworthy, the mice lacking neuronal cell adhesion molecule (NCAM) share a few common characters with the abl-/- mice, such as a reduced PPF at the NMJ (Rafuse, Polo-Parada et al. 2000; Polo-Parada, Bose et al. 2001), supporting the notion that NCAM and c-Abl might be functionally linked at the synapse. Furthermore, activation of FAK/paxillin by Aβ can induce tau phosphorylation, cell cycle activation, and the loss of cell adhesions can lead to subsequent cell death, suggesting focal adhesion and c-Abl may contribute to AD pathogenesis together. However, no evidence exists showing the interaction of NCAM and c-Abl either *in vitro* or *in vivo*. Our study is the first time to show preliminary evidence of the interaction of c-Abl with one of NCAM, paxillin in human hippocampus. The hypothesized model is shown in Figure 22. Upon Aβ binding to integrin receptor on the membrane, the integrin signal pathway is activated and c-Abl is recruited and activated by interacting directly with paxillin, which can bind to integrin receptor. Activated c-Abl phosphorylates tau directly at tyr 394 or indirectly by enhancing other serine/threonine

kinases, such as GSK-3β and cdk5, eventually causing tau hyperphosphorylation and neuronal cell death during AD. Further work will be done in the near future to test this hypothesis. It is worth examining the functional relationship between paxillin and c-Abl during AD.

Under normal conditions, activation of the integrin signal pathway regulates cell survival and neuronal growth. It has been suggested that in cell culture the early effects of fibrillar Aβ are the induction of FAK and paxillin tyrosine phosphorylation, which mimic early events in integrin signaling during adhesion and/or growth factor signaling. Therefore, FAK and paxillin are rapidly phosphorylated by fibrillar Aβ, leading to downstream signaling events that can regulate cell viability. However, incomplete activation of these pathways fails to enhance cell survival and may lead to cell death. Fibrillar Aβ does not activate the complete Fyn/FAK/PI3-K/MAPK pathways and therefore incompletely mimics this survival pathway. Fibrillar Aβ activated integrin signaling also induces tau phosphorylation and cytoskeleton dysregulation, competing with the normal signal from ECM, causing a loss of connection between synapse and ECM, which may contribute to neuronal cell death during AD (Caltagarone, Jing et al. 2007).

In addition to the integrin signal pathway, c-Abl may also mediate Aβ toxicity through other receptors. A recent study demonstrated that c-Abl is required for the agrin-induced clustering of acetylcholine receptors (AchR) on the postsynaptic membrane of the neuromuscular junctions (NMJ) in cultured myotubes (Finn, Feng et al. 2003). AchR has been shown to be significantly reduced in brain regions affected in AD (Sabri, Kendziorra et al. 2008) Most important, AchR also binds to monomeric and fibrillar forms of Aβ (Verdier, Zarandi et al. 2004). Therefore, it will be interesting to determine whether c-Abl is associated with both of these two signal pathways during AD. If so, what is the relationship of these two signal pathways, competitive or cooperative?

9.3 THE SUBCELLULAR LOCALIZATION OF C-ABL

c-Abl is a non-receptor tyrosine kinase that shuttles between the cytoplasm and nucleus and functions in multiple physiological and pathological processes (Woodring, Hunter et al. 2003). In the cytoplasm, c-Abl regulates the cytoskeleton through interactions with actin, whereas in the nucleus it can induce apoptosis in response to DNA damage (Van Etten 1999). Although our immunohistochemistry results demonstrated that c-Abl was predominantly in the cytoplasm of pyramidal neurons, we did observe a nuclear immunostaining of c-Abl, p-Y412 and T735 within some hippocampal neurons in AD. This result is consistent with prior studies showing a minor pool of c-Abl interacting with nuclear DNA in mouse neuroepithelial cells, while the majority of c-Abl was located in the cytoplasm (Koleske, Gifford et al. 1998). Our results do not exclude the possibility of the involvement of nuclear c-Abl in the pathogenesis of AD, as c-Abl may participate in nuclear events contributing to apoptosis during AD (Caltagarone, Jing et al. 2007). Alternatively, c-Abl may contribute to APP/Fe65-mediated gene transcription by direct interaction with Fe65 (Perkinton, Standen et al. 2004). In addition, activated c-Abl may only transiently enter the nucleus, thereby making its detection in post-mortem tissues much more difficult. Our data suggests a preferential sequestration of c-Abl, p-Y412 into GVD. Sequestering c-Abl in GVDs may represent a neuroprotective response as has been suggested for other proteins located in GVDs (Mott and Hulette 2005).

In the cytoplasm c-Abl can also be activated by the platelet-derived growth factor (PDGF) receptor. PDGF induces the beta-/gamma-secretase mediated cleavage of amyloid precursor protein. It would be interesting to study the functional relationship of PDGF receptor and c-Abl in AD. c-Abl interacts with and mediates the signal pathway induced by multiple cell surface receptors, including integrin and PDGF receptor. However, it is not clear how c-Abl

participates and modulates these signal pathways. One possibility is that c-Abl activates downstream targets, and these target proteins are transported to and function in different regions of the cell; another possibility is that the subcellular localization c-Abl determines its function. The second possibility is more likely as we observed a cytoplasmic punctate staining pattern of c-Abl in hippocampal neurons, indicating c-Abl is accumulated at specific area of cells, not diffusely within cells.

Prior studies examining Abl and Arg expression in mice brain showed that c-Abl was located at the cell membrane of presynaptic terminals (active zone) and postsynaptic density of dentritic spines (Moresco and Koleske, 2003). By performing IHC for c-Abl, we observed that both total and phospho-c-Abl were predominantly localized in neuronal cell body. However, light microscopy is not capable of determining the synaptic localization of c-Abl. This issue could be addressed by performing EM or double staining to determine the co-localization of c-Abl with synapse in AD brain using specific presynaptic markers, such as synaptophysin (a well characterized presynaptic protein) (Murphy, Rueter et al. 2000) or vesicular GABA transporter (VGAT) (Saito, Iwata et al. 2005); or postsynaptic marker, such as postsynaptic density zone protein, PSD-95 (Gylys, Fein et al. 2004).

The subcellular localization of c-Abl is regulated by its interaction with 14-3-3, which is controlled by the phosphorylation of 14-3-3. Once 14-3-3 is phosphorylated by JNK, it losses the ability to bind to c-Abl and allows c-Abl to enter the nucleus (Yoshida, Yamaguchi et al. 2005). This idea is supported by prior studies that reported an increased expression of JNK in AD brain. In the future, it is interesting to determine the interaction of 14-3-3 and c-Abl and whether increased JNK in AD modulates this interaction.

9.4 LIMITATION OF EXPERIMENTAL APPROACHES

9.4.1 Human subjects:

Postmortem human brain tissue was assigned to 3 groups: 5 subjects with Alzheimer's disease (AD) Braak stage V-VI (late AD group), 5 subjects with no clinical history of dementia, but with AD pathology (early AD group, Braak stage II-IV), and 5 subjects with no clinical history of a neurodegenerative disease and no AD pathology (Aβ) and only rare tangles in the hippocampus (healthy control group, Braak stage 0-I). Braak stage is based on the distribution pattern of neurofibrillary tangles and neuropil threads, which correlates to AD progression and permits the differentiation of six stages. A Bielschowsky stain was also performed on all cases. The diagnosis of AD was determined by the combined use of Consortium to Establish a Registry for AD (CERAD), the National Institute of Aging (NIA) and the Reagan Institute (RI) criteria (Mirra, Heyman et al. 1991; Hyman and Trojanowski 1997).

Braak staging, the criteria we used to define early and late AD stages, is a neuropathologic staging system. A measure of cognition in living patients would be better. The reason we use the Braak and CERAD criteria are that our ADRC has few banked cases with cognitive assessments while the patient was living. Therefore we must use a post-mortem assessment for our studies. However, in order to determine the change of c-Abl during the AD progression, a better system would be to use MCI cases as early stage AD, as these should progress to AD. Such a system has been used in a recent study, in which MCI cases were defined by a multi-step approach based on cognitive and functional status during the last 6 months of life, which was applied to the assignment of clinical dementia rating (CDR) scores (Meuser, 2001). Subjects were divided into groups on the basis of their CDR scores as follows: 0, non-

demented; 0.5, questionable dementia (MCI); 1, mild dementia; 2, moderate dementia; 5, very severe dementia (Zhao, Xiang et al. 2007). Approximal 62% MCI cases progressed to dementia in their study (Zhao, Xiang et al. 2007). In some of the results of our study, although there is a trend toward changes between different groups, it fails to show a significant difference, which might be caused by the limitation in the number of cases for each group.

In addition, there are also other factors to cause the variation between individuals in this study. First, the ages of the subjects we used in this study vary from 50-90, which is not an ideally age-match comparison. The second factor is cause of death. Other pathological changes in patients may cause an alteration of c-Abl's expression and distribution, since c-Abl is a ubiquitous protein and involved in many distinct physiological and pathological processes. Therefore it may influence the correlation of c-Abl and AD progression. Unfortunately, we only have the final cause of death for some cases in this study and the information about final cause of death for other cases is not available. A complete clinical history would be helpful to better interpret our data. The third factor is PMI (post mortem interval). Delayed fixation may result in diffusion of antigen-containing components causing false interpretation of such immunohistochemical reactions (Reid, Branch et al. 1987). Ideally, a pre-experiment should be done to test if PMI influence on the IHC for c-Abl.

In addition, 5 cases for each group is the minimum number for statistical analysis in human subjects studies. However, the level of c-Abl of each individual varies dramatically. This may be the most important factor that contributes to the lack of statistical significance for most comparisons in our study. Increasing the number of cases would be helpful to determine whether a significant difference of c-Abl exists between different sample groups.

Another important issue is that because of the specific neuronal staining of c-Abl in human brain, using microdissection or laser capture microdissection would be a better way to perform protein analysis with only the cell types of interest.

9.4.2 Antibody specificity and efficiency:

Since the immunostaining pattern of p-T735 c-Abl in AD hippocampus is very similar to p-tau in AD, we tested the cross-reaction between the anti-p-T735 antibody we used for IHC and p-tau. By performing western blot analysis using AD hippocampus tissue lysate (Fig. 11), the anti-p-T735 antibody recognized a few bands, but none of them appears like the classic ladder of p-tau as shown in Fig 18 between 50 and 75 KD, suggesting that the anti-p-T735 antibody does not react with p-tau epitope. However, this antibody does recognize additional bands by western blot in the insoluble fractions, which may correspond to degraded c-Abl or other proteins. The presence of multiple bands by western blot may reduce our confidence of the IHC results for p-735, since the antibody may detect multiple proteins in tissue samples. However we currently do not have evidence that multiple proteins are detected by IHC, and the lack of immunostaining in control subjects would argue that non-specific or multiple proteins are not detected by this antibody during IHC. It is possible that this antibody may cross-react with other proteins when denatured and analyzed by gel electrophoresis. A separate experiment could be performed to help address this important issue. Phosphorylation at Thr735 can be induced in cultured cells by sorbitol (Date sheet of phospho-c-Abl (T735) antibody, catalog # 2864, Cell signaling tech). We could perform western blot of lysated prepared from cultured cells overexpressing c-Abl +/- sorbitol to determine if these additional bands on the western appear upon sorbitol induced phosphorylation of c-Abl.

Ideally, in order to test the specificity of an antibody, first, the antibody should be tested in different species tissue slides to see if there is a cross reaction between species; second, the primary amino acid sequence of the epitope recognized by this antibody should be checked if exists in other proteins of this species by screening protein primary sequence database. The best way to do this is to test the antibody using the antigen knockout transgenic animal tissue.

Although this anti-p-T735 c-Abl antibody we used immunolabeled a few AD pathologies, such as amyloid plaques, tangles, GVDs and Hirano bodies by performing IHC, it predominantly recognized very intense punctate dots, like GVDs visualized by immunofluorescent staining. It recognized p-T735 c-Abl in tangles only when the concentration of antibody increased.

Since one of our hypotheses is that c-Abl activation during early AD contributes to tau phosphorylation, it is very important to show the co-localization of c-Abl, p-Y412 (the activated form) and p-tau. Unfortunately, the antibody we used for c-Abl, p-Y412 does not work well for immunofluorescent staining. Although we used TSA method to amplify the signal for c-Abl, p-Y412 and showed that all the c-Abl, p-Y412 positive neurons were p-tau positive in CA1 of late AD, most of p-tau positive neurons are c-Abl, p-Y412 negative because the weak signal of c-Abl, p-Y412. Another antibody for c-Abl, p-Y412, if available, should be tested for more efficient staining using immunofluorescence. Alternatively, consecutive tissue slides staining for c-Abl, p-Y412 and p-tau could also be helpful to show the co-localization. The tissue slides we used for c-Abl, p-Y412 and p-tau are not consecutive, and thus we could not do that analysis in this study.

The specificity of anti-total c-Abl antibody refers to the discussion of Chapter 5.

9.4.3 Reagents and protocols:

In order to unmask the antigens as much as possible, we tried different antigen retrieval buffers, the normal citrate buffer and high pH buffer (pH 9.9) that is effective for particular antigens, as well as we tried a different way to retrieve the antigens, microwave or steaming. Antibodies against total c-Abl or phospho-c-Abl only work when high pH antigen retrieval buffer + steaming.

We used RIPA-DOC buffer (50mM Tris at pH 7.4, 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease and phosphatase inhibitor cocktails) to lysate brain tissue for this study. A previous published paper, in which they detected β-catenin in GVDs using this buffer, suggests that this buffer contains a relatively higher concentration of detergent which be enough to get the proteins in GVDs dissolved (Ghanevati and Miller 2005). Since IHC results indicated that phospho-c-Abl was predominantly located in GVDs in AD hippocampus, we choose this buffer for our co-IP experiments. However, we should have compared this buffer with other milder buffers, such as NP40 buffer. RIPA buffer gives the lowest background, but can denature some kinases. It also has the potential to disrupt protein-protein interactions. NP40 buffer is less denaturing, but gives a higher background. It is less likely to inhibit kinase activity and disrupt protein complexes. Although our co-IP data showed a detectable interaction between c-Abl and other proteins, we cannot exclude the possibility that the high concentration detergent in this buffer interrupts the protein-protein interaction. These co-IP data also might suggests the interaction of c-Abl with paxillin and p-tau is kinase-activity independent (kinase activity of c-Abl in RIPA-DOC buffer should be tested).

We used Tris-triton X buffer to lysate brain tissue for western blot analysis according to a prior published paper (Ghanevati and Miller 2005). In this paper, the authors found that phospho-

β-catenin was located in GVDs in AD brain, similar to phospho-c-Abl. Using this Tris-triton X buffer, they detected an increase of protein level of phospho-β-catenin in the detergent insoluble fraction, suggesting that GVDs is the detergent insoluble fraction using this lysis buffer and centrifuge speed, and is soluble in SDS for PAGE. We modified this buffer with no salt, because by direct comparison, c-Abl, p-Y412 dissolved better in no salt Tris –triton X buffer (data not shown).

9.4.4 Technical limitations

One important finding in this study is that we detected the activated form of c-Abl in GVDs during AD. However, we defined GVDs just based on morphological characters. This conclusion requires further validation by other methodologies such as electron microscopy (EM). We initiated EM studies but were not successful using the available post-mortem tissue samples. Unfortunately, the tissue slides we obtained from the brain bank were embedded in paraffin, which is good for protein fixation but often causes damage to lipid membrane structures. Future studies will require proper preparation of fresh tissue samples for EM studies.

In this study, we measured the overall immunoreactivity of c-Abl within certain areas, including all the cell types, such as neuron, neuropil and glial cells, although c-Abl was predominantly located in neuronal cell body and neuropil. Considering this, the decreased immunoreactivity of c-Abl in AD compared to healthy controls might be caused by the neuronal loss during AD. The more accurate method to quantify the immunoreactivity of c-Abl would be randomly pick 100 neurons within a certain area and then manually circle the neuronal cell body, and calculate the staining intensity within these circles.

Homogenization of the whole tissue probably also lead to the inaccuracy of western blot analyses. Possibly better results would be obtained by microdissecting out only the specific hippocampal regions of interest, or by using laser capture microdissection to remove only pyramidal neurons from a specific region of the hippocampus.

9.4.5 Experimental design

Our study for the first time examines the expression and distribution of c-Abl in human hippocampus, which is helpful to further understand its function in AD pathogenesis. It would be helpful to examine c-Abl in other brain regions, such as frontal cortex, which is also severely affected during AD. However, the limitation of any post-mortem study is the difficulty of testing protein alterations during disease progression. We used tissue samples with different Braak stages of AD, which helped us to examine alterations of c-Abl distribution or its interaction with other proteins during AD progression.

For the correlation study, we used the AT8 antibody to perform IHC for p-tau, which is against the phospho-tau at serine 202 and commonly used to label NFTs. However, since the goal of this correlation analysis was to test if activated c-Abl contributes to tau phosphorylation in AD, an antibody against phospho-tau Y394, the phosphorylation site of tau by c-Abl, should be used to perform IHC for p-tau. Unfortunately, such antibody is not yet commercially available. Future studies using such an antibody will determine if p-tau-Y394 co-localizes with phospho-c-Abl and would further test our hypothesis. Interestingly, once Tau is phosphorylated at serine/threonine by CDK5 and GSK-3β, it is degraded by UPS (Hsp70-Chip-UbcHe5 signal pathway) (Kosik and Shimura 2005). It raises the question if tyrosine phosphorylation of tau also induces tau degradation? Thus, determining the physiological and pathological meanings of tau

phosphorylation at tyr 394 is helpful to understand the function of c-Abl in AD pathogenesis as well.

Our hypothesis is that c-Abl mediates Aβ facilitated tau pathology via the integrin signaling pathway. However, our confocal images did not show a co-localization of c-Abl and Aβ, although c-Abl positive staining was observed in amyloid plaques. This inconsistency might be caused by the tissue slides we used to examine the co-localization of c-Abl and Aβ, which are late-AD hippocampus. Because c-Abl's activation may occur at the early stage of AD based on our IHC results, it might be helpful to examine the co-localization of these two proteins using the early AD hippocampal tissue.

For the co-IP data examining the association of total c-Abl with p-tau, although we did reverse co-IP to further confirm the association between these proteins, these experiments only was performed once (expect indicated as "n=2"). Repetitions of this experiment are needed to make any conclusions. In addition, the experiments also lacked some of the proper controls. The blots should be stained for the protein immunoprecipitated with to make sure the IPs worked, as well as to give a way to standardize the protein amount on the blot. An input control also should be included to identify the correct band for the protein of interest.

9.5 FUTURE DIRECTIONS

In this study, we hypothesize that c-Abl is associated with Aβ facilitated tau phosphorylation via integrin binding and activation, and contributes to the generation of AD pathology through direct phosphorylation of tau. We tested this hypothesis by examining the

expression and distribution of c-Abl in the human hippocampus and by characterizing c-Abl interacting proteins in AD brain.

Our results demonstrate that levels of total c-Abl (unphosphorylated form) were highest in control hippocampus. An activated form of c-Abl, c-Abl, p-Y412, exhibited highest levels during early stage AD. However, another phosphorylated form of c-Abl, c-Abl, p-T735, which is involved in regulation of c-Abl subcellular localization, exhibited statistically significant increases in the CA1 during late stage AD, which may represent an additional c-Abl phosphorylation that occurs upon c-Abl accumulation in NFTs and GVDs (Figs. 8, 10, and 12). This study represents the first to examine the expression and distribution of c-Abl in human AD brain, and indicates the functional status and subcellular distribution of c-Abl is altered during AD progression. We also showed an association of activated c-Abl and p-tau in AD, by performing correlation analysis, confocal microscopy and co-IP. However, further experiments are needed to confirm these co-IP data.

Our study found that c-Abl locates to AD pathology by IHC and confocal microscopy. However, such results are not sufficient to suggest a potential role of c-Abl contributing to AD pathology. Further functional studies are needed to make a precise conclusion.

Our *in vivo* data highlight the association of two phosphorylated form of c-Abl, Y412 and T735, with AD pathology, especially with tau pathology. Further studies are required to examine the physiological and pathological functions of these two phosphorylated isoforms by expressing mutated c-Abl in cell lines or transgenic mice. The essential role of c-Abl in Aβ facilitated tau pathology should be further determined in primary neuronal cultures, utilizing specific c-Abl inhibitors or dominant negative c-Abl mutant.

Importantly, the phosphorylation site of tau by c-Abl, Tyr 394 has just been identified recently (Derkinderen et al. 2005). The physiological and pathological meaning of this phosphorylation site is not clear yet. Understanding this must be helpful to study the function of c-Abl in AD.

In addition, the kinase that is responsible for c-Abl activation in AD is not known yet. The known upstream activators of c-Abl, such as ATM and DNA-PK, should be examined as required co-factors for c-Abl's contributions to AD pathology. ATM has been shown to be required for neuronal cell cycle control (Yang and Herrup 2005). Also, DNA-PK mediated DNA repair is impaired in AD (Shackelford 2006) and the protein level of DNA-PK is correlated with synapse dysfunction in AD (Davydov, Hansen et al. 2003). Interestingly, DNA-PK phosphorylated tau *in vitro* (Wu, Chen et al. 1993).

Further functional studies of c-Abl and downstream signaling proteins are currently underway and may provide novel therapeutic targets for AD and other neurodegenerative diseases.

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