A study of putative p25 modulated synaptic molecules - CYFIP1, CYFIP2 and CSPα - in Alzheimer's Disease

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A study of putative p25 modulated synaptic molecules - CYFIP1, CYFIP2 and CSPα - in Alzheimer's Disease

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Abstract

Alzheimer's Disease (AD) is a devastating neurodegenerative condition with synaptic impairment at the onset of disease. Previously, our group have shown that the p25 molecule is changed in the post mortem brain of mild stage AD patients. A mass spectrometric analysis of p25 transgenic mouse brain provided us with a set of potential p25 downstream molecules. Three of these candidate molecules which had synaptic/dendritic localization − CYFIP2, CYFIP1 and CSPα were selected to be studied in the post-mortem brain of Alzheimer's patients. CYFIP2 is a dendritically localised molecule with biological role in local translation modulation and cytoskeleton remodelling. Our case −control studies revealed that CYFIP2 is downregulated in severe stages of disease in hippocampus. We showed a similar CYFIP2 downregulation in Tg2576 mouse model of AD. We performed functional studies of this molecule, using CYFIP2 heterozygous knockout mice. We found that these mutants suffer from memory loss after Pavlovian conditioning. CYFIP1 has similar cellular function as CYFIP2. Our studies showed that CYFIP1 is upregulated in AD hippocampus. However, this upregulation is unlikely to be compensation for CYFIP2 downregulation, as it was not observed in superior temporal gyrus. CSPα, a synaptic vesicle protein that has been implicated in neurodegeneration in Kufs disease, was found to be downregulated in AD hippocampus, but, surprisingly, upregulated in cerebellum. This suggests that CSPα may protect neurons from degeneration. In agreement, we found that CSPα upregulation in htau mutant mice correlates with absence of neuronal loss. Taken together, analysis of candidate p25-regulated synaptic proteins have provided novel insights into mechanisms underlying synaptic degeneration and memory impairment in AD.

Table of Contents

Index of figures and tables -

<u>**Publication**</u>

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Abbreviations

1) AD - Alzheimer's disease

2) AMPAR- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

3) APP- Amyloid precursor protein

4) sAPPα - soluble amyloid precursor protein alpha

5) sAPPβ - soluble amyloid precursor protein beta

6) BCA - Bicinchoninic acid

7) Cdk5 -Cylin dependent kinase-5

8) CSP - Cysteine String Protein

9) CYFIP1 - Cytoplasmic FMRP interacting protein 1

10) CYFIP2 - Cytoplasmic FMRP interacting protein 2

11) ECL -Enhanced Chemiluminescence

12) EIF4E - Eukaryotic translation Initiation Factor 4E

13) FMRP -Fragile-X Mental Retardation Protein

14) FTLD - Frontotemporal Lobar Degeneration

15) FXR - Fragile-X related protein

16) NFT - Neurofibrillary Tangeles

17) NMDAR -N-methyl-D-aspartate receptor

18) NSE- Neuron Specific Enolase

19) PWS- Prader Willi Syndrome

20) STG - Superior Temporal Gyrus

21) TBST - Tris-Buffered Saline Tween 20

22) WAVE - WASP family Verprolin-homologous protein

23) WASP - Wiskott–Aldrich syndrome protein

24) WRC -WAVE regulatory complex

Chapter 1: Introduction

1.1. Alzheimer's Disease

Alzheimer's Disease (AD) was reported first by the German psychiatrist Alois Alzheimer in 1906 in Tübingen, Germany (Alzheimer et al., 1995). He reported the correlation between cognitive deficits of a 51 year-old woman and cortical histopathology of plaques and neurofibrillary changes on her death at the age of 55 years. 106 years after this revelation, much has been discovered about the pathology of the disease and yet a lot remains to be understood in terms of the molecular mechanisms leading to AD and associated dementia.

1.1.1. Significance of AD research

Alzheimer's disease (AD) is a neurodegenerative disorder and it is the most prominent cause of dementia in the elderly. The prevalence of AD is evident from the epidemiological statistics for the year 2011 in the US population (Alzheimer's Association statistics) –

- 5.4 million Americans irrespective of the age group have been diagnosed with AD. This group includes 5.2 million people above the age of 65 years and 200,000 below the age of 65 years diagnosed with early onset AD. Therefore approximately 1 in every 8 individuals (or 13%) above the age of 65 years has been diagnosed with AD. If untreated, the prevalence of this disease in people above 65 years is projected to triple by 2050 to about 11 to 16 million.

- It is the sixth leading cause of death amongst all ages in the US population

- With the current growth in prevalence of AD in the US population, the annual economic burden of the disease is estimated to inflate from \$183 billion in 2011 to \$1.1 trillion by 2050.

Due to the current lack of knowledge about the mechanisms underlying AD aetiology and poor diagnostic as well as therapeutic tools for AD, it is emerging as a major social and economic burden in our modern ageing society. Hence there is an urgent need to understand this disease in as much detail as possible to develop better early stage diagnostic markers as well as therapies.

1.1.2. Disease - Pathology and Diagnosis

AD is characterized by the presence of extracellular amyloid β plaques, intracellular neurofibrillary tangles (NFT), and loss of synapses and neurons. AD progression has been divided into three stages based on the spread of hyperphosphorylated tau protein – mild (Braak stages 1-2), moderate (Braak stages 3-4) and severe (Braak stages 5-6) (Braak and Braak, 1991b). The mild stage lasts 2-5 years and is characterized by the onset of memory impairment with impairment in short-term memory, deficits in problem solving ability, depression, aphasia (inability to effectively communicate) and other cognitive impairments (Holtzman et al., 2011). However, the patient at this stage can perform daily tasks and has perfect motor co-ordination. In the moderate stage of AD, lasting 2-4 years, there is progressive memory impairment that now includes long-term memory deficits, agnosia (inability to recognize others) and apraxia (loss of motor skills). The patient becomes more and more dependent on friends and relatives for performing everyday work (Holtzman et al., 2011). In the severe stages, the patient is

totally dependent on others and has severe cognitive and memory deficits, problems with swallowing, bowel movement and bladder function (Holtzman et al., 2011). When the patient is alive, diagnosis of AD is still not conclusive with post-mortem neuropathological analysis required to unequivocally diagnose AD. Apart from lack of efficient biomarkers of the disease, there is also no cure or effective symptomatic treatment available for AD.

1.1.3. Mechanistic understanding of Disease

The cause of the development of AD is still unknown. Prior the 1970s AD was considered to be a case of presenile dementia (affecting people aged < 65 years), distinct from senile dementia (with age onset > 65 years). However, by the 1970s it was realized that the biology behind both dementias is the same with identical pathological hallmarks and symptoms. There are several hypotheses that have been proposed to explain the causes of early onset and late onset forms of AD. One of the first hypotheses was based on the observation that cholinergic transmission is essential for memory formation (Bartus, 1979) and loss of cholinergic neuron occurs in AD (Davies and Maloney, 1976). Thus, impaired cholinergic transmission was proposed to cause AD. This hypothesis has led to the development of a widely used acetylcholinesterase inhibitor-based therapy, which provided moderate symptomatic relief in clinical use (Farlow et al., 2008). However, in AD most of the cortical pyramidal neurons, which are glutamatergic, are affected and hence the cholinergic neuron-based hypothesis and neurotransmitter replacement therapies have their limits as cause and cure, respectively.

Being the pathological hallmarks of AD, NFT and amyloid β plaques attracted significant attention as possible causes for the disease. Intracellular NFTs are composed of aggregates of paired helical filaments (Kidd, 1963) and these filaments are made up of hyperphosphorylated tau protein (Kosik et al., 1986, Wood et al., 1986). The normal function of tau protein is to stabilize the microtubule assembly (Weingarten et al., 1975). Amyloid plaques are extracellular aggregates of amyloid β peptide fragments and are derived from the cleavage of transmembrane amyloid precursor protein (APP) (Golde et al., 2000, Hardy and Selkoe, 2002). The cleavage of APP to amyloid β peptide is mediated by β-secretase and γ-secretase (Holtzman et al., 2011) as shown in **Fig.1.1.** The cellular function of APP is not yet well known. The *APP* gene has been localized to chromosome 21 in humans (and chromosome 16 in mouse) (Goldgaber et al., 1987, Beyreuther et al., 1993, Cheng et al., 1988) and explains the high correlation between trisomy 21 (Down's syndrome) and AD pathology of amyloid plaques.

1.1.3.1. APP – production and processing

As a key protein implicated in AD it is important to understand APP production and processing mechanisms **(Fig.1.2).** APP production goes through a series of steps from gene transcription, posttranscriptional modification, translation and finally posttranslational modification during its production pathway (Westmark, 2013, Westmark and Malter, 2012). This is then followed by complex processing by enzymes, which leads to production of amyloid peptide amongst other products.

There are several isoforms of APP ranging in size from 695 to 775 amino acids, which includes the fragment from which the amyloidβ is derived. APP695 is the most abundant isoform in brain (Kummer and Heneka, 2014). APP mRNA binds to Fragile

X Mental Retardation Protein (FMRP) – a protein important for mRNA transport into dendrites and regulation of local dendritic/synaptic mRNA translation. FMRP represses mRNA translation. This repression is released through an $mGluR_5$ –dependent signalling pathway at synapses (Sokol et al., 2011, Lee et al., 2010, Darnell et al., 2011). APP is reported to be synthesized in a developmentally regulated manner with expression reaching maximum levels during neuronal differentiation, particularly synaptogenesis, and declining with the final establishment of major neural circuits (Loffler and Huber, 1992, Moya et al., 1994). Changes in the expression profile (both quantitative levels and localization) of RNA binding proteins like FMRP are expected to influence APP and amyloidβ synthesis and expression during the course of development. This has been shown in neonatal brains, whereby the APP levels reach their maximum during postnatal weeks 2-6, a period when crucial sensory neural circuits are being established in rodents, and the FMRP levels decline after the 1st postnatal week (Berardi et al., 2000, Lu et al., 2004). A decrease in FMRP levels (or other dendritic translation regulators) will eventually lead to an increase in APP levels during synpatogenesis as reported by Westmark's group (Westmark and Malter, 2012, Westmark, 2013) since FMRP levels regulate the translation of APP mRNA. During synaptogenesis this translational repression is removed by a decrease in FMRP levels leading to elevation of APP levels (Westmark and Malter, 2007). At the other extreme of the development phase, Prasad's group has reported that FMRP levels decrease in an age-dependent manner in rodents (Singh et al., 2007). Hence, with ageing, the decrease in FMRP levels could be contributing to an age-dependent increase of APP or amyloidβ production.

At the posttranslational level, APP processing happens through two pathways – amyloidogenic and non-amyloidogenic (**Fig1.1 and Fig.1.2**). In the amyloidogenic pathway, APP is cleaved by β-secretase (BACE-1) followed by γ-secretase (the

catalytic component of which is presenilin) leading to amyloidβ synthesis. Presenilin 1 (PS1) and Presenilin 2 (PS2) are gamma-secretases that cleave APP (Takasugi et al., 2003). They are multiple transmembrane proteins and span membrane 6-9 times(Kim and Schekman, 2004, Oh and Turner, 2005). Both β-secretase and γ-secretase are transmembrane proteins that have aspartic protease catalytic domains that cleave APP to generate an internal fragment – amyloid β (Westmark, 2013). BACE1 expression is high in brain and BACE1 knockout mice do not show detectable amyloidβ levels which may have therapeutic use (Vassar et al., 1999, Bennett et al., 2000, Vassar and Citron, 2000)). Amyoid β begins about 99 residues from the C-terminus of APP and extends from the extracellular domain into middle of membrane spanning domain (Westmark, 2013). The γ secreatse activity in both amyloidogenic and nonamyloidogenic pathway releases APP intracellular cytoplasmic domain (AICD) apart from amyloidβ in amyloidogenic pathway and p3 fragment in non-amyloidogenic pathway (Selkoe, 2002). AICD binds to different proteins and may be involved in gene regulation, neuronal growth and apoptosis (Raychaudhuri and Mukhopadhyay, 2007). The best characterized of these AICD binding proteins are Fe65 family members. These protein contain two domains that physically interact with C- terminal of APP regulating AICD formation (Fiore et al., 1995, Duilio et al., 1998, Huysseune et al., 2007). Mint/X11 family bind to AICD and modulate AICD mediated transcription in isoform specific manner (Borg et al., 1996, Biederer et al., 2002). Members of JIP family activate AICD-mediated signalling (Scheinfeld et al., 2003). AICD modulates calcium homeostasis , cellular trafficking and cell death (Hamid et al., 2007, Ghosal et al., 2009, Passer et al., 2000). Finally, AICD undergoes two step proteolytic fate – rapid inactivation by endosomal insulin degrading enzyme – insulysin (Farris et al., 2003) and cleavage at C-terminal end by caspase 3 activity to yield C31 fragment (Lu

et al., 2000). Both the pathway contribute to certain extent in the final AICD degradation.

The formation of amyloidβ peptide is a multiple step process. Amyloidβ is a mix of peptides of 39 to 43 amino acids (Wang et al., 1996, Lamb et al., 1997). The endoproteolysis is believed to occur in stepwise manner cleaving the C-terminal stub multiple times within its transmembrane domain. The cleavage occurs approximately 3 amino acid apart – the first one at position 48/49 (ε-cleavage site) , followed by at position 45/46 (ζ-cleavage site) , and the last one at position 38/40 or 42 (γ-cleavage site) (Takami et al., 2009, Qi-Takahara et al., 2005, Gu et al., 2001, Sastre et al., 2001). So, ε cleavage is a limiting step for γ cleavage occurrence. Depending on the cleavage site of γ-secretase at the C-terminus end of amyloidβ, different forms of amyloidβ are generated (Steiner et al., 2008) and some of them are shorter isoforms than the ones mentioned before (Amyloidβ - 17,18) depending on the γ secretase activity(Portelius et al., 2011). The ε cleavage site is considered equivalent to S3 site in Notch 1(Schroeter et al., 1998), Also , a new study revealed that S4 cleavage site in Notch1 could be homologous to γ cleavage site in APP(Okochi et al., 2002). This suggest that the intramembrane cleavages that APP and Notch 1 undergo are similar atleast at two sites : ε/S3 cleavage and γ /S4 cleavage , releasing amyloidβ/notch-1β and APP intracellular cytoplasmic domain(AICD)/ Notch-1 intracellular cytoplasmic domain (NICD)(Okochi et al., 2002, Tagami et al., 2008). Just as NICD transcription factor , there are increasing evidence that AICD is also trafficked to nucleus where it could be acting as a transcription factor (Kopan, 2002, Goodger et al., 2009, Roncarati et al., 2002). Though amyloidβ peptide is produced throughout life, it has been recently reported that the production of amyloid β_{42} (both the soluble and insoluble form) increases with ageing relative to amyloid β_{40} (after 50 years of age), possibly contributing to AD pathology (Miners et al., 2014). The increase in amyloidβ$_{42}$ with

respect to amyloidβ$_{40}$ levels have been reported in familial AD as well as transgenic animal models of AD (Wolfe, 2007, Selkoe, 1998). The alternate mechanism of APP processing is the non-amyloidogenic pathway whereby another secretase, α-secretase (ADAM 17), cleaves within the amyloidβ domain (between position 16 and 17) of APP leading to formation and release of an 82kDa neuroprotective protein, soluble APPα, into the extracellular matrix , an αC-terminal fragment stub and avoids the formation of the amyloidβ fragment (Gandy et al., 1993, Westmark, 2013). APP overexpression leads to increased processing of APP through the amyloidogenic pathway (Mattson, 1997) leading to increased production of amyloidβ. Though amyloidβ is present in body fluids under physiological conditions, an increased production or reduced clearance of amyloidβ leads to toxic formation of amyloid oligomers and amyloid plaques (Klein et al., 2004, Westmark, 2013).

Amyloidβ species undergo various kind of post-translational modifications. Pyroglutamylation is an important modification , which was identified at the glutamic acid on position 3 at N-terminal end (Mori et al., 1992) . This species was weakly soluble (Saido et al., 1995) and was present in small amount in plaques. Another amyloid species with pyroglutamate modification showed the pyroglutamylation at aspartate 11(Naslund et al., 1994, Liu et al., 2006). Conversion of glutamate to pyroglutamte is a dehydration reaction which can be catalyzed by the enzyme glutaminyl cyclase (Schilling et al., 2004). In AD glutaminyl cyclase expression has been reported to be increased (Schilling et al., 2008). Both *in vitro* and *in vivo*, reduced glutaminyl cyclase results in reduced pyroglutamate amyloidβ formation(Schilling et al., 2008) (Cynis et al., 2008, Jawhar et al., 2011). The in vitro toxicological profile of amyloidβ$_{42}$ and amyloidβ pyroglutamated at position 3 is same in neuronal cells. Various mouse models of AD show the presence of pyroglutamated AD but its time of first appearance varies (Christensen et al., 2008, Kawarabayashi et al., 2001).

Oxidation of amyloidβ occurs at methionine at position 35. Methionine 35 is oxidized to methionine sulfoxide and was reported in AD by Greengard's group (Naslund et al., 1994) There are three potential phosphorylation sites on amyloidβ – serine at position 8 and 26 , tyrosine at position 10. Amyloidβ serine 26 phosphorylation has been reported in AD brains (Milton, 2001). Serine 8 phosphorylated species is found in plaques as well as intracellularly and increases oligomeric aggregate formation (Kumar et al., 2011, Kumar et al., 2013a). Many post-translational modifications in amyloidβ are induced by Nitric oxide, like the dityrosine formation and nitration at tyrosine residues, S-nitrothiols at cysteine residues (Radi et al., 2002, Butterfield et al., 2007, Castegna et al., 2003). In APP/PS1 mice, nitrated amyloidβ initiates plaque formation which may have a role in early phase of AD (Kummer et al., 2011). The presence of O-glycosylated amyloidβ species in the CSF of AD patients have been shown by mass spectrometric analysis (Halim et al., 2011). Isomerization at asparagine residue and racemization at aspartly residues are other mode of post translational modification in amyloidβ species (Szendrei et al., 1994, Roher et al., 1993).

Familial, early onset forms of AD are due to point mutations in the gene encoding APP, PS1 or PS2 (Murrell et al., 1991, Levy-Lahad et al., 1995a, Sherrington et al., 1995). These point mutations shift APP cleavage from the non-amyloidogenic pathway to the amyloidogenic pathway, leading to toxic amyloid production. These point mutants are sufficient to induce AD pathology. Thus, abnormal processing can lead to tau hyperphosphorylation and formation of neurofibrillary tangles.

Amyloidβ species activate a number of intracellular signalling pathways (Sheng et al., 2012). They may directly or indirectly activate a mitchondrial apoptotic pathway leading to neuronal toxicity or synaptic impairments by caspase-3 activation (D'Amelio

et al., 2011). They also trigger a number of signalling pathways that lead to increased Ca^{2+} influx, impaired energy metabolism and increased oxidative stress, all of which will contribute to synaptotoxicity and neurodegeneration (Bezprozvanny and Mattson, 2008). Pharmacological studies reveal an amyloidβ induced Ca^{2+} influx by interaction with NMDA receptors that leads to the formation of reactive oxygen species (De Felice et al., 2007). Amyloidβ also stimulates glycogen synthase kinase 3 (GSK3), which has been implicated in AD due to its role in tau phosphorylation (Bhat et al., 2004). In the hippocampus, GSK3 activation has been reported to lead to increased NMDA receptor dependent long term depression (LTD) and inhibition of LTP, which is similar to the activity of amyloidβ (Peineau et al., 2007, Sheng et al., 2012). A recent report has also shown that inhibition of GSK3 leads to an increase in lysosomal number, causing autophagic degradation of APP (Parr et al., 2012). According to the most recently accepted form of the amyloid cascade hypothesis of AD **(Fig.1.1 and Fig.1.2),** proposed in 1991 by Hardy and Selko (Hardy and Selkoe, 2002), it is most likey the soluble oligomeric forms of amyloidβ that lead to neurodegeneration in AD and that the toxic amyloid species could be propagated by a prion-like mechanism from one cell to another (Sheng et al., 2012).

1.1.3.2. Tau and APP

Hyperphosphorylation of tau contributes to AD pathology. Tau belongs to a family of microtubule–associated proteins. Apart from its well known function in microtubule polymerization and stabilization, tau is also implicated in the regulation of axonal transport by motor protein regulation. Mutations in the *Tau* gene have linked tau abnormalities to neurodegenerative diseases; however, there is no report of a *Tau* mutation in AD (Poorkaj et al., 2001). The human *Tau/MAPT* gene has 16 exons and is

located on chromosome 17 at band position 17q21 (Neve et al., 1986, Andreadis et al., 1992). Alternative splicing of the human tau gene gives rise to six tau isoforms (Goedert et al., 1989a, Goedert et al., 1989b). The inclusion or exclusion of a coded exon 10 region determines the classification of these isoforms as 3 repeat (3R) or 4 repeat (4R) (Poorkaj et al., 2001). In the adult human brain the 3R and 4R levels are almost equal whereas adult mouse brain contains the 4R isoforms exclusively. There are 14 amino acids difference at the N-terminal end between mouse and human tau sequences (Andorfer et al., 2003). Tau is a neuronal protein with axonal localization and the primary sequence of tau can be sub-divided into an amino-terminal region, a proline-rich domain followed by microtubule-binding repeat motifs and the carboxy-terminal tail (Weingarten et al., 1975, Schweers et al., 1994). There are 79 potential phosphorylation sites on the longest tau isoform (441 amino acids) (Buee et al., 2000). About 20 protein kinases are reported to phosphorylate tau, which includes glycogen kinase 3 (GSK3) and cyclin-dependent protein kinases 5 (Sergeant et al., 2008, Hamdane et al., 2003, Tomizawa et al., 2001). In AD, tau pathology follows a cortico-cortical connection sequential pathway starting from entorhinal cortex and ending in motor-sensory cortex (Braak and Braak, 1991b). Mice overexpressing human tau show synaptotoxicity even in the absence of NFT, leading to the conclusion that soluble oligomeric tau protein could be an important synaptotoxic molecule downstream of amyloid β (Pooler et al., 2014). Hyperphosphorylated tau aggregates have been shown to be interacting with post-synaptic signalling complexes modulating axonal mitochondrial transport and glutamatergic receptor levels in dendritic spines (Ittner et al., 2010, Shahpasand et al., 2012), providing clues to the mechanism of tau-mediated synaptotoxicity.

1.1.3.3. Other factors implicated in the onset of AD

There are various other risk factors that have been considered important for the onset of AD. Positional cloning strategies have revealed a number of other genes that are risk factors or contributors to AD onset. For the early onset forms of AD (age of onset <65 years) that are mostly familial, *presenilin1* and *presenilin 2* on chromosomes 14 and 1 respectively, have been identified as a major locus linked to the AD (Schellenberg et al., 1992, St George-Hyslop et al., 1992, Levy-Lahad et al., 1995a, Levy-Lahad et al., 1995b). Presenilin, along with other components, form the core functional complex of γ-secretase, which is important for cleavage of Notch, APP and other transmembrane protein (Edbauer et al., 2003).

Apolipoprotein E is an amyloid β binding protein and it is proposed to bind soluble forms of amyloid peptide (Kim et al., 2009). An allele of the *apolipoprotein E* (*apoE*) gene on chromosome 19 has been linked with late onset AD (onset age >65 years), which is mostly sporadic (Pericak-Vance et al., 1991, Strittmatter et al., 1993). The *apoE4* variant of this gene is considered a major risk factor for late onset AD (Corder et al., 1993). About a quarter of the population carries this allele, raising the question of the contribution of this risk factor to AD. Another variant, apoE2, is thought to have a protective function against the disease as it negatively correlates with AD (Chartier-Harlin et al., 1994).
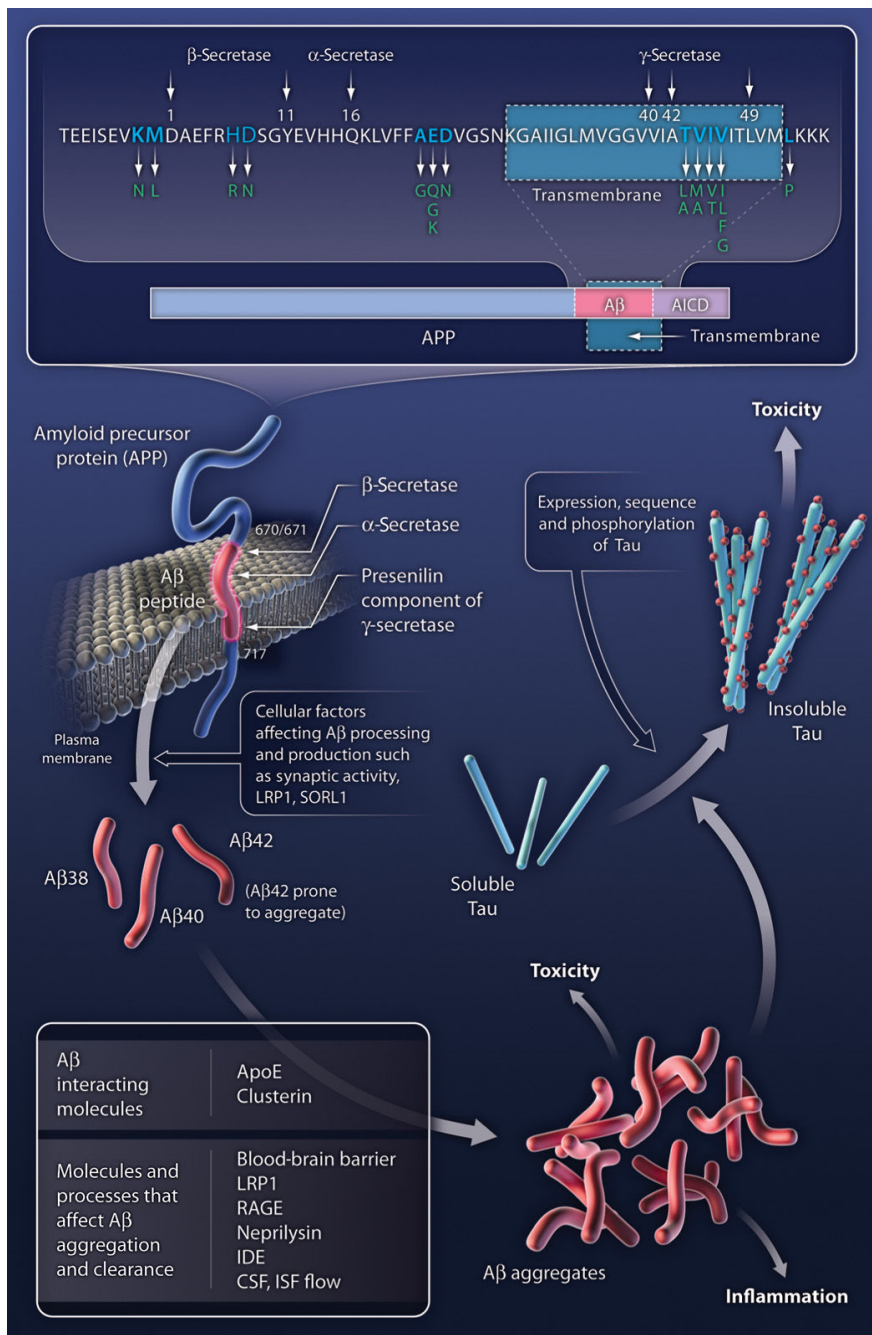
Figure 1.1. Interaction of amyloid β, Tau and other factors involved in AD pathogenesis. The cleavage of APP by β secretases and γ secretases in the amyloidogenic pathway leads to formation of amyloid peptides of various lengths. These amyloid peptides aggregate to form plaques. The plaques (possibly acting as a reservoir of toxic amyloid species) and the oligomers are the possible cause of subsequent toxicity, inflammation and downstream toxic tau hyperphosphorylation.

This is the most basic pathway of Hardy-Selko's amyloid cascade hypothesis. Taken from (Holtzman et al., 2011).
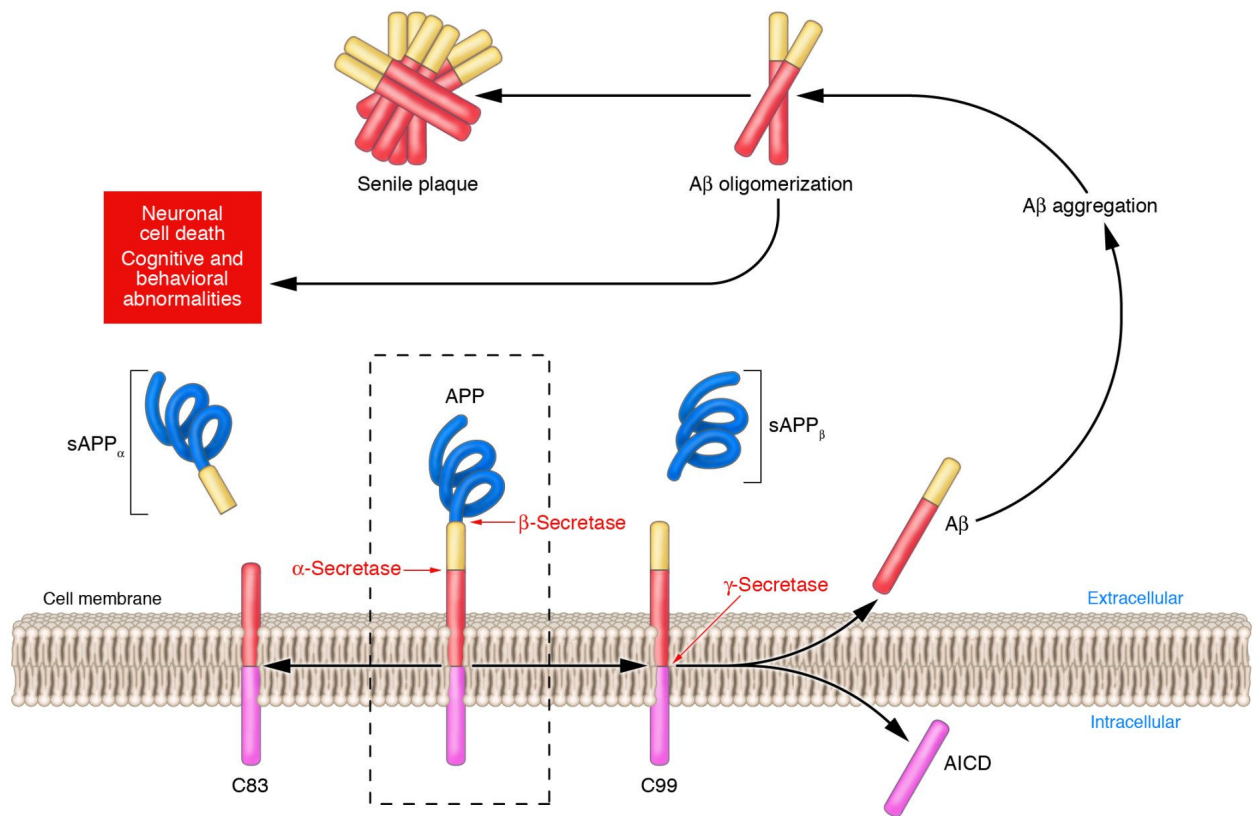
Figure 1.2. APP processing and amyloid β accumulation. Mature APP (center, inside dashed box) is metabolized by 2 competing pathways, the α-secretase pathway that generates sAPP$_\alpha$ and C83 (also known as CTFα; left) and the β-secretase pathway that generates sAPP$_\beta$ and C99 (right). Some β-secretase cleavage is displaced by 10 amino acid residues and generates sAPP$_{\beta'}$ and C89. All carboxy terminal fragments (C83, C99, and C89) are substrates for γ-secretase, generating the APP intracellular domain (AICD) and the secreted peptides p3 (not shown), Aβ (right), and Glu^{11} Aβ. Aβ aggregates into small multimers (dimers, trimers, etc.) known as oligomers. Taken from (Gandy, 2005).
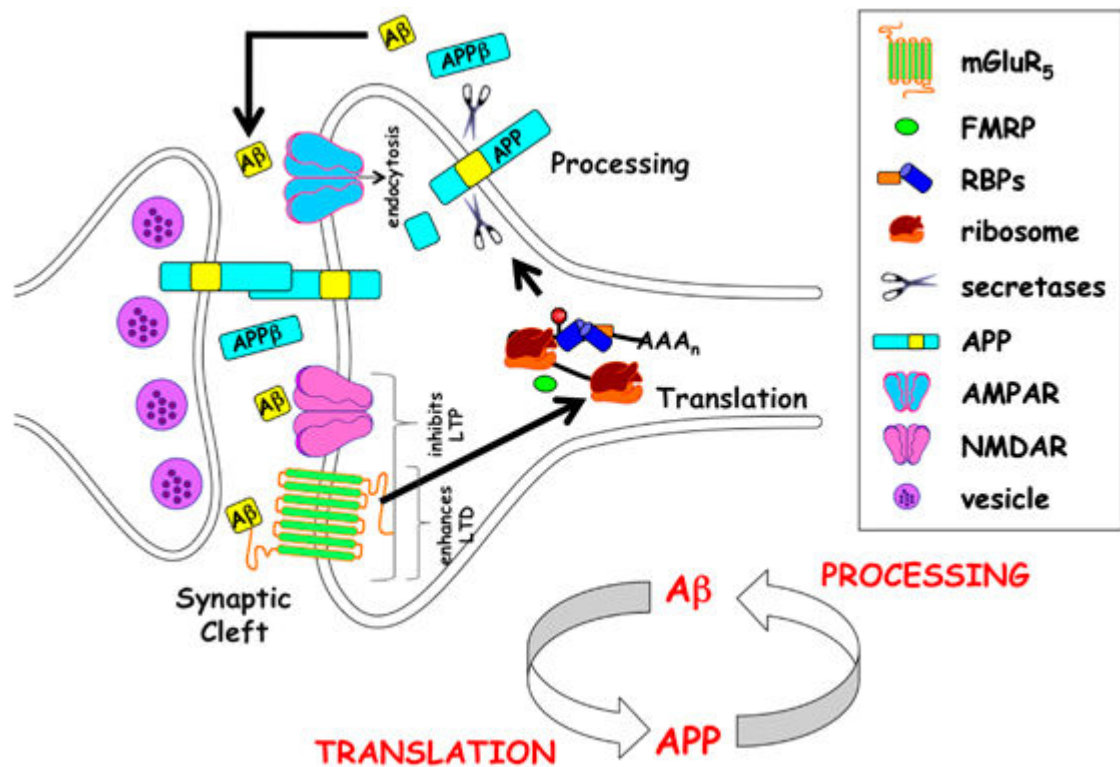
Figure 1.3. APP processing and feed forward model. This model has been proposed by Westmark on the basis of their findings that indicate that amyloid β stimulates dendritic APP synthesis and can be inhibited by anisomycin or MPEP, indicating the relevance of an mGlur5 dependent and protein translation dependent pathway for APP synthesis at dendrites. Thus APP processing and cleavage leads to amyloid β formation, which in turn initiates a feed forward loop resulting in further APP synthesis through an mGluR$_5$ and protein translation dependent mechanism. This generates more APP molecules to be processed by the amyloidogenic pathway into amyloid β peptides. Taken from (Westmark, 2013).
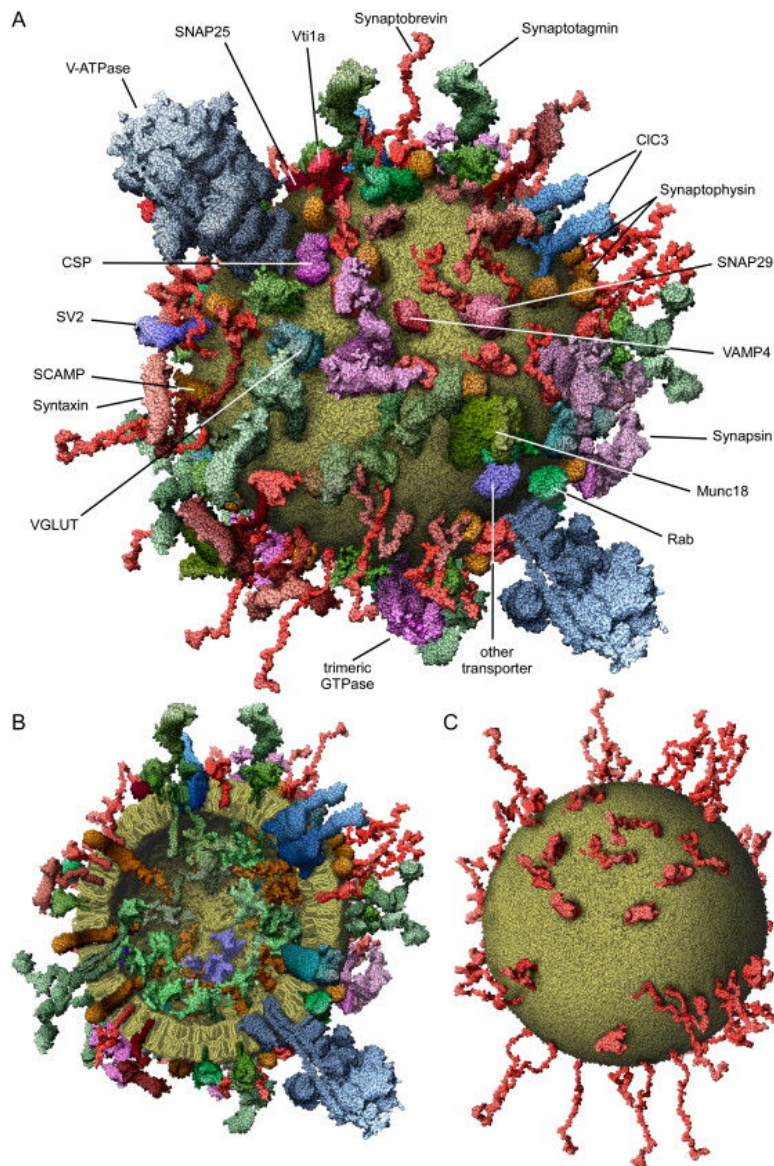
Figure 1.4. Molecular model of an average synaptic vesicle containing CSP protein.

The model is based on space-filling models of all macromolecules at near atomic resolution.

(A) Outside view of a vesicle. CSP is one of the proteins visible on the surface.

(B) View of a vesicle sectioned in the middle (the dark-colored membrane components represent cholesterol).

(C) Model containing only synaptobrevin to show the surface density of the most abundant vesicle component. Taken from (Takamori et al., 2006).

1.2. The Synapse and Alzheimer's disease

Loss of synapses is an important pathology of AD, which occurs at the earliest stages of disease preceding the neuronal loss (discussed in detail later). To understand synaptic degeneration in AD was the primary objective of this PhD study. Which mechanisms lead to synaptic loss in AD is an important unanswered question in neurodegeneration research. To better understand the factors leading to synaptic and subsequent neuronal losses in AD it is essential to understand broadly the significance of APP (and its catabolites) and Tau at the synapses or dendrites.

1.2.1. Role of APP and its catabolites at synapses

1.2.1.1. APP at synapse

APP has been reported to play a role in synapse formation, synaptic transmission, dendritic spine formation, learning and memory (Hoe et al., 2012). Apart from the important amyloid β (discussed in section 1.2.1.2), other catabolites of APP have distinct functions. Soluble APPα (sAPP α, produced as a result of α secretase cleavage of APP in the non-amyloidogenic pathway) interacts with and disrupts APP dimers in the membrane preventing starvation-induced cell death, hence performing a neuroprotective function (Gralle et al., 2009). CPEB (Cytoplasmic Polyadenylation Element Binding) factor is anchored to membrane by APP promoting polyadenylation induced translation (Cao et al., 2005). sAPPα also has been reported to enhance LTP and enhance the *de novo* protein synthesis in rat synaptoneurosomes (Claasen et al., 2009, Taylor et al., 2008). Whereas sAPPα is neuroprotective, soluble APPβ (sAPPβ)

on the other hand could be neurotoxic. Tessier-Lavigne's group has reported that sAPPβ binds to death receptor 6 (DR6) causing axonal degeneration (Nikolaev et al., 2009). In the amyloidogenic pathway of APP processing, β-secretase cleavage of APP produces a 104 amino acid long fragment at the carboxy terminal end, which has been shown to be important for spatial learning and LTP maintenance (Nalbantoglu et al., 1997). γ-secretase further cleaves this fragment producing different lengths of amyloid β and an intracellular carboxy terminal fragment. The latter intracellular fragment of APP has been shown to be important in cellular signaling, protein-protein interactions and apoptosis (Zheng and Koo, 2011).

1.2.1.2. Amyloid β at synapse

Amyloid β has been reported to be involved in increasing LTD, decreasing LTP, enhancing calcium influx and enhancing membrane depolarization (Koffie et al., 2011, Blanchard et al., 2002). Amyloid β binds to number of cell surface receptors, which also includes APP and NMDA receptors (Verdier et al., 2004). At the excitatory synapses, the lateral diffusion and accumulation of Amyloid β causes peptide clustering leading to a decreased mobility but increased activity of $mGluR_5$ at synapses (Renner et al., 2010). Amyloid β also activates two signaling molecules, GSK3β and mTOR, both of which are relevant to AD pathology (Takashima et al., 1996, Caccamo et al., 2011, Mines et al., 2011). Westmark et al. have shown that Amyloid β stimulates dendritic APP synthesis (Westmark et al., 2011). Primary cultured neurons that were treated with Amyloid $β_{42}$ showed increased expression of APP, MAP1B and RhoB. The Amyloid $β_{42}$ mediated overexpression of dendritic APP can be blocked by MPEP (an $mGluR_5$ antagonist) or anisomycin (a translation inhibitor) indicating that the $mGluR_5$ and the translation dependent pathway are involved. Amyloid β has been reported to increase

the levels of APP in neuronal hybrid cells as well as induce secretion of amyloid β in rat cortical neurons (Le et al., 1995, Marsden et al., 2011). On the basis of the above facts, Westmark has proposed a feed forward loop for amyloid β action at the synapses whereby increased APP translation leads to increased release of amyloid β (by the amyloidogenic pathway), which stimulates mGluR$_5$ signalling dependent dendritic translation of APP mRNA **(Fig.1.3)** (Westmark et al., 2011, Westmark, 2013).

1.2.1.3. Soluble oligomers and plaques at synapse

In earlier versions of the amyloid cascade hypothesis, amyloid plaques were considered to be the toxic entity responsible for synaptic and neuronal losses (Hardy and Higgins, 1992). However, reports have shown the presence of soluble active oligomeric forms (dimers, trimers, tetramers, dodecamers, etc.) of amyloid β in synthetic amyloid β peptide preparation (Kayed et al., 2003), in APP transgenic mouse brains (Shankar et al., 2009) and in AD brain tissue (Shankar et al., 2008), which is potentially neurotoxic. In addition, the insoluble amyloid fibrils as well as their aggregate plaques might be inactive, acting simply as reservoir of these oligomers. Protofibrils from synthetic amyloid β which are thinner than classical 8nm amyloid fibrils have been reported to be neurotoxic in *in vitro* condition (Hartley et al., 1999). However, the plaques acting as reservoirs at the synapse do lead to distortions of neurites in their vicinity (Hyman et al., 1995). A recent array tomography study by Spires-Jones et al in APP transgenic mice , shows a radial gradient of excitatory synapse loss and neuritic dystrophy (Spires-Jones et al., 2007). This synaptic loss and dystrophy is highest in the region closest to the plaques and reduces in a radial fashion, reaching normal levels at about 30-50 μm away from plaque core edge (Spires-Jones et al., 2007). The same group used array tomography with post-mortem tissue and they shows that oligomeric amyloid β

binds to the pre- and post-synapse. Further the signal is much higher in close proximity to a plaque (Koffie et al., 2012). These morphological studies reveal that insoluble plaques may be having synaptotoxic and neuritic effects by acting as a reservoir of toxic soluble oligomers. Biochemical isolation of soluble amyloid oligomers and insoluble plaques from same AD cortex and electrophysiologically assay of their effects on mouse brain slices have shown that soluble oligomers block LTP whereas washed amyloid cores do not (Shankar et al., 2008). However, washing the amyloid core in harsh solvent (formic acid) before the assay made them toxic as it releases their constituent oligomers (Shankar et al., 2008). Hence, it is becoming more widely accepted that insoluble plaques or amyloid fibrils might be locally neurotoxic by being in a dynamic equilibrium with the soluble oligomers and protofibrils in the vicinity.

1.2.2. Synaptic defects in AD

Synaptic loss can be detected in the earliest stages of AD. Various groups have reported the loss of pre-synaptic proteins like VAMP2 and SNAP25 and post-synaptic proteins like PSD95, and Shank1 (Pham et al., 2010, Arendt, 2009). Microscopy studies have reported alterations in synaptic structure in the early stages of AD as well as APP transgenic mice (Scheff et al., 2013, Masliah et al., 1994, Alonso-Nanclares et al., 2013). Studies have shown more severe losses of glutamatergic terminals but not GABAergic terminals in AD hippocampus and animal models (Canas et al., 2014, Mitew et al., 2013). At the same time Cuello's group suggest that GABAergic synapses degenerate equally to glutamatergic synapses in AD from animal model studies (Bell et al., 2006). Consistent with studies using neuropathological and structural studies, gene expression studies have revealed a number of genes that are altered in early AD that includes the genes involved in synaptic vesicle trafficking, postsynaptic density

scaffolding, neurotransmitter receptors, etc. (Berchtold et al., 2013, Chang et al., 2013). Synaptic losses in the cortex and limbic system have been shown to correlate best with memory impairments in AD and the synaptic losses precede neuronal losses as suggested by a greater extent of synaptic loss compared to neuronal loss during AD. Post mortem brain immunohistochemical studies have shown that neurons in AD have reduced synaptic staining (Terry et al., 1991, DeKosky and Scheff, 1990, Scheff et al., 1990, DeKosky et al., 1996, Ingelsson et al., 2004). An increased level of amyloid β_{42} is expected to be involved in synaptic losses during AD (Sisodia and Price, 1995, Selkoe, 1989, Selkoe, 1993). However the mechanism by which amyloid β and other APP metabolites lead to synaptotoxicity is not yet known (Overk and Masliah, 2014). Monomeric amyloid β aggregates to form amyloid fibrils and smaller order oligomeric species (Glabe, 2008, Selkoe, 2008). The oligomers of amyloid β organize into dimers, trimers and higher order arrays (Mucke and Selkoe, 2012, Tsigelny et al., 2014). More recent studies have reported these oligomers to be the toxic species and a trigger for the synaptic pathogenesis in AD (Klein, 2002, Glabe, 2005). However, understanding the precise nature and mode of action of this oligomeric species is an active field of research.

Based on the positive feedback loop model **(Fig.1.3),** Westmark has suggested that increased processing of APP through the amyloidogenic pathway, at the expense of non-amyloidogenic pathway, promotes amyloid β accumulation and synaptic loss before plaque accumulation in AD (Westmark, 2013). Excessive amyloid β could be leading to synaptic failure in AD by altering the molecular composition of postsynaptic density (a site where scaffolding protein recruit and anchor receptors) and thus altering the downstream signaling (Westmark, 2013). Experiments by Dineley's group have strengthened this hypothesis and had shown that the form of the oligomeric species as well as the treatment time determines the downstream signaling (Bell et al., 2004).

Acute treatment with oligomeric amyloid β_{42} activates ERK (extracellular regulated kinase) mitogen activated protein kinase (MAPK^{ERK}) and its downstream target, ribosomal S6 kinase, but not c-JNK (Jun N-terminal kinase) mitogen activated protein kinase (MAPK^{JNK}). On the other hand, chronic treatment with oligomeric form or high molecular weight aggregates of amyloid β_{42} leads to MAPK^{ERK} downregulation and MAPK^{JNK} activation (Bell et al., 2004, Westmark, 2013). Hence, amyloid β induced alteration at PSD could be leading to synapse failure in AD.

1.2.3. Molecular Mechanism of synaptic degeneration in AD

Dysregulation of glutamate receptors has been shown to be one of the processes upstream of synaptic degeneration that could cause alterations in axonal transport of synaptic vesicles and mitochondria leading to dendritic and spine alterations (Mota et al., 2014, Hsieh et al., 2006). Therefore aberrations in synaptic function may precede the loss of pre-synaptic terminals and dendritic spines culminating into synaptic loss. Neuronal loss occurs in the late stages of AD. Downstream of amyloid β accumulation at synaptic sites, many receptors and signaling cascades have been identified those are affected. mGluR5 (Renner et al., 2010), ephrin (ephR2) (Cisse et al., 2011) and prion protein (PrP) (Lauren et al., 2009) are some of the molecules that have been reported to be acting as amyloid β oligomer receptors. At the PSD, binding of extracellular amyloid β oligomers to lipid anchored PrP(C) activates intracellular Fyn kinase affecting synaptic activity (Chin et al., 2005, Um and Strittmatter, 2013). Strittmatter's group has shown that this activation requires mGluR5, where mGluR5 interacts with an amyloid β oligomer-PrP(C) complex (Um et al., 2013). This amyloid β-PrP(C)-mGluR5 complex activates a signaling pathway causing eEF2 phosphorylation and

ultimately dendritic spine loss (Um et al., 2013). Binding of amyloid β oligomers at the synapse leads to the dysregulation of activity and expression of NMDA and AMPA receptors, which in turn leads to defects in synaptic activity (Paula-Lima et al., 2013, Sivanesan et al., 2013). Lipton's group has shown that amyloid β induces the glutamate release from astrocytes. This causes activation of extrasynaptic NMDA receptors (eNMDAR) on neurons and eNMDA is the glutamate receptor system involved in synaptotoxicity in AD (Talantova et al., 2013). eNMDAR activation causes synaptic transmission dysregulation, caspase-3 activation and tau phosphorylation, which leads to spine loss (Talantova et al., 2013).

Downstream of amyloid β, tau is also considered to be an important factor leading to synaptic loss. Reports of the relevance of tau interacting proteins like spastin and α1 Takusan in amyloid-induced synaptic and spine loss further supports this point (Zempel et al., 2013, Nakanishi et al., 2013). Apart from indirect interactions between amyloid β and Tau mediated by receptors/ signaling pathways (spastin, α1 Takusan, GSK3, CDK5, Fyn Kinase), the monomeric and oligomeric amyloid β directly interacts with Tau in AD affected neurons. These interactions increase in number as cognitive decline and synaptic loss increase with disease progression (Manczak and Reddy, 2013).

Studies in transgenic mouse lines carrying mutant forms of human APP have been important for understanding mechanisms related to synaptotoxicity in AD, although these mouse models do not show overt neuronal loss (Gandy et al., 2010, Malthankar-Phatak et al., 2012, Wirths and Bayer, 2010). However, they do show substantial synaptic loss and neuritic dystrophy. Also, the manipulations that rescue synaptic loss also rescue memory impairment in these models again suggest that β causes cognitive impairments in AD by inducing synaptic deficits (Roberson et al., 2011). Since, the AD mouse models do not have significant neuronal loss and develop synaptic loss as well

as memory impairment before the appearance of plaques, it can be suggested that the soluble amyloid β entities are the ones causing synaptic deficits. Also, the impairments in the earlier stages of AD are primarily due to synaptotoxicity that leads to neuronal degeneration.

1.3. Relevant mouse models in the study

Mouse model of disease are an excellent system to study specific disease mechanisms, in spite of the drawbacks of not being able to fully replicate the entire AD pathology. There are several mouse model of AD that has been generated in past decades. For our project we have used the following animal systems: the hTau mouse model of AD(Andorfer et al., 2003), the Tg2576 mouse model of AD (Hsiao et al., 1996) and the CYFIP2$^{+/-}$ mouse model (Kumar et al., 2013b) .

1.3.1. Htau mouse model

In humans, the alternative splicing of a single *Tau* gene leads to six isoforms (Goedert et al., 1989a, Goedert et al., 1989b, Kosik et al., 1989). These isoforms are categorized as 3R or 4R based on inclusion or exclusion of a nonessential region coded by exon 10 (Hutton et al., 1998, Poorkaj et al., 2001). Mouse and human tau protein sequences differ by 14 amino acids at their N-terminal end. Possibly this species difference in tau alternative splicing could be the reason as to why the AD mouse models do not have NFTs. In 2003 Peter Davies's group developed a mouse model that expresses the 6 human tau isoforms and does not express the 3 mouse tau isoforms. The htau

transgenic mice from Davies's group express a human tau transgene under the control of a tau promoter and have a null mutation obtained by the insertion of eGFP cDNA into exon1 of the mouse tau gene (Andorfer et al., 2003). Htau transgenic mice were obtained by crossing 8c mice that carry the human tau gene (Duff et al., 2000) with tau knockout mice with a disrupted mouse tau gene (Tucker et al., 2001), leading to an F1 generation that was backcrossed to tau knockout mice. This produced htau transgenic mice on a C57BL/6 background that are homozygous for mouse *tau* disruptions but homozygous for a human tau transgene. The mice express all six isoforms of human tau but none of the mouse tau isoforms.

These mouse models have been shown to undergo age-related accumulation of AD relevant phosphorylated tau in the cell bodies and neuronal dendrites, also showing an accumulation of aggregated paired helical filaments. Cell body accumulation (redistribution from axons) of phosphorylated tau was detected by 3 months of age and the levels increase with age (Andorfer et al., 2003). By 9 months of age the levels of phosphorylated tau in htau mice model resemble early stage NFT pathology in human brain. The majority of tau pathology in htau mice is located in hippocampus and neocortex and is minimal in brain stem and spinal cord (Andorfer et al., 2003). Polydoro et al. reported age-dependent synaptic and cognitive impairments in these mice model. Basal synaptic transmission as well as LTP induction is impaired in the hippocampal CA1 region in 12 month-old mice but not 4 month-old mice (Polydoro et al., 2009). Further, spatial memory formation (water maze test) and object recognition memory formation (disruption of visual recognition memory of novel object) are impaired in 12 month-old htau mice. At this ageing point the mutants have a moderate tau pathology compared to 4 month-old mice (Polydoro et al., 2009).

1.3.2. Tg2576 mouse model

Familial AD is an inherited form of AD and its onset is earlier in comparison to sporadic AD. In 1996 Karen Hsiao's group created the Tg2576 mouse line to model familial AD using an APP transgene from a large Swedish family with early onset AD (Hsiao et al., 1996). Tg2576 mice express human APP_{695} (K670N, M671; APP_{770} numbering), under control of hamster prion protein (PrP). This model was developed on a background of C57BL/6 and SJL mouse strains. The mice express the mutant human APP at 5.5 times the level of endogenous murine APP (Hsiao et al., 1996). The hAPP transgene had a double mutation (amino acid substitution) in APP, with Lys 670 to Asn and Met671 to Leu.

These mutations lead to amyloid β overproduction. The mice display several characteristic neuropathologies of AD – plaques, activated microglia, inflammation, synaptic deficits, increased amyloid β soluble as well as insoluble) (Hsiao et al., 1996, Chapman et al., 1999, Frautschy et al., 1998, Benzing et al., 1999, Smith et al., 1998). The soluble oligomeric forms of amyloid β are considered to be the primary toxic species as discussed above. This soluble form is present as early as 4 months of age in these mice (Fodero et al., 2002). By 10-11 months, amyloid plaques start to form (Hsiao et al., 1996). However, there are many neuropathological features of AD that are not faithfully replicated in this model. These include negligible change in the cholinergic system (Gau et al., 2002), neurofibrillary tangles are missing (Irizarry et al., 1997) and neuronal loss or brain atrophy are not detectable (Irizarry et al., 1997). Also, the physico-chemical organization of amyloid peptides in Tg2576 mice has been reported to be different than that found in AD in humans (Kalback et al., 2002). Furthermore, it has been suggested that the overexpression of the C-terminal APP fragment may cause unwanted phenotypes in this mouse model (Saito et al., 2014).

Taken together, the Tg2576 model does not exactly replicate AD but it models amyloid β production and plaque deposition – an important pathology in both sporadic and familial forms of AD. The neuropathology in the Tg2576 models is reported to be present at 10-12 months of age (Hsiao et al., 1996, Kawarabayashi et al., 2001, Pratico et al., 2001) although there, is already a loss of dendritic spines at 3 months of age (D'Amelio et al., 2011).

The behavioural and memory impairments reported in the Tg2576 animals emerge at time points before plaque deposition. Hsiao et al. report an impairment in spatial memory (from Morris water maze tests) in these models at 9-10 months of age (Hsiao et al., 1996), whereas Westerman et al. report it at 6 months (Westerman et al., 2002). In Y-maze tests, Hsiao et al. report an impairment at 9-10 months of age (Hsiao et al., 1996) while Ognibene reports it at 7-12 months age (Ognibene et al., 2005). In the novel arm recognition test reported by Park et al, the young mice (3-4 months) do not show any difference, whereas the aged Tg2576 mice (12-15 months) show impairments (Park et al., 2008).

1.4. Target molecules of the study

In this project, we have studied the role of two novel neuronal proteins that are dysregulated in AD and they may have a role in synaptic degeneration during AD – CYFIP1/2 (Chapter 3) and CSPα (Chapter 4). Previous studies in the laboratory have shown both of these molecules to be regulated by another neuronal molecule – p25.

1.4.1. p25

p25 is a 209 amino acid long proline rich cleavage product of the cyclin-dependent kinase 5 (Cdk5) activator p35. p35 protein's cleavage into the C-terminus 25kDa p25 fragment and N-terminus 10kDa p10 fragment is mediated by calpain, a calcium-dependent protein protease (Patrick et al., 1999, Tang et al., 1997). p25 is a more stable Cdk5 activator protein with a longer half life in comparison to ubiquitin degradation prone p35 (Patrick et al., 1999). Also whereas p35 is membrane bound, p25 is localized in the cytosol and nucleus, leading to the suggestion of p25 acting as a signal between the synapse and the nucleus (Patrick et al., 1999, O'Hare et al., 2005). Not much is known about the function of p25 at the synapse. Tsai's group has reported that p25 is upregulated in AD (Patrick et al., 1999). However, reports from several groups, including ours, have shown that p25 might be downregulated in the earlier stages of AD and continue to be so until the severe stages (Engmann et al., 2011, Tandon et al., 2003, Yoo and Lubec, 2001). Studies with a p25 transgenic mouse model have shown that p25 overexpression is neurotoxic and leads to memory impairments (Fischer et al., 2005). However, our group has shown in AD postmortem tissue that p25 levels are downregulated in milder stages of AD, so p25 overexpression-based models are not relevant for AD (Engmann et al., 2011). p25 has been reported to be involved in LTP, synaptic functions and memory formation (Fischer et al., 2005, Engmann et al., 2011, Ris et al., 2005, Angelo et al., 2003), however the pathway by which it influences these processes is under active investigation.

p25 has been reported to be essential for memory formation and synaptogenesis. Both the Giese group and Tsai group have shown the importance of p25 in memory formation. Overexpression of p25 leads to improved spatial memory in mouse models (Angelo et al., 2003, Fischer et al., 2005) and p25 transgenic mice have enhanced late

phase hippocampal CA1 LTP as well as increased synapse density (Angelo et al., 2003, Ris et al., 2005, Engmann et al., 2011). Hence, p25 has been shown to be involved in regulation of molecules essential for synaptogenesis or synaptic functioning. A proteomic study from syaptosomes of p25 transgenic mice yielded a set of synaptic proteins (about 20) that are regulated by p25 and includes post synaptically located Cytoplasmic FMR interacting proteins2 (CYFIP2) and pre-synaptically located Cysteine string protein (CSPα) (Engmann et al., 2011). These molecules might be involved in synaptogenesis and LTP during memory formation. At the same time it could be speculated that these molecules may be downregulated along with a decrease in p25 levels in the initial stages of AD (Tandon et al., 2003, Engmann et al., 2011). This has been demonstrated in Optic atrophy 1 (OPA1) – a mitochondrial protein involved in mjtochondrial fusion as well as spine formation.(Wang et al., 2009). OPA1 was also one of the candidate p25 regulated proteins obtained in the aforementioned proteomic study from the Giese group that was downregulated in the hippocampus in early stages of AD (Engmann et al., 2011). Hence p25 downregulation possibly leads to dysregulation of synaptic proteins in the initial stages of AD affecting the synaptic structure and function (Giese, 2014).

1.4.2. CYFIP2/ CYFIP1

Cytoplasmic FMR Interacting Protein -1 (CYFIP1) and Cytoplasmic FMR Interacting Protein -2 (CYFIP2) are FMRP co-activators (Schenck et al., 2001). They are members of a highly conserved protein family in humans (Schenck et al., 2001). CYFIP1/2 colocalize with FMRP and are found in the cytoplasm as well as synaptosomal extracts (Schenck et al., 2001). Even though the amino acid sequence is 87.7% identical between CYFIP1 and CYFIP2, the binding affinities of these proteins differ; CYFIP1 binds only with FMRP whereas CYFIP2 binds FMRP

and the FMRP related proteins - FXR1 and FXR2 (Schenck et al., 2001). CYFIP1 acts as an EIF4E binding protein (4E-BP) inhibiting the translation of FMRP bound mRNA (Napoli et al., 2008). CYFIP family proteins are also part of the actin cytoskeletal modulating WAVE complex (Cory and Ridley, 2002, Derivery et al., 2009). The small Rho GTPase, Rac1, binding to CYFIP1/2 removes CYFIP1/2 from the WAVE complex so that WAVE can activate actin polymerization via Arp2/3 (De Rubeis et al., 2013). CYFIP2 has been shown to be a p53-inducible protein that causes apoptotic cell death in non-neuronal cells (Jackson et al., 2007). CYFIP1 on the other hand is not p53 inducible (Jackson et al., 2007). In Drosophila there is just one orthologue of CYFIP family molecules that is exclusively expressed in the nervous system (Schenck et al., 2003), suggesting that during the course of evolution CYFIP1 and 2 might have acquired different functions in humans. The *CYFIP1* gene is located on chromosome 15 (Nowicki et al., 2007) with *CYFIP2* located on chromosome 5 (NCBI Gene ID: 26999). CYFIP1 and CYFIP2 have not been reported to have any non-neuronal expression in the brain.

1.4.3. CSPα

CSPα (Cysteine String Protein α) is a 34 kDa synaptically located J-domain containing protein (Zhao et al., 2008) **(Fig.1.4)**. The CSPα structure contains an N-terminal J-domain and a string of 13-15 cysteine residues in the middle region (Braun and Scheller, 1995). CSPβ and CSPγ are two other protein variants that are homologous to the CSPα sequence in the mammalian genome (Evans et al., 2003). However, these proteins are not expressed in brain (Fernandez-Chacon et al., 2004).

CSPα has been shown to be involved in the following functions in neurons: (1) Exocytosis - it acts as a co-chaperone of a trimeric complex by interacting with two other proteins - Heat Shock Protein 70 (Hsc 70) and small glutamine–rich TPR-containing protein (SGT). Thomas Sudhof's group has shown that this trimeric complex is involved in exocytosis by interacting and co-chaperoning the SNARE proteins leading to synaptic vesicle fusion in presynaptic terminals (Sharma et al., 2011). SNARE proteins are a set of synaptically localized membrane fusion proteins involved in vesicle recycling mechanism in presynaptic terminals. In CSPα knockout mice, the levels of SNARE proteins (like α Synuclein and SNAP-25) are reduced (Chandra et al., 2005). (2) Endocytosis – CSPα interacts with dynamin 1 and facilitates the polymerization of dynamin, which is important for endocytotic vesicle fission (Zhang et al., 2012). (3) Modulation of calcium dependent K^+ channels (BK channels) where CSPα is important for modulation of pre-synaptic BK expression (Kyle et al., 2013). CSPα knockout mouse show upregulation of BK channels and hence aberrant synaptic activity. (4) Modulation of presynaptic calcium levels by regulating calcium channels (Ranjan et al., 1998). CSPα have not been reported to have any non-neuronal expression in the brain.

1.5. Overall objective of the study

As described before, synaptic losses are important feature of AD pathology. However, the molecular mechanisms leading to the synaptic losses or synaptic impairments are not precisely known. Based on the previous studies in the Giese lab using AD post mortem brain as well as a p25 transgenic mouse model (Engmann et al., 2011, Angelo

et al., 2003, Giese, 2014), we obtained a set of novel, putative p25 regulated candidate molecules that could be relevant to synaptic and neuronal losses in AD. After screening these molecules, we narrowed down our targets for further study in AD to three synaptic molecules - CYFIP1, CYFIP2 and CSPα.

So, the broad aim of this study was –

<u>To investigate the role of three putative p25 regulated molecules in AD – CYFIP1, CYFIP2 and CSPα.</u>

The methodology and experimental design used to complete this PhD study have been described in **Chapter 2**. Since, CYFIP1 and CYFIP2 belong to the same family of proteins, their study has been described in a single chapter (**Chapter 3**), with the study of CSPα described in a separate chapter (**Chapter 4**). The specific aims and results of each of these two parts have been stated in the respective chapters. The broader discussion on the overall implications and outlook from the results of this PhD study has been presented in the final chapter (**Chapter 5**).

Chapter 2: Materials and methods

2.1. Samples

2.1.1. Postmortem Human Brain Samples

Human brain samples were obtained from the London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry, King's College London. They were received in two set, which were treated separately. The first set contained hippocampal tissue from control subjects, subjects with mild Alzheimer's disease (AD; Braak stages I-II) and subjects with severe AD (Braak stages V- VI) [n = 7 for each group], as well as superior temporal gyrus (STG) samples from control and severe AD [n = 7 and n = 9, respectively]. The second set comprised hippocampus, STG and cerebellum samples from control, mild and severe AD patients (n = 5 for each group). To increase the sample size of cerebellum, a new cohort (n = 5 per group) was added later to the analysis. Additionally, cerebellum samples (n=5) were obtained from the post mortem brains of patients with Frontotemporal lobar degeneration (FTLD) to analyze the levels of CSPα in FTLD, where the cerebellar pathology has been reported. Moreover, to analyze the level of CSPα in ageing , cerebellar tissues were obtained from subjects who died less than 30 year old - young (n=6) and subjects who died more than 90 year old – aged (n=7). The causes of death were not related to neurodegenerative disease in these subjects (see **Table 2.1** for details). All human tissue samples were handled according to the regulations of Human Tissue Authority and King's College London brain bank for neurodegenerative diseases.

2.1.2. Tg2576 Mouse Brain Samples

APPswe (Tg2576) mice, expressing mutant human APP (K670N/M671L) under the control of the hamster prion promoter (Hsiao et al., 1996) were obtained from Taconic farms (Germantown, NY, USA). Mice were maintained by breeding Tg2576 males in C57BL/6 x SJL F1 genetic background with C57BL/6 x SJL F1 wild-type females, as recommended by the supplier.

Mice were housed on 12 h light: 12 h dark cycles with food and water available *ad libitum*. Mice were killed by cervical dislocation; the brains removed, tissue dissected and immediately snap frozen on dry ice. All animal procedures were conducted in accordance with the UK Animal Scientific Procedures Act 1986. Mice were genotyped by PCR using primer set 5′-CGACTCGACCAGGTTCTGGGT-3′, 5′-ATAACCCCTCCCCCAGCCTAGA-3′.The amplification conditions were as following : PCR Reaction mixture - 1X buffer, H_2O - 10.7μl, 2mM Mg_2Cl_2, 0. 2 mM dNTP, 0.75μM APP Forward primer, 0.75μM APP Reverse primer, 0.025μM Taq polymerase (Invitrogen) , 2μl/reaction DNA (Total volume made to 20 μl by addition of H_2O) . PCR Program - (1)Initialization - 94^0C for 3 minutes (2) 35 cycles – Denaturation - 94^0C for 30 seconds , Annealing - 60^0C for 60 seconds, Extension - 72^0C for 60 seconds (3) Final hold – 4^0 C. Cortico-Hippocampal tissue from 4 month (n=3) and 12 month (n=4) old Tg2576 mutants as well as wild type (WT) littermate mice (4 month ,n=4; 12 month ,n=4) were used for analysis by immunoblotting. Sexes of the animals were balanced.

2.1.3. Htau Mouse Brain Samples

Htau transgenic mice expressing a human tau transgene under control of tau promoter and having a null mutation obtained by the insertion of eGFP cDNA in exon1 of the mouse tau gene were studied to analyse tau-related neurodegeneration (Andorfer et al., 2003) . The htau transgenic mice were obtained by crossing 8c mice with human tau gene (Duff et al., 2000) and tau knockout mice with disrupted mouse tau gene (Tucker et al., 2001) , leading to an F1 generation which was backcrossed to tau knockout mice, that produces htau transgenic mice on a C57BL/6 background which were obtained from Jackson Laboratories (Bar Harbour, Maine, USA; B6.Cg-Mapttm1 (EGFP) Klt Tg(MAPT)8cPdav/J. Stock number: 005 491). These mice express all six isoforms of human tau, but none of the mouse tau isoforms. Mice were genotyped by PCR as described in (Andorfer et al., 2003) to confirm the presence of the human *MAPT* (Tau) transgene and the mouse *Mapt null* background using primers for the human *MAPT* gene (forward 5′-ACTTTGAACCAGGATGGCTGAGCCC-3′, reverse 5′-CTGTGCATGGCTGTCCCTACCTT-3′), and the mouse *Mapt* gene (forward 5′-CTCAGCATCCCACCTGTAAC-3′, reverse 5′-CCAGTTGTGTATGTCCACCC-3′), as described in (Andorfer et al., 2003). The primers for the disrupted *Mapt* gene were: Forward:5′AAGTTCATCTGCACCACCG3′, Reverse:5'TCCTTGAAGAAGATGGTG CG3'. The amplification conditions were as following : PCR Reaction mixture - 1X buffer, H_2O - 10.7μl, 2mM Mg_2Cl_2, 0. 2 mM dNTP, 0.75μM APP Forward primer, 0.75μM APP Reverse primer, 0.025μM Taq polymerase (Invitrogen) , 2μl/reaction DNA (Total volume made to 20 μl by addition of H_2O) . PCR Program - (1)Initialization - 94^0C for 3 minutes (2) 35 cycles – Denaturation - 94^0C for 30 seconds , Annealing - 60^0C for 60 seconds, Extension - 72^0C for 60 seconds (3) Final hold – 4^0 C.

Hippocampal, frontal cortex and cerebellar tissue were isolated from 3-4 months-old and hippocampal-cortical tissue from 24 month-old htau transgenic mice as well as wild-type littermates. Sample size for each category is described in the relevant chapters.

Mice were housed on 12 h light: 12 h dark cycles with food and water available *ad libitum*. Mice were killed by cervical dislocation; the brains removed, tissue dissected and immediately snap frozen on dry ice. All animal procedures were conducted in accordance with the UK Animals Scientific Procedures Act 1986

2.1.4. CYFIP2$^{+/-}$Mouse Brain

CYFIP2 null mutants were generated by European Conditional Mouse Mutagenesis Program (EUCOMM) having a neo gene insertion in an intron of the *CYFIP2* gene. This mutant uses a knockout first design. The knockout allele contains an IRES:*lacZ* trapping cassette. A floxed promoter driven neo cassette inserted into the intron of *CyFIP2* gene disrupts the gene function **(Fig. 2.1.)(Kumar et al., 2013b, Skarnes et al., 2011)**. CYFIP2 heterozygote and wild type mouse with C57BL/6 N background were obtained from Wellcome Trust Sanger Institute (Wellcome Trust Genome Campus, Hinxton Cambridge, UK). The mice were subsequently bred by crossing CyFIP2 $^{+/-}$ males with wild type female in the animal breeding facility at the James Black Center, King's College London. Mice were genotyped by PCR, using genomic DNA isolated from ear or tail samples. The mutants were detected by mutant allele specific primer (Forward CYFIP2 primer -5'TTCCTTCCTTCCCTTGTCCC3', Reverse CASR1 primer - 5'TGCCAGGAGAGACAGTGGTG3') and wild type were detected using wild allele specific primer (Forward,CYFIP2primer

5'TTCCTTCCTTCCCTTGTCCC3';Reverse,CYFIP2primer5'TCGTGGTATCGTTAT

GCGCC3') (All primers synthesized by Sigma Aldrich).
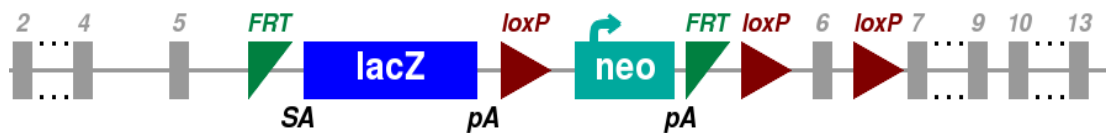
Figure 2.1. CYFIP mutant allele cassette. The *lacZ-neomycin* cassette is knocked in

an intronic sequence within *CYFIP2* allele.
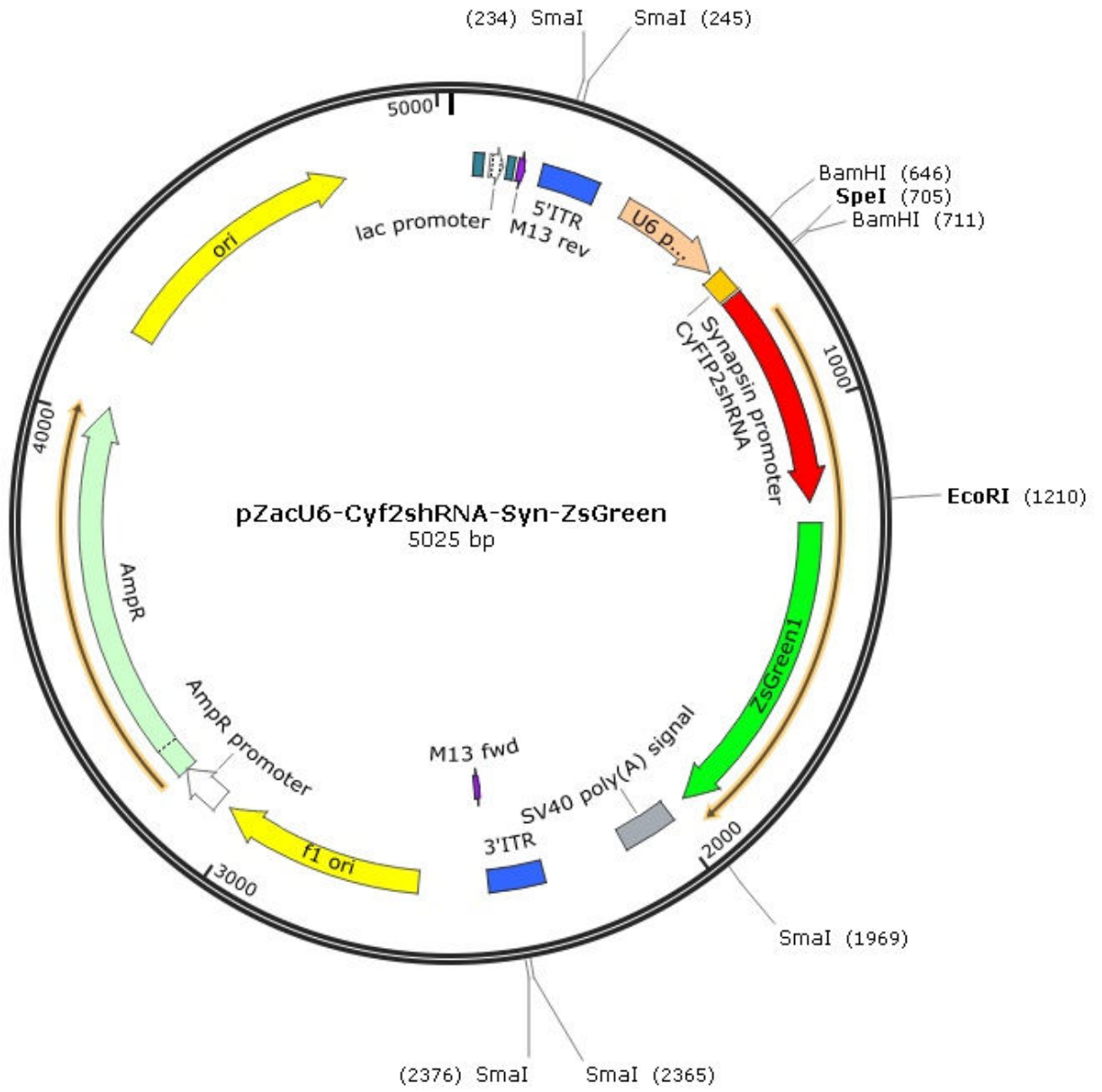
Figure 2.2. The CYFIP2 shRNA cloned in pZacU6 plasmid
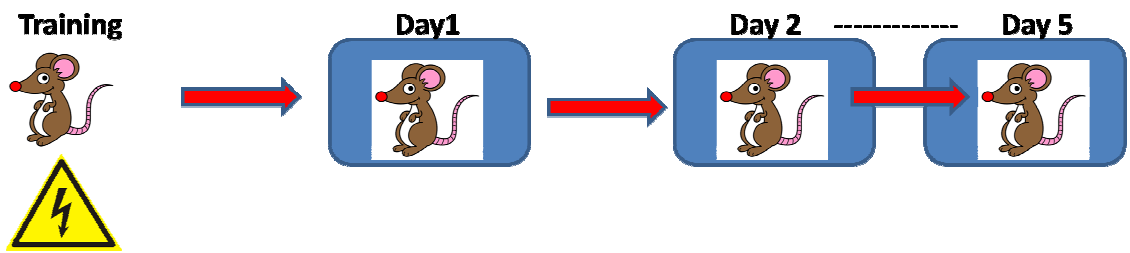
Figure 2.3. CYFIP2$^{+/-}$ mice have no deficit in forming contextual fear memories
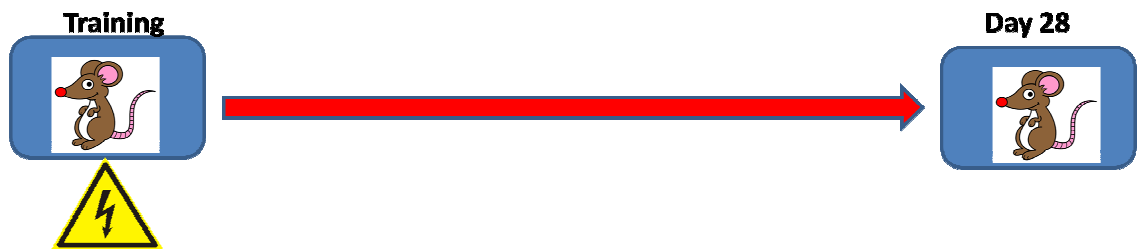
Figure 2.4. CYFIP2$^{+/-}$ mice have no deficit in retention of contextual fear memories
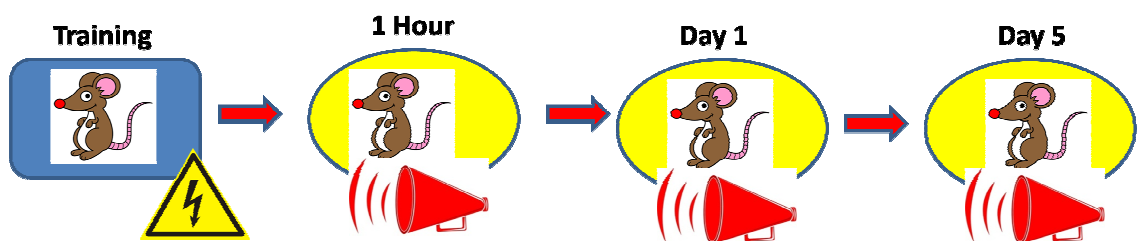
Figure 2.5. CYFIP2$^{+/-}$ mice show memory impairment in cued fear conditioning paradigm at 5 days interval

The amplification conditions were as following: The wild type band was 461 base pairs and mutant band is 248 base pairs. PCR Reaction mixture - 1X buffer, (0.5μl) 50mM Mg_2Cl_2 (0.5μl) 10mM dNTP , (1.5μl) 10μM CYFIP2 Forward primer, (1μl) 10 μMCYFIP2 Reverse primer, (0.5μl) 10 μM CASR1, 0.125μl Taq polymerase (Invitrogen) 1μl/reaction DNA (Total volume was made 25 μl by addition of 17.3μl H_2O) . PCR Program - (1)Initialization - $93^{o}C$ for 2 minutes (2) 35 cycles – Denaturation - $93^{o}C$ for 30 seconds , Annealing - $58^{o}C$ for 30 seconds, Extension - $72^{o}C$ for 30 seconds (3) Final extension - $72^{o}C$ for 10 minutes (4) Final hold – 4° C.

Hippocampi from 3-4 months old CYFIP2 heterozygote mice and wild-type littermates were isolated. Samples sizes are – n= 9 for CYFIP2$^{+/-}$ mice and n= 7 for wild type mice. Mice were housed on 12 h light: 12 h dark cycles with food and water available *ad libitum*. Mice were killed by cervical dislocation; the brains removed, tissue dissected and immediately snap frozen on dry ice. All animal procedures were conducted in accordance with the UK Animals Scientific Procedures Act 1986.

2.2. Protein preparation

2.2.1. Lysate preparation from Human Brain Sample

The frozen samples were lysed at 4°C in a RIPA lysis buffer system (Santa Cruz Biotechnology, Inc., USA). The RIPA buffer contained 0.1 % sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.004% sodium azide in Tris buffered saline (TBS; composed of 6.0 g/l Tris and 8.7 g/l NaCl, adjusted to pH 7.5 with 37% HCl). Protease inhibitors cocktail, sodium orthovandate, and α-toluenesulphonyl fluoride were added to the buffer, diluted to 1:100. The SDS

concentration was increased by adding 0.25% SDS in the final volume of buffer. About 100 mg of brain tissue was lysed in 300 µl buffer volume. Samples were homogenized using a dounce homogenizer (12 strokes, 700 rpm) and centrifuged at 3,000 rpm for 10 minutes. Supernatants were then isolated for immunoblotting.

2.2.2. Protein preparation from mouse brain tissues

Frozen tissue was homogenised at 100 mg/ml in 2 x sample buffer (0.5 M Tris-HCl, pH 6.8, 4.4 % (w/v) sodium dodecyl sulfate (SDS), 20 % (v/v) glycerol, 2 % (v/v) 2-mercaptoethanol, 0.01 % (w/v) bromophenol blue, and Complete mini-protease inhibitor cocktail (Roche Products Ltd., UK), using a mechanical homogenizer. Following brief sonication, homogenates were centrifuged at 25,000 g for 20 minutes at 4ºC, and the supernatant was collected. For mouse samples, a BCA based protein quantification procedure (Thermo Fisher) was used to determine the protein amount.

Crude synaptosomes were isolated from hippocampus as following – Frozen tissue was homogenized at 100 mg / ml in lysis buffer (0.32 M sucrose, 10mM pH7.4 Tris-HCL, 2mM EDTA, 2mM EGTA, 0.2 mM phenyl methyl sulfonyl fluoride). Following centrifugation at 1000g for 10 minutes, 4^0C to remove nuclei and cell debris, the resulting supernatant was centrifuged at 10,000 g for 20 minutes to obtain crude synaptosomal pellet. To resuspend the synaptosomal pellets (P2 fraction), for each 10 mg of starting tissue, 10 µl of 2X sample buffer (No- EC886, Protein loading buffer, Blue 2X, National Diagnostics) was added.

2.3. Western Blot Analysis

Comparable protein amounts (for mouse samples according to protein determination; for human samples an equal volume) were separated on a criterion TGX precast gels (BioRad-continous gradient, 4-15 %) and the protein was transferred onto a methanol activated Polyvinylidene (PVDF) membrane (BioRad), using standard protocols. One hour blocking was carried out in 5% w/v milk powder (Merck) in 1X TBST (Tris-Buffer saline - 60g/l Tris, 87 g/l NaCl, 0.05% Tween-20, pH 7.5). Subsequently, membranes were incubated in primary antibody solution prepared in blocking buffer overnight at 4ºC. After three ten-minute washes in 1X TBST at room temperature, the membrane was incubated with horse-radish peroxidase conjugated secondary antibodies in blocking buffer for two hours at room temperature. After three ten-minute washes with 1X TBST , the membrane was incubated for 3 minutes in enhanced chemiluminescence (ECL) reagent (Thermo Scientific) and then exposed to an X-ray film (Amersham) in the linear range. To probe the membranes with other primary antibodies, they were washed in western blot stripping buffer (Santa Cruz Biotechnology) for one hour followed by three washes with TBST of 10 minutes duration each and incubation as described above.

Antibody details are given in **Table 2.2**. CSPα antibody specificity was checked by performing western blot analysis of forebrain and cerebellar tissues from CSPα knockout mouse. The knockout mouse tissue was a gift from Dr.Fernandez-Chacon (IBiS, Seville, Spain). The CSPα band was missing in the knockout forebrain and cerebellar tissue , and was prominently and very specifically visible in equal amount of protein fraction from wildtype mouse forebrain and cerebellum **(Fig.4.3).** The CSPα band in wildtype mouse forebrain showed up as double bands as observed in our studies.

Our CYFIP2 and CYFIP1 antibodies were highly specific. **Fig.3.4.** is a full blotscan from the same membrane with post-mortem hippocampal tissue lysates, showing the CYFIP2, CYFIP1 and NSE bands (internal control). This blot shows the highly specific binding of the CYFIP antibodies used by us. Further specificity of CYFIP2 antibody was demonstrated by the western analysis of the forebrain tissues from CYFIP2 heterozygous mice that showed 50 percent downregulation when compared to wild type mice as expected assuming the antibodies were CYFIP2 specific **Fig.3.11**. To study if the CYFIP2 is also expressed in the glial cells, lysates from mixed glial cell culture (as explained below) were probed with CYFIP2 antibodies. There was no expression of CYFIP2 in glial cells **(Fig.3.16).**

Neuronal Specific Enolase are neuron specific enzymes and are used as neuronal markers (McAleese et al., 1988). NSE was used as loading control in our studies and as a neuronal protein to normalize the amount of studied proteins. Synaptophysin are presynaptic proteins that are widely used as synaptic markers (Sudhof et al., 1987). In our studies, we have used synaptophysin as loading control and normalizing marker protein for synapse specific analysis.

Signals were analyzed with ImageJ software (NIH). For standardization of protein in each lane, the proteins of interest were normalized against the neuronal house-keeping marker protein neuron-specific enolase (NSE) and the synaptic vesicle protein synaptophysin.

Glial cultures: Glial cells were isolated from post-natal day 1-4 (P1-P4) Cln3-/- or WT mouse cerebral cortices, as previously described (Williams and Price, 1995) and cultured on poly-D-lysine (PDL, 25µg/ml, Sigma) coated T75 (Corning, Costar) flasks at a density of 2-3 cortices per flask in Dulbecco's Modified Eagles Medium (DMEM, Gibco), supplemented with 10% foetal bovine serum (FBS, Biosera) and

penicillin/streptomycin (50 U/ml / 50µg/ml, P/S, Sigma). Once these cultures reached confluence (12-14 days) they were composed of a base layer of non-dividing astrocytes and an upper layer of dividing microglia and a few oligodendrocytes.

2.4. Immunohistochemistry

Human brain sections from sample described in the section under post-mortem human brain samples were used for immunohistochemistry.   Sections of human brain of 7 µm thickness were cut from paraffin-embedded tissue blocks. Sections were deparaffinised in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation of sections with 2.5% H_2O_2 in methanol. To enhance antigen retrieval sections were exposed to citrate buffer (2.94 g/L, pH 6.0) for 16 minutes microwave treatment (6 minutes high, two 5 minutes simmer). After blocking in normal swine serum (DAKO Ltd), primary antibodies against CSPα (1:500, AB1576 Merck Millipore), and synaptophysin (1:100, SY38 DAKO Ltd) were applied overnight at 4°C. Following rinsing and two five minutes washes in TBS, sections were incubated with appropriate biotinylated secondary antibodies (1:100,Swine anti-rabbit immunoglobulin/biotinylated , E0353 DAKO Ltd), followed by incubation with avidin:biotin enzyme complex (Vectastatin Elite ABC kit, Vector Laboratories, Peterborough, UK). Following washing, sections were incubated for 10 –15 min with 0.5 mg/ml 3,3′-diaminobenzidine chromogen (Sigma-Aldrich Company Ltd, Dorset UK) in Tris-buffered saline (pH 7.6) containing 0.05% H_2O_2. Sections were counterstained with Harris's haematoxylin.

See **Table 2.2** for list of antibodies and concentrations used.

2.5. Cloning of CYFIP2 shRNA plasmid

A 65 basepair CYFIP2 shRNA as described by Anitei *et al* (Anitei et al., 2010) (Sense strand:5'CCTTCCTCCATCATGTACC3';Antisensestrand:5'GGTACATGATGGAGG AAGG3') was designed with Bam H1 sites at the terminal positions and Spe1 site inside the sequence to confirm the presence of insert (Forwardsequence:5'GATCC-CCTTCCTCCATCATGTACCTTCAAGAGAGGTACATGATGGAGGAAGGTTTTT TACTAGTG3';Reversesequence:5'GATCCACTAGTAAAAAACCTTCCTCCATCA TGTACCTCTCTTGAAGGTACATGATGGAGGAAGG-G3'). The CYFIP2 shRNA oligonucleotide strands were designed by Sigma. The two strands were annealed together as following –Forward oligonucleotide (2μl,100μM)and Reverese oligonucleotide(2μl,100μM)were added in 5μl,10X Buffer 2 (New England Biolab)and the volume was made to 50μl by addition of deionized water .This mixture was heated at 95^{0}C for 3 minutes,80^{0}C for 1 minute,70^{0}C for 1 minute,60^{0}C for 1 minute, 50^{0}C for 1 minute and finally placed in ice.The oligonucleotide starnd were annealed to form an shRNA complex. The annealed shRNA was ligated in a pZacU6 plasmid and was inserted downstream to a U6 promoter using Bam H1 sites **(Fig.2.2)**. The plasmid also had a GFP marker protein downstream to a Synapsin promoter. The resulting plasmid correctness was verified by sequencing facility at Santa Cruz Biotechnology using the followingprimersequences,Forwardprimer:5'ACGATACAAGGCTGTTAGAGAGA3', Reverseprimer:5'GGTGCTGAAGCTGGCAGT3'(IntegratedDNATechnologies). This plasmid(pZacU6CYFIP2) was packaged into an Adeno Associated Viral delivery sytem (AAV2/9) by Penn Vector Core Facility , Univeristy of Pennsylvania.

2.6. CYFIP2$^{+/-}$ Mouse Genotyping

For genotyping the mouse, the genomic DNA was isolated from tail tissues (or ear clips). About 1 cm of tail tissue (approx 30 mg) or ear clips from each mice were placed in 500 µl (100µl for ear clips) of lysis buffer (1M Tris-HCL,pH8.5 ; 0.5M EDTA ;1% SDS; 5M NaCl ; The volume was made up by adding deionized distilled water (MilliQ) with an addition of Proteinase K (18.6 mg/ml) to a final concentration of 0.1µg/µl. This mix was incubated at 55˚C overnight. The digested tail mix was vortexed for 1 minute and then centrifuged at 13,000 rpm for 10 minutes. The supernatant was decanted in a new tube , followed by addition of 500 µl of isopropanol at room temperature. The precipitate was obtained after inverting the tube several times. The tube was centrifuged at 13,000 rpm for 2 minutes and the supernatant is discarded. The pellet were washed with 500 µl 70 % ethanol (100 µl for the ear clips) by spinning for 1 min and the ethanol was decanted followed by 5 minutes of air drying. The DNA was resuspended in about 100 µl deionized water.

The isolated genomic DNA was used for CYFIP2 mouse genotyping using the mutant and wild type specific primer as described before. The primers were synthesized by Integrated DNA Technologies. The PCR mix per sample consisted of 2.5µl 10X buffer, 18.375 µl water, 0.5 µl MgCl$_2$ (50 mM), 0.5 µl dNTP (10mM) and 0.125µl *Taq* DNA polymerase (Invitrogen). The PCR protocol consisted of a 2 minute heating at 93^{0}C, followed by 35 cycle of denaturation (30 second, 93^{0}C), annealing (30 second, 56^{0}C) and extension (30 second, 72^{0}C). At the end of PCR cycle, the sample were heated for 10 minutes at 72^{0}Cand then held at 4^{0}C until recovered.

2.7. Fear Conditioning

 For the fear conditioning trial mouse were handled for three days prior to training by the person performing the experiment. The mice were habituated to the experimentalist by placing them on hand for 2 minutes per animal per day. The experimentalist was blinded to the genotype of the animals. Each mouse was placed in a soundproof conditioning chamber (Med Associates Inc, USA). The fear conditioning protocol used was carried out simultaneously to observe the contextual memory and cued memory. 3-4 months aged CYFIP2 mutant and wild type were trained and the same animals were trained for tone conditioning and context conditioning. The animal was placed in the fear conditioning chamber for 3 minutes duration in total; after 2 minutes a tone (75dB, 10KHz) was presented for 30 s and the last 2 seconds of tone co-incided with a mild foot shock (0.75 mA). After the tone/ foot shock pairing the animal was left in the conditioning chamber for 30 s and then returned back to its home cage.

2.7.1. Testing for Contextual Conditioning

The testing for contextual fear memory was carried out in the training chamber for 5 minutes duration without any tone presentation or foot shock being provided. The readout for the memory was a state of freezing during a pre assigned two second slot in every 5 second interval of entire 5 minutes recording. The scoring was done by an unbiased genotype blinded experimenter. The testing for memory of contextual conditioning at 1 to 5 days interval was carried out with one set of mouse (Wild type n= 13; CYFIP2$^{+/-}$ n= 15) from the same generation of litters. The testing for memory of contextual conditioning at 28 days interval was carried out with another set of mice (Wild type n= 18; CYFIP2$^{+/-}$, n= 12)

2.7.2. Testing for Tone conditioning

As mentioned previously the mice used for testing the contextual memory were used for testing the tone memory as well. They were tested for the tone memory in the same fear conditioning chamber, but the contextual cues (Interiors of the box, colour of light and odour) inside the chamber were modified. Each animal was placed in the chamber for 6 minutes. The first 3 minutes were meant for acclimatization to the chamber environment and for testing whether the animal had a fear response to the modified context. In the last 3 minutes the tone used for training (75 dB, 10 KHz) was introduced. The readout for the memory was a state of freezing observed during a pre assigned two second slot within every 5 second interval (of entire 6 minutes recording). The scoring was done by a genotype blinded experimenter. The testing for tone conditioning at 1 hour and 1 day interval post training was carried out with one set of animals from same generation of litters (Wild type ,n= 18 : CYFIP $2^{+/-}$, n= 12). The testing for tone conditioning at 5 day interval post training was carried out with a different set of animals from same generation of litters (Wild type , n= 13 ;CYFIP2$^{+/-}$, n=15)

2.8. Morris Water maze test and Visible platform test

The spatial reference memory will be analyzed using Morris watermaze paradigm (D'Hooge and De Deyn, 2001) . The watermaze trial was performed in a pool of 1.5 m diameter, with water temperature of 24˚C degrees. The platform diameter was 0.1

meter and the level of water was 0.1 cm above platform surface. The water was made opaque by addition of non toxic white paint (ELC ready mix paint, 44800 White 284).

Both CYFIP2$^{+/-}$ (n= 12) and Wild type (n=18) mouse aged between 3-4 months were handled for 2 minutes each for 8 days before the training commenced. The mouse cages were placed half an hour before the experiment in the watermaze training room. On the first day of the trial, each mouse was habituated to the platform conditions. 4 trials per day per animal from four different directions were performed. Each trial lasted a maximum of 90 seconds, with 60 seconds of interval in between each trial whereby the animal stayed on the platform. If the animal was unable to locate the platform by the end of 90 seconds, it was guided to the platform. On sixth day, a probe trial was carried out for 60 seconds by removing the platform and recording the time spent in each quadrant including the target quadrant.

The results from water maze revealed that the mice haven't learned the task during the training **(Fig.3.14a)** until day 6 and further training was discontinued due to time limitations. The test will be repeated in future studies.

After a resting interval of two days, a visible platform test was carried with the same conditions as described in the hidden platform test to avoid any error in the water maze hidden platform result by the defects in sensory - motor responses in animal to due to genetic manipulation. The water pool was covered on all sides by curtains to avoid exposure to any spatial cues and the platform position was highlighted by placing a white sphere of about 4 cm diameter on platform as a distinguishable visible object. The animal was released in the pool from the farthest location from the visible platform. The recording was made in two trials separated by 60 seconds interval where the animal stayed on the platform. The person performing the experiment was

blind to the genotype of the mouse. The recording was done by HVS Image tracking software (HVS Image 2013).

2.9. Statistical analysis

<u>For the post mortem human brain studies</u> –

Statistical analysis for the samples where the data wasn't pooled from two different time point was performed using an unpaired t-test analysis. The pooling of data from tissue samples procured from patients in two sets at two different time point was performed using a linear regression model based on following equation -

$$\left(\frac{CSP}{Synaptophysin}\right)_i = \beta_0 + \beta_1 \times X_{1i} + \beta_2 \times X_{2i} + \varepsilon_i$$

Where X_{1i} is the categorical predictor coding for the group difference (e.g. Control versus Severe), and X_{2i} is the categorical predictor coding for the different experiments ("1st cohort" versus "2nd cohort")

This regression model helps us to pool the CSP or CYFIP score from two different set of patients eliminating the contribution made by the difference in experimental conditions (like the scanner used) to the final result. This analysis was performed using SPSS (version 20). SPSS provides the output as an ANOVA score. The final result is displayed as an ANOVA score indicating the overall significance. The contribution and the significance of the factor of interest e.g. the disease pathology to the overall significance is subsequently provided by a t-test analysis. Hence, this analysis strategy helps to identify the significant change of a CSP/CYFIP score in diseased patients as compared to control eliminating any effect induced by the different

experimental conditions when the different set of tissue samples was processed. The level of significance for the analysis was 0.05 and the outliers were decided by using mean ± 4*SD as threshold.

Since the data used in this project involved pooling the data from two different sets , and hence utilized the regression analysis model for statistical studies , it was not suitable to perform a co-relation study between different aforesaid parameters and the protein levels observed in post-mortem tissues. Hence, I adopted the strategy of comparing the significant difference between the age, PMD and Gender (nonparametric analysis) of patient samples grouped into different pathological state of disease– control , mild , severe AD. The analysis for pH couldn't be performed as some of the samples I used in my study were from a previous published study in lab where the pH data was not recorded or analysed.

Hippocampus AD samples –

A) Effect of Gender – Pearson's chi-square test was performed to discover the relationship between the categorical variable (Gender and Pathological state) . The p = 0.627 , shows there was no statistically significant association between the individuals gender and its pathological state.

B) Effect of Age – One way ANOVA showed no changes in the age between control , mild and severe groups , $F(2,33) = 0.961$, p=0.393.

C) Effect of Postmortem Delay – One way ANOVA showed an association between post-mortem delay and the pathological state of patients, $F(2,33) = 4.812$, p=0.015. Tukey's posthoc test revealed that the post-mortem delay was not significantly different between the control and severe pathological state group (p=0.091). It was also not different between the mild and severe pathological state group (p=0.686) . However,

the post-mortem delay between control and mild pathological state showed significant difference (p= 0.014). The post-mortem delay in control (21.4 ±2.3 hours) was significantly higher than mild state AD patient hippocampus (12.7±1.9 hours) .

Gender and age do not show any statistically significant difference in various pathological states of AD in hippocampus. This result along with the fact that none of the molecule (CYFIP1/2 and CSPα) used in this study have any sex linked inheritance pattern , leads us to conclude that gender does not have any impact on the results. Also , the statistical output concludes that the age of the patients across the different pathological states was same and hence did not had any impact on the protein level analysis in post mortem tissues. The post mortem delay did not had any effect on the result between control and severe , as it was not significantly different between the two groups. However, the post-mortem delay between the control and mild group was significantly different, with an increased delay in control group. This difference could have impacted the protein expression profiles. It is unlikely that this delay may have impacted our results as there was no change observed in CYFIP1 , a trend of increase of CYFIP2 and a significantly increased CSP α in control hippocampus group as compared to mild stage AD hippocampus. This shows that the proteolytic degradation of hippocampal proteins under investigation was not significant to effect the read out from control tissues as compared to mild tissues within the given time frame of post-mortem delay.

STG AD Samples –

A) Effect of Gender - Pearson's chi-square test was performed to discover the relationship between the categorical variable (Gender and pathological state) . The p =

0.018 , showed a statistically significant association between the individuals gender and its pathological state.

B) Effect of Age – Independent sample two tailed T test showed there was no significant difference in the age between the control and severe STG groups (t= -0.959 , p=0.347)

C) Effect of Postmortem delay – Independent sample two tailed T test showed there was no significant difference in the post-mortem delay between the control and STG groups (t= 1.046 , p=0.306)

Gender showed significant association with the pathological state of STG. However, since none of the studied proteins have been reported to have sex linkages, the possibility of gender differences having an impact on results is negligible. Unfortunately, the exact co-relation study cannot be performed with our scores as they were pooled together from different experimental sets. Age and post-mortem delay was not significantly different in the control and severe AD STG.

Cerebellum AD Samples –

A) Effect of Gender - Pearson's chi-square test was performed to discover the relationship between the categorical variable (Gender and pathological state) . The p = 0.585 , showed no statistically significant association between the individuals gender and its pathological state.

B) Effect of Age – One way ANOVA showed there was no significant difference in the age between the control , mild and severe AD cerebellum , $F_{(2,27)} = 0.756$, p=0.479.

C) Effect of Postmortem delay – One way ANOVA showed that there was no significant difference in the post-mortem delay between the control , mild and severe AD cerebellum. $F_{(2,27)}=2.066$, p=0.146.

Gender, age or post-mortem delay do not effect the results obtained from cerebellum AD tissue.

Cerebellum FTLD samples –

A) Effect of Gender - Pearson's chi-square test was performed to discover the relationship between the categorical variable (Gender and pathological state) . The p = 1.0 , showed no statistically significant association between the individuals gender and its pathological state.

B) Effect of Age – Independent sample two tailed T-test showed there was no significant difference in the age between the control and FTLD cerebellum , t = -0.361,p=0.728.

 C) Effect of Postmortem delay – Independent sample two tailed T-test showed that there was no significant difference in the post-mortem delay between the control and FTLD cerebellum. t=1.309, p=0.227.

Gender, age or post-mortem delay do not effect the results obtained from cerebellum FTLD tissue.

Cerebellum ageing study samples –

Since age was different in the two groups within this study (young and old cerebellum) , the effect of gender and post-mortem delay was ascertained.

 A) Effect of Gender - Pearson's chi-square test was performed to discover the relationship between the categorical variable (Gender and pathological state) . The p = 0.048, shows a statistically significant association between the individuals gender and its pathological state.

B) Effect of post-mortem delay - Independent sample two tailed T-test showed that there was no significant difference in the post-mortem delay between the young control and old control cerebellum. t=-0.701, p=0.498.

Since CSPα does not shows sex linked inheritance , it is possible that this slight significance is due to the small sample size and this significance will disappear with increase in the number of cerebellar samples. Post mortem delay is not significantly different in the young and old cerebellar tissues.

For the studies with mice model ,

For normality distribution test - Levene's test and Histogram normality plots were used (refer to Box G.E.P. (1953) - "Non-Normality and Tests on Variances". *Biometrika* **40** (3/4): 318–335). Independent T-test, one way ANOVA and Two –way repeated measure ANOVA were performed based on the design of experiments. SPSS version 20 (IBM) was used to perform statistical analysis. For Non-parametric analysis of independent group Mann Whitney U test and for related samples Wilcoxon Signed rank test was used. 2-way repeated measure ANOVA was employed for analyzing the impact of time and genotype on the behaviour on post training scoring at different intervals. The level of significance for the analysis was 0.05 and the outliers were decided by using mean $\pm$ 3*SD as threshold.

Table 2. 1. Details of post-mortem brain tissues. PMD refers to post-mortem delay

Hippocampus -

S.No	Pathological state	Sex	Age(Years)	PMD(Hours)
1	Control	M	81	18
2	Control	F	92	17
3	Control	M	78	10
4	Control	M	85	16
5	Control	F	76	28
6	Control	M	65	24
7	Control	M	86	6
8	Control	F	72	24
9	Control	F	55	24
10	Control	F	80	31
11	Control	F	71	30
12	Control	M	77	29
13	Mild AD	M	81	12
14	Mild AD	F	92	9
15	Mild AD	F	80	3
16	Mild AD	F	55	12
17	Mild AD	F	81	17
18	Mild AD	F	81	16.5
19	Mild AD	F	82	13
20	Mild AD	M	64	16

21	Mild AD	F	83	24
22	Mild AD	M	81	3
23	Mild AD	M	90	5.5
24	Mild AD	F	94	21
25	Severe AD	M	64	23
26	Severe AD	F	68	11
27	Severe AD	M	80	15
28	Severe AD	F	69	16
29	Severe AD	M	77	10
30	Severe AD	F	69	16.3
31	Severe AD	F	79	24
32	Severe AD	F	71	21
33	Severe AD	F	82	4.5
34	Severe AD	F	80	4.3
35	Severe AD	F	88	19
36	Severe AD	M	75	17

Superior Temporal Gyrus -

S.No	Pathological state	Sex	Age(Years)	PMD(Hours)
1	Control	F	55	24
2	Control	M	55	24
3	Control	M	65	24
4	Control	M	69	24
5	Control	M	86	6
6	Control	M	65	24
7	Control	M	71	5
8	Control	M	81	18
9	Control	F	92	17
10	Control	M	78	10
11	Control	M	85	16
12	Control	F	76	28
13	Severe AD	F	69	16.3
14	Severe AD	F	71	21
15	Severe AD	F	80	4
16	Severe AD	F	81	24
17	Severe AD	F	82	4.5
18	Severe AD	F	82	12
19	Severe AD	F	88	19
20	Severe AD	F	91	23
21	Severe AD	M	75	17
22	Severe AD	M	64	23

S.No	Pathological state	Sex	Age(Years)	PMD(Hours)
23	Severe AD	F	68	11
24	Severe AD	M	80	15
25	Severe AD	F	69	16
26	Severe AD	M	77	10

Cerebellum -

S.No	Pathological state	Sex	Age(Years)	PMD(Hours)
1	Control	M	73	23
2	Control	F	92	23
3	Control	F	55	12
4	Control	M	77	11
5	Control	M	54	30
6	Control	M	81	18
7	Control	F	92	17
8	Control	M	78	10
9	Control	M	85	16
10	Control	F	76	28
11	Mild AD	M	81	12
12	Mild AD	F	92	9
13	Mild AD	F	80	3
14	Mild AD	F	55	12
15	Mild AD	F	81	17
16	Mild AD	M	93	14
17	Mild AD	F	84	24

18	Mild AD	F	92	20
19	Mild AD	M	92	11
20	Mild AD	M	63	16
21	Severe AD	M	70	20
22	Severe AD	F	71	18
23	Severe AD	F	92	11
24	Severe AD	F	61	3
25	Severe AD	F	95	13
26	Severe AD	M	64	23
27	Severe AD	F	68	11
28	Severe AD	M	80	15
29	Severe AD	F	69	16
30	Severe AD	M	77	10

Cerebellum (FTLD) -

There were no known mutations in any of the patient samples. One patient showed Tau-positive frontotemporal dementia with parkinsonism (FTDP-17) and another showed tau negative , ubiquitin immunoreactive neuronal changes (FTLD – U). The ubiquinated protein is now known to be TAR- DNA- binding protein 43 (TDP-43). Two patients had FTD caused by FTLD-TDP43. Fifth one had FTLD-TDP43 with Motor Neuron Disorder (FTLD-MND-TDP43)

Cerebellum (FTLD)						
S.No	Pathological state	Autopsy No.	Sex	Age(Years)	PMD(Hours)	FTLD status
1	Control	A127/11	M	73	23	
2	Control	A144/10	F	92	23	
3	Control	A358/08	F	55	12	
4	Control	A053/11	M	77	11	
5	Control	A130/09	M	54	30	

					No known mutations
6	FTLD-U	A103/08	F	85	24	
7	FTLD-TDP43	A013/10	M	69	6	No known mutations
8	FTLD-MND-TDP43	A120/11	M	71	14	No known mutations
9	FTLD-TDP43	A403/08	F	70	16	No known mutations
10	FTLD (FTLDP-17)	A099/08	M	70	7	No known mutations

Cerebellum (healthy ageing) -

S.No	Age Category	Sex	Age(Years)	PMD(Hours)
1	Young Control	M	18	24.5
2	Young Control	M	22	45
3	Young Control	M	21	37
4	Young Control	M	16	14
5	Young	M	25	18

	Control			
6	Young Control	F	26	10
7	Old Control	F	102	44
8	Old Control	M	97	44
9	Old Control	F	99	32
10	Old Control	F	96	16
11	Old Control	F	92	9
12	Old Control	F	92	23
13	Old Control	M	95	44

Table 2.2. Details about the antibodies used

Serial No.	Primary Antibody	Dilution	Secondary Antibody	Primary Antibody manufacturer
1	CSPα (for immunoblotting)	1:50,000	1:50,000, Peroxidase conjugated, GoatAntirabbit(P0448,DAKO Ltd)	AB1576, Merck Millipore
2	CSPα (for immunohistochemi-stry)	1:500	1:100, Biotinylated Swine,Antirabbit (E0353,DAKO Ltd)	AB1576, Merck Millipore
3	CYFIP1	1:1000	1:2000, Peroxidase conjugated, Goat-Antirabbit (p0448,DAKO Ltd)	07-531, Merck Millipore
4	CYFIP2	1:1000	1:2000, Peroxidase conjugated, Goat-Antirabbit p0448, DAKO Ltd)	GTX110897, GeneTex
5	Synaptophysin (for immunoblotting)	1:1000	1:5000, Peroxidase conjugated, Goat-Antirabbit p0448,DAKO Ltd)	4329, Cell Signalling Technology

6	Synaptophysin (for immunohistochemi-stry)	1:100	1:100, Biotinylated Swine,Antirabbit(E0353,DAKO Ltd)	4329, Cell Signalling Technology
7	NSE	1:60,000	1:5000, Peroxidase conjugated, Goat-Antirabbit p0448, DAKO Ltd)	AB951,Merck Millipore

Chapter 3 - CYFIP1/2 are dysregulated in Alzheimer's disease

3.1. Introduction

3.1.1. What is CYFIP1/2?

Cytoplasmic FMR Interacting Protein -1 (CYFIP1) (also called Sra1) and Cytoplasmic FMR interacting Protein -2 (CYFIP2) (also called PIR121) are co-activators of FMRP (Fragile-X Mental Retardation Protein) and members of a highly conserved protein family in humans (Schenck et al., 2001). CYFIP1/2 co-localizes with FMRP and is found in the cytoplasm as well as synapses/dendrites (Schenck et al., 2001). Even though the amino acid sequence is 87.7% identical between CYFIP1 and CYFIP2, the binding affinities of these proteins differ. CYFIP1 binds only to FMRP whereas CYFIP2 binds FMRP and the FMRP related proteins FXR1 and FXR2 (Schenck et al., 2001). CYFIP1 acts as a EIF4E (Eukaryotic Initiation Factor 4E) binding protein (4E-BP) inhibiting the translation of FMRP bound mRNA (Napoli et al., 2008). CYFIP family proteins are also part of the actin cytoskeletal modulating WAVE complex, binding to the constituent of WAVE complex (Cory and Ridley, 2002, Derivery et al., 2009). Binding of the small Rho GTPase Rac1 to CYFIP1 and 2 removes these proteins from the WAVE complex so that WAVE can activate actin polymerization via Arp2/3 (De Rubeis et al., 2013). Additionally in non-neuronal cells (human colorectal adenocarcinoma cell line), CYFIP2 was shown to be a p53 inducible protein which causes apoptotic cell death (Jackson et al., 2007). CYFIP1 on the other hand is not p53 inducible in these cells (Jackson et al., 2007). In Drosophila there is a single orthologue of the CYFIP family of molecules, which is exclusively expressed in the nervous

system (Schenck et al., 2003), implicating that during the course of evolution CYFIP1 and 2 might have acquired different functions in humans. CYFIP1 gene is located on chromosome 15 (Nowicki et al., 2007) and CYFIP2 gene is located on chromosome 5 (NCBI Gene ID: 26999).

3.1.2. Discovery of CYFIP1/2

Tabata's group cloned the CYFIP1 gene while they were sequencing a cDNA library from an immature myeloid cell line (KG-1) (Nomura et al., 1994) . This was designated as KIAA0068. The same group mapped the gene to chromosome 15. In subsequent years the CYFIP1 gene was mapped to the locus- chromosome 15q11.2 (Chai et al., 2003). Jean-Louis Mandel's group in 2001 isolated CYFIP1 and CYFIP2 proteins when they tried to identify novel proteins that interact with Fragile X-mental Retardation Protein (FMRP) (Schenck et al., 2001). They used a yeast 2-hybrid screen based on a mouse embryonic expression library using the N-terminus of FMRP as bait.

The CYFIP 2 gene was mapped on chromosome 5 by International Radiation Hybrid Mapping Consortium (Map element – stSG9917). The precise location is at 5q33.3 locus.

3.1.3. What is the expression pattern of CYFIP1/2?

Both the members of CYFIP family are expressed in the hippocampus and forebrain. However, in the cerebellum, only CYFIP1 and not CYFIP2 is expressed (Hoeffer et al., 2012). Both CYFIP1 and CYFIP2 show co-localization with FMRP and ribosomes in

neurons. Apart from the perinuclear space, they are also reported to be present in synapses and dendrites (Schenck et al., 2001, Cajigas et al., 2012) .

3.1.4. What are the functions of CYFIP1/2 at the synapse?

CYFIP1/2 were discovered relatively recently and the precise function of these molecules in normal physiology is not well known. However, there are two distinct roles in which both these molecules have been implicated (though with slight variations between CYFIP1 and CYFIP2 within these). Two differing schools of thought arising from the common pioneer group of Mandel ((Schenck et al., 2001) stress differently on the contribution of the CYFIPs to each of these functions. Whereas De rubies *et al* (De Rubeis and Bagni, 2011, Napoli et al., 2008) have reported on the local translation modulatory functions of CYFIP , Bardoni *et al* have reported on the role of CYFIP1/2 as more relevant to the regulation of actin cytoskeletal dynamics (Abekhoukh and Bardoni, 2014, Schenck et al., 2003). However, a recent paper from Claudia Bagni's group reveals a link between both these reported functions of CYFIPs (De Rubeis et al., 2013). The detailed descriptions of both the functions assigned to CYFIP1/2 are as following -

1) **Actin cytoskeletal remodeling** - CYFIP1/2 are components of the WAVE regulatory complex (WRC) **(Fig.3.1)** that also consists of WAVE protein , NAP1 (NCKAP1) subunit, ABI1/ABI2 protein and BRK1 (also known as HSPC300) (Cory and Ridley, 2002, Derivery et al., 2009). The transduction of Rac Signalling by WAVE complex to trigger Arp2/3 dependent actin nucleation is mediated by CYFIP1 (Derivery et al., 2009) (and possibly CYFIP2) – a process important for modulating actin dynamics, leading to proper cell polarity and migration. The

WAVE proteins are part of WASP family (Wiskott-Aldrich syndrome protein), which consists of 5 members – WASP, N-WASP, WAVE1, WAVE2 and WAVE3 proteins. All these proteins can activate Arp2/3 by a VCA (Verprolin homology, Central and Acidic region) domain present in all the WASP members. The interaction of CYFIP1/2 with the small RhoGTPase Rac1 leads to cleavage of the sub-complex consisting of CYFIP1/2,NAP1, ABI1 from the inactive WAVE holocomplex This cleavage results in availability of WAVE holocomplex for interaction with Arp2/3 (leading to actin nucleation), and the availability of CYFIP1/2 for interactions with other proteins, such as FMRP (as discussed in second function) (Abekhoukh and Bardoni, 2014, Schenck et al., 2001, Schenck et al., 2003). The WAVE complex has been shown to be involved in actin cytoskeletal dynamics by assisting the remodeling of lamellipodia via interaction with CHC (Clathirin Heavy Chain Protein) (Gautier et al., 2011). In MCF10A cells (an immortalized mammary epithelial cell line), CYFIP1 activation leads to abnormal acini structures. A similar phenotype is observed following knockdown of the WRC component – WAVE2 and NCKAP 1 (Silva et al., 2009). However knocking down FMRP (the CYFIP interacting partner) in the same cell line doesn't lead to a similar phenotype (Silva et al., 2009). Chen *et al* have recently reported a study which emphasizes the relevance of CYFIP1/2 in actin cytoskeletal dynamics in Drosophila. A new class of motif – WIRS (WRC-interacting receptor sequence) - was identified in drosophila and defines a new class of ligands for Wave regulatory Complex. The WIRS peptide specifically interacts with the surface formed from the complex between CYFIP1/2 and Abi2. Alterations in these interaction lead to disruption of actin reorganization and egg morphology in Drosophila causing female sterility (Chen et al., 2014, Abekhoukh and Bardoni, 2014).
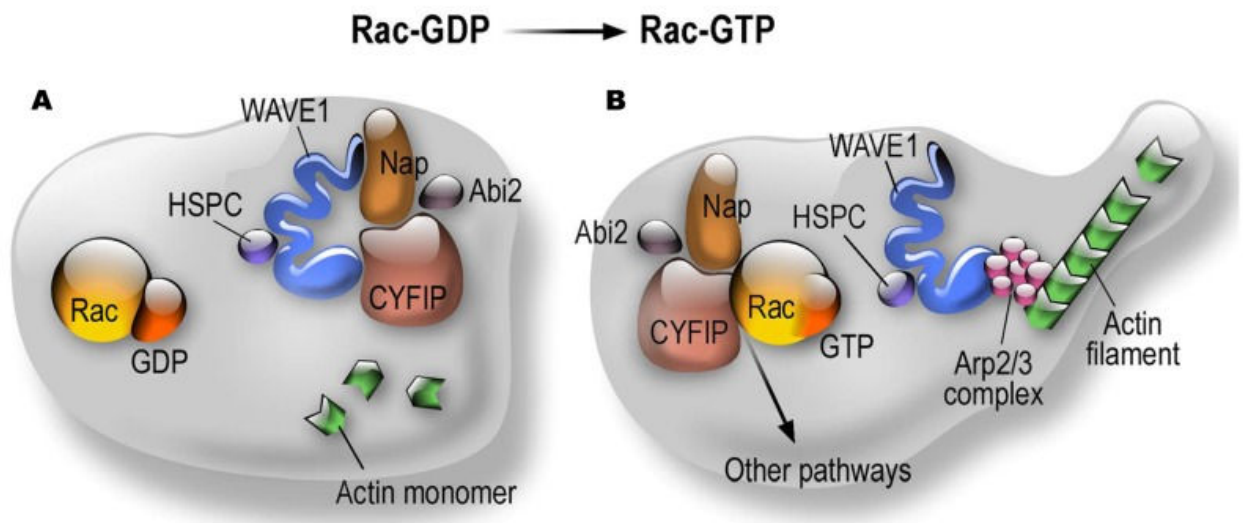
Figure 3.1. The WAVE regulatory complex. Under Rac-GDP condition CYFIP is a component of WAVE regulatory complex (WRC). Rac GTP interacts with CYFIP leading to cleavage of CYFIP along with Abi2 and Nap from WRC leaving behind a WAVE holocomplex which interacts with Arp2/3 causing actin nucleation. Taken from (Abekhoukh and Bardoni, 2014) .
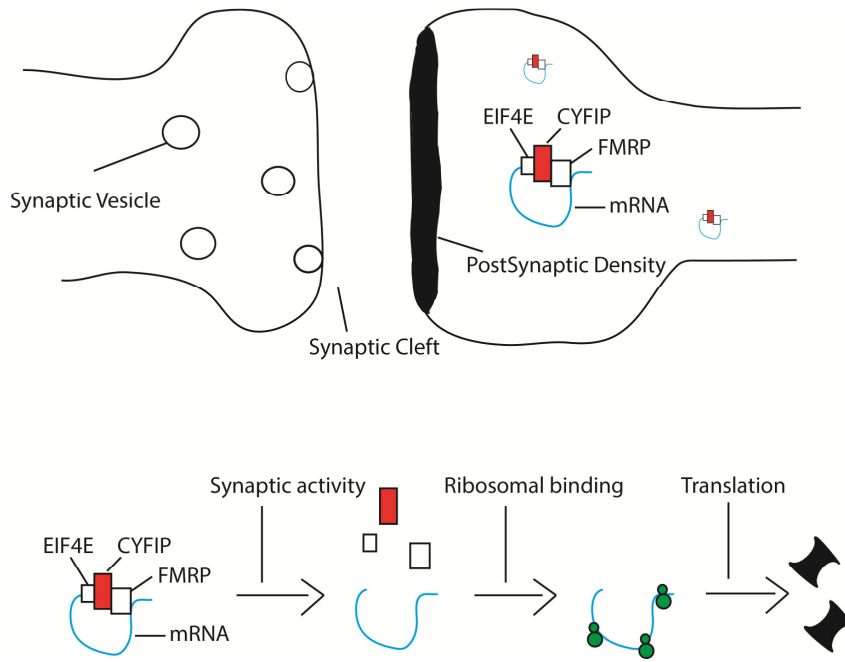
Figure 3.2. The translational repression complex. *CYFIP binds to EIF4E and FMRP to form a translation repression complex at the postsynaptic end. This complex binds to locally regulated pool of mRNA molecules at synapse/dendrites and keeps them repressed in an activity dependent manner. During synaptic activity, this complex breaks down liberating mRNA to be accessed and translated by polyribosomes leading to protein synthesis at synapse.*
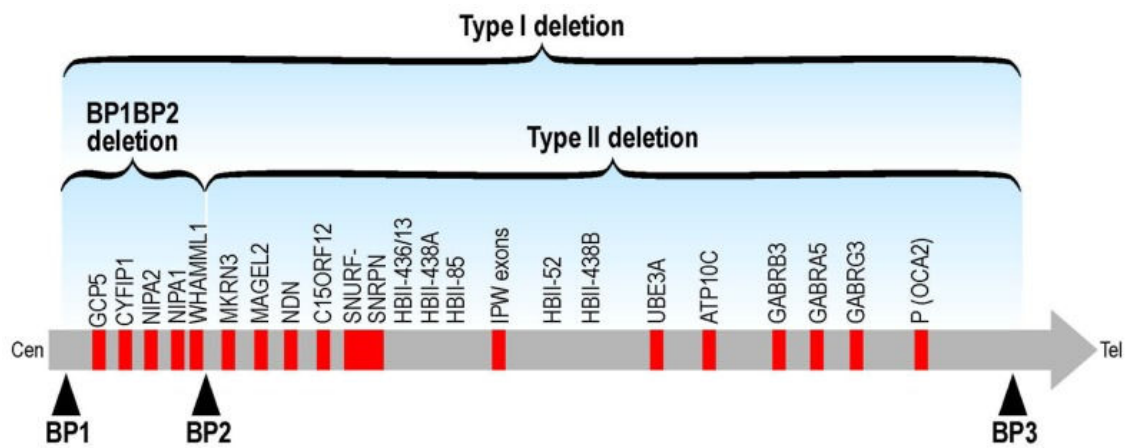
Figure 3.3. Chromosome locus 15q11- q13.*CYFIP* is present in the region between breakpoint 1 (BP1) and breakpoint 2 (BP2).The BP1-BP2 deletion is present at 15q12region. Taken from(Abekhoukh and Bardoni, 2014).
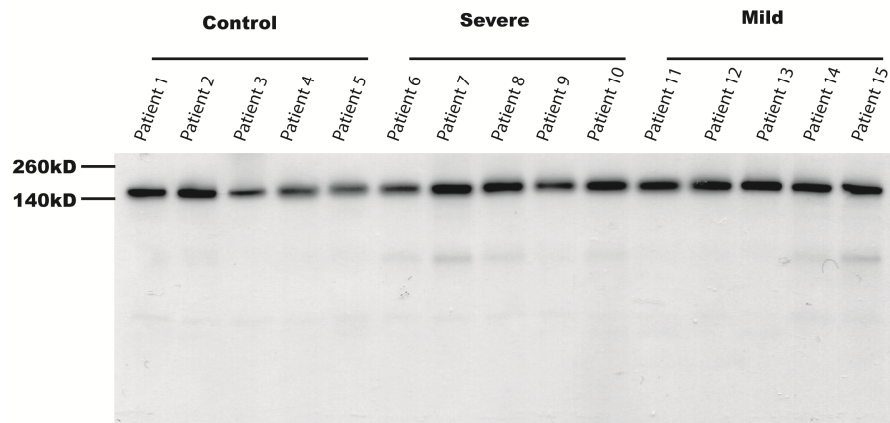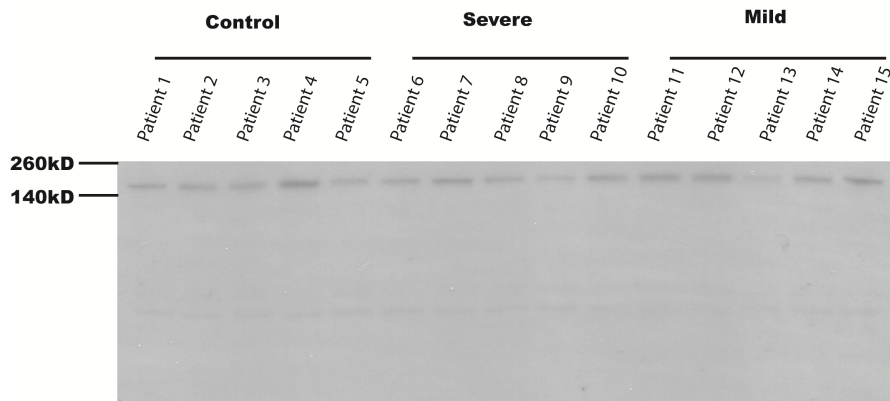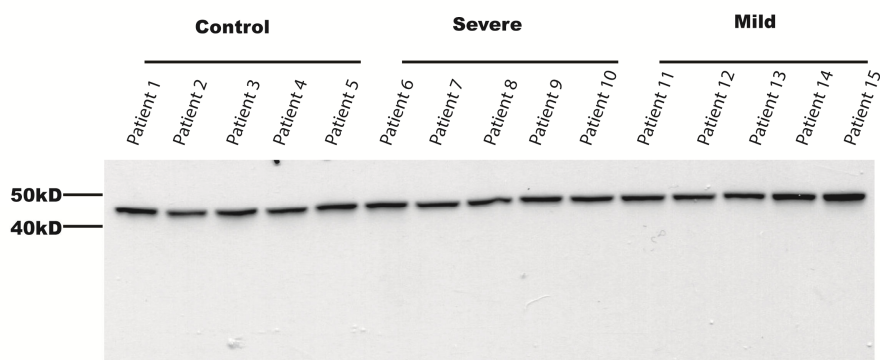
Figure 3.4. Specific binding of anti-CYFIP1/2 antibodies. The full blot containing the human hippocampal lysates showing the specific binding of anti-CYFIP1 , anti-CYFIP2 and loading control anti-NSE antibodies. For all three antibodies, bands are from the same membrane and same set of patients.
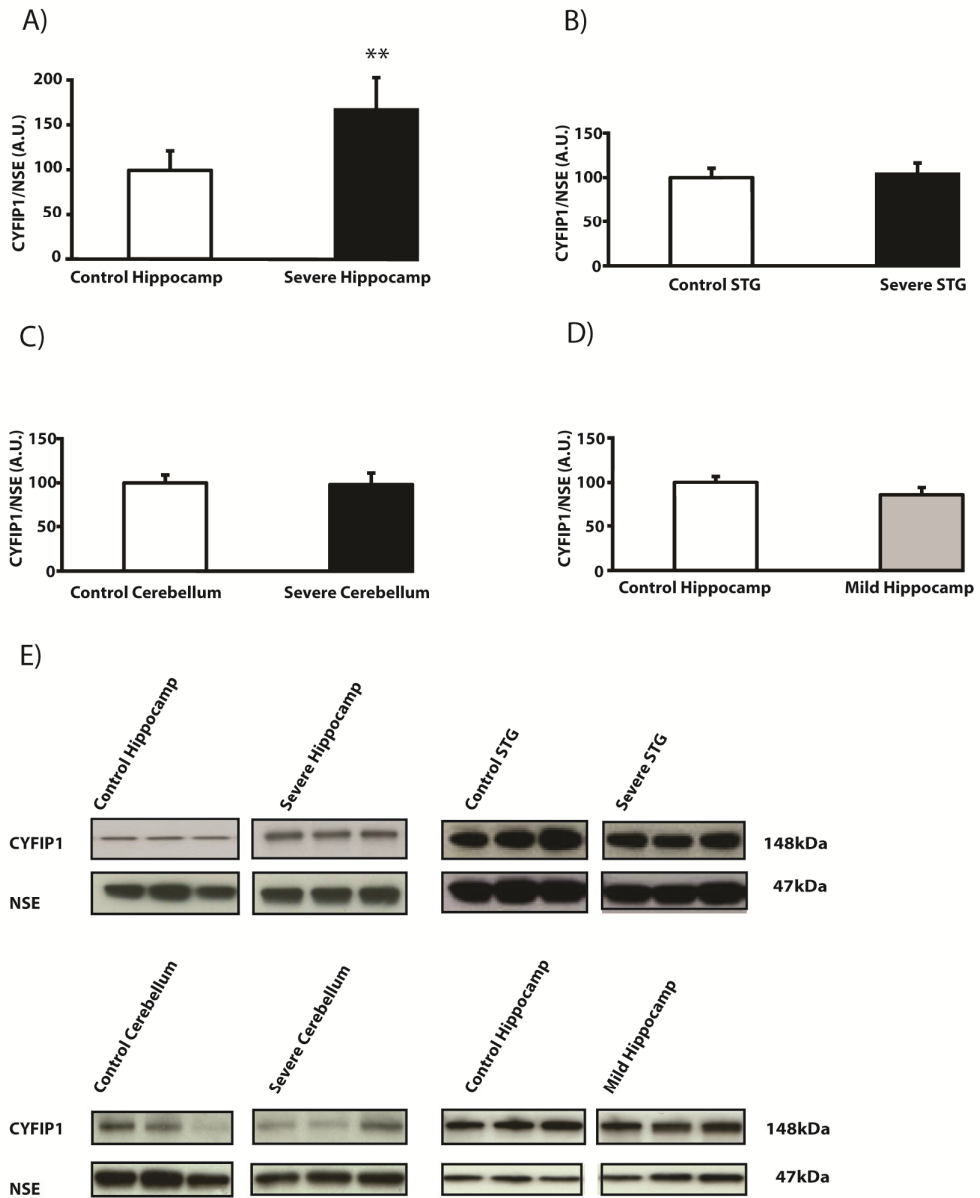
Figure 3.5. CYFIP1 expression is specifically increased in hippocampus in severe, but not mild AD. (a) Significant up-regulation of CYFIP1 expression in hippocampal lysates from severe AD patients (n=12) and control subjects (n=12). **(b)** CYFIP1 expression in lysates of superior temporal gyri from severe AD (n=13) and control subjects (n=12). **(c)** CYFIP1 expression in cerebellar

lysates from severe AD (n=10) and control subjects (n=10). **(d)** Similar levels of CYFIP1 expression in hippocampal lysates from mild AD patients (n=12) and control subjects (n=12). All the samples were normalized against neuron specific marker NSE. Panel **(e)** shows the representative blots. Means ± s.e.m. are shown. **, $p<0.01$.
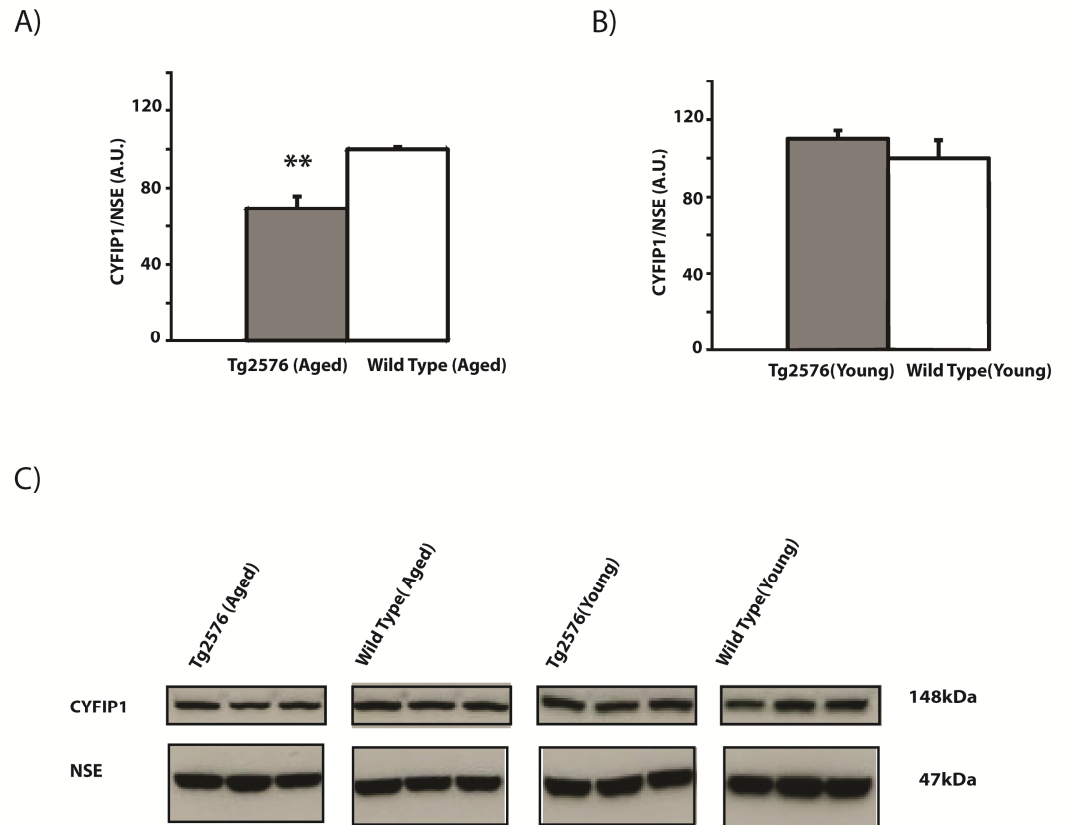
Figure 3.6. Age-dependent down-regulation of CYFIP1 in forebrain of Tg2576 mice. (a) CYFIP1 expression in hippocampal cortical lysates of 12 month-old wild type (n=4) and Tg2576 (n=3) mice. **(b)** The expression of CYFIP1 in hippocampal cortical lysates of 4 month -old wild-type mice (n=4) and Tg2576 (n=4) mice. Panel **(c)** shows representative western blots. All the samples were normalized against NSE. Means ± s.e.m are shown. **,p<0.01.
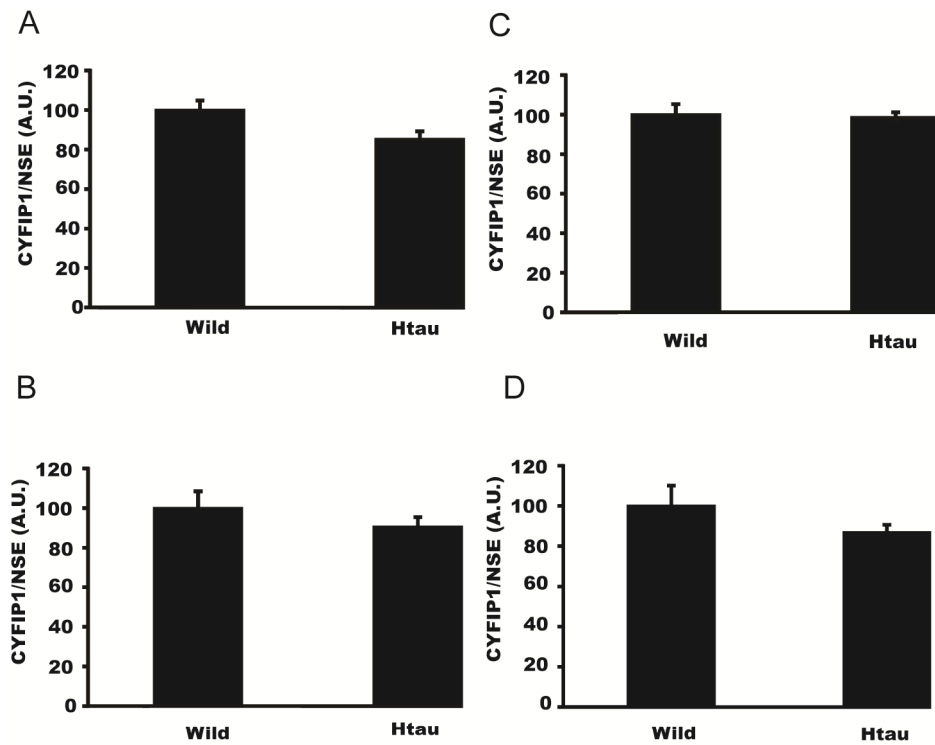
Figure 3.7. CYFIP1 levels in Htau mouse brain are not changed. Hippocampus **(A)** from young mouse (3-4 months), frontal cortex **(B)** from young mouse (3-4 months) , cerebellum **(C)** from young mouse and hippocampal frontal cortical region **(D)** from aged Htau mouse (24 months) were probed with CYFIP1 antibody and there was no change detected as compared to the levels in the wild type (**t= 1.456, p= 0.179).**
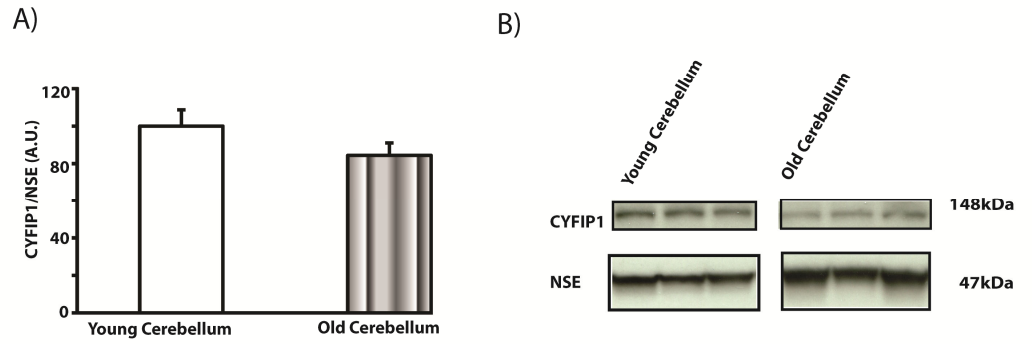
Figure 3.8. CYFIP1 expression does not change in cerebellum with normal ageing. CYFIP1 expression was normalized against NSE in cerebellum of young (15-30 years, n=6) and old (90-105 years, n=7) control patients. Means ± s.e.m are shown.
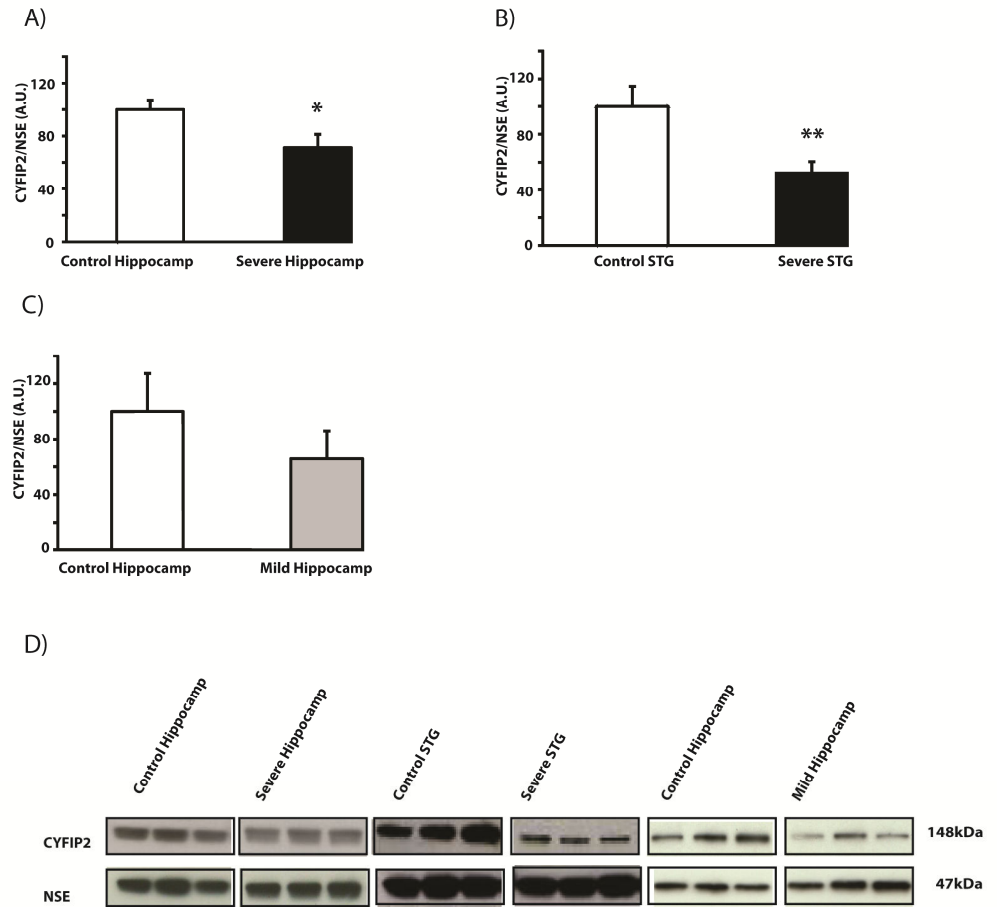
Figure 3.9. CYFIP2 expression is decreased in forebrain of severe AD patients. (a) CYFIP2 expression in hippocampal lysates from severe AD patients (n= 9) and control subjects (n=11). **(b)** CYFIP2 expression in lysates of STG from severe AD patients (n=13) and control subjects (n=12). **(c)** CYFIP2 expression in hippocampal lysates of mild AD (n=12) and control subjects (n=12). **(d)** Representative western blots. CYFIP2 protein expression was normalized against NSE. Means ±s.e.m. are shown. *, $p < 0.05$; **, $p < 0.01$.
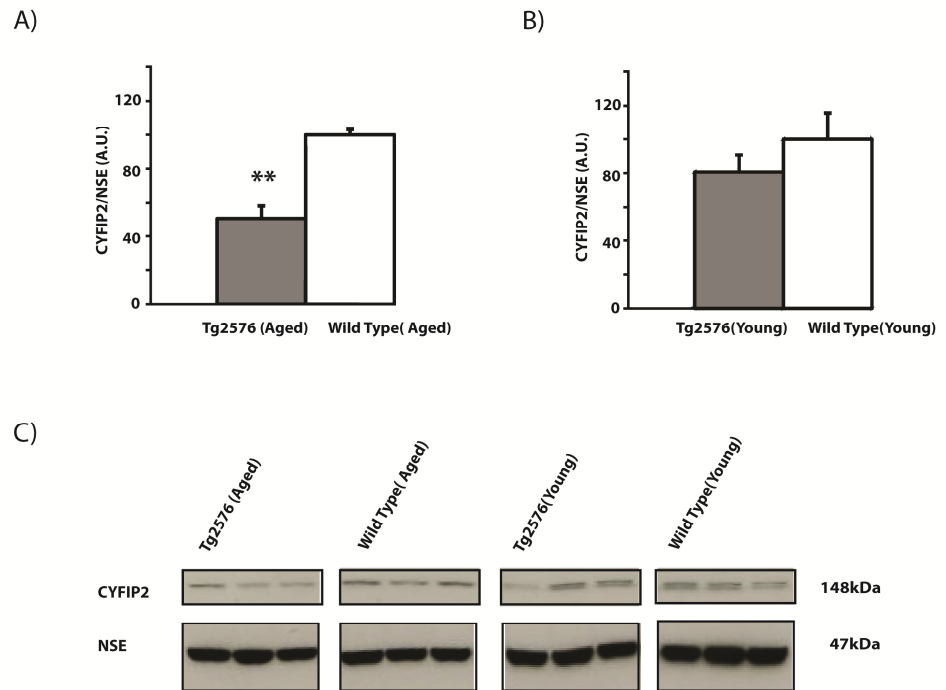
Figure 3.10. Age-dependent decrease of CYFIP2 expression in forebrain of Tg2576 mice. (a) CYFIP2 expression in hippocampal –cortical lysates of 12 month-old wild-type mice (n=4) and Tg2576 (n=3). **(b)** CYFIP2 expression in hippocampal-cortical lysates of 4 month-old wild-type (n=4) and Tg2576 mice (n=4). Representative western blots are shown in panel **(c)**. In all the panels, the CYFIP2 expression was normalized against NSE. Means ± s.e.m. are shown. **, $p < 0.01$.

A)

B)

C)
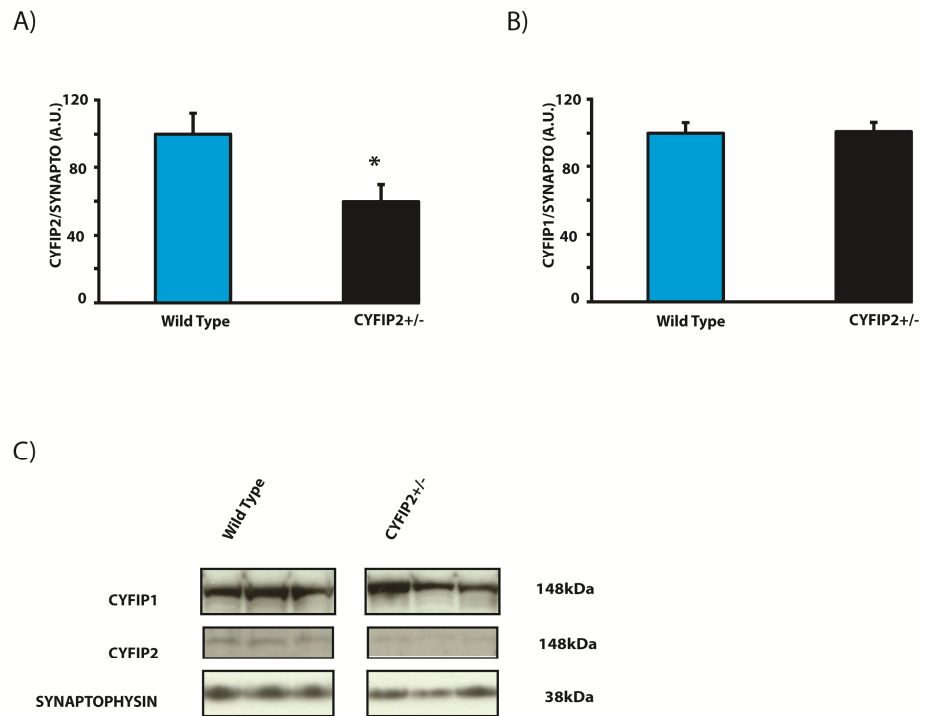
Figure 3.11. CYFIP1 expression is not altered in crude hippocampal synaptosomes of CYFIP2$^{+/-}$ mice. **(a)** CYFIP2 expression in crude synaptosomal (P2) fraction of hippocampal lysates from wild-type controls (n=7) and CYFIP2$^{+/-}$ mice (n=9). The same blot as in panel **(a)** was used to probe for CYFIP1 expression as shown in panel **(b)**. The house keeping synaptic marker protein synaptophysin was used to normalize against CYFIP expression in both panels. Panel **(c)** shows the representative western blot. Means ± s.e.m. are shown. *, $p < 0.05$.
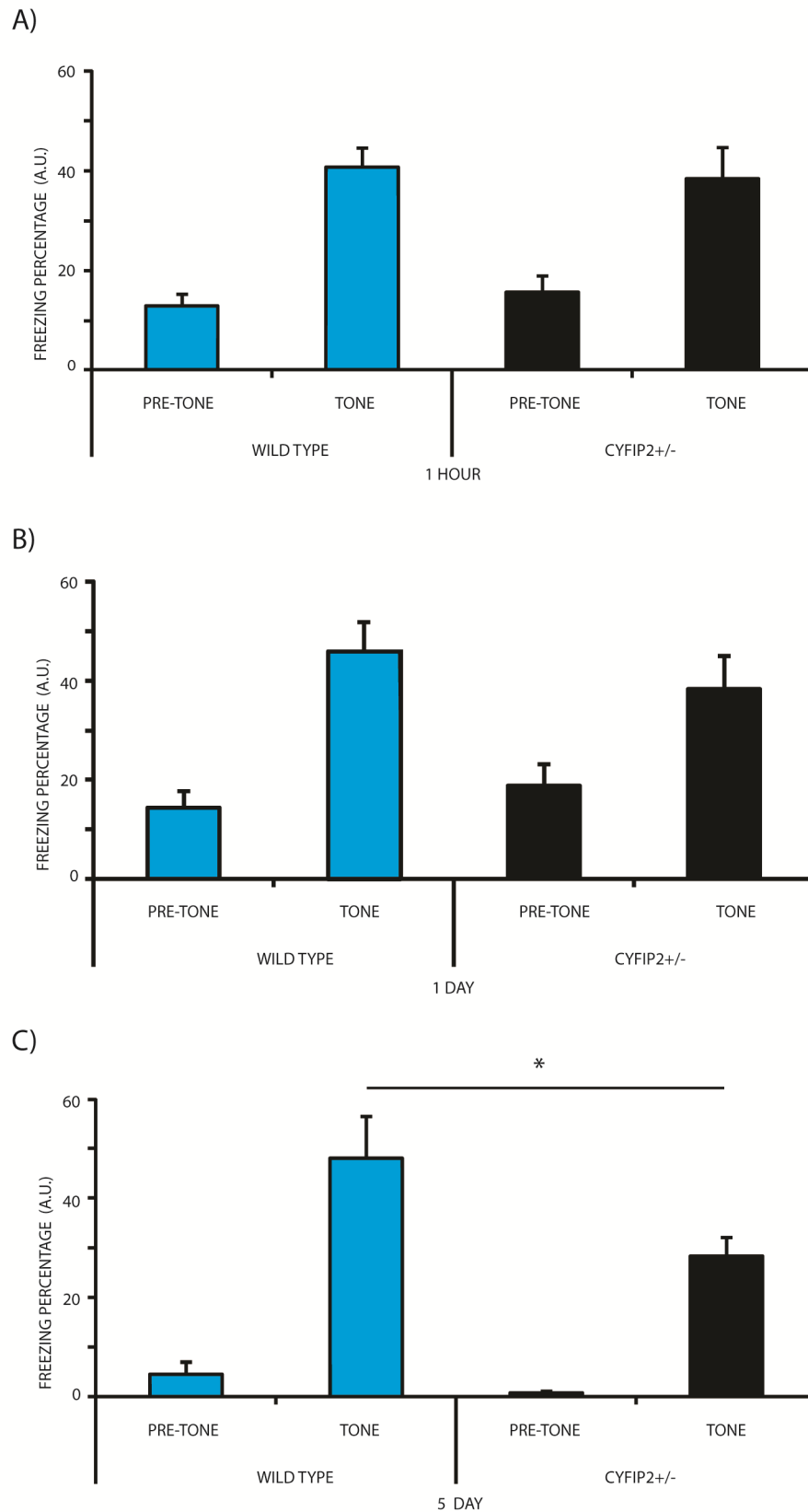
Figures 3.12. CYFIP2$^{+/-}$ animals are impaired in maintaining cued fear memory. (a) Freezing score before (Pretone) and during tone stimulus (Tone)

presentation of wild-type mice (n=18) and CYFIP2$^{+/-}$ mice (n=12) 1 hour after training. **(b)** The same mice as in panel **(a)** were tested 1 day after training for freezing score before and during the exposure to tone stimulus. **(c)** Freezing score (Pretone and tone) for the wild type (n=13) and CYFIP2$^{+/-}$ mice (n=15) 5 days after training. Means ± s.e.m. are shown. *, $p < 0.05$.
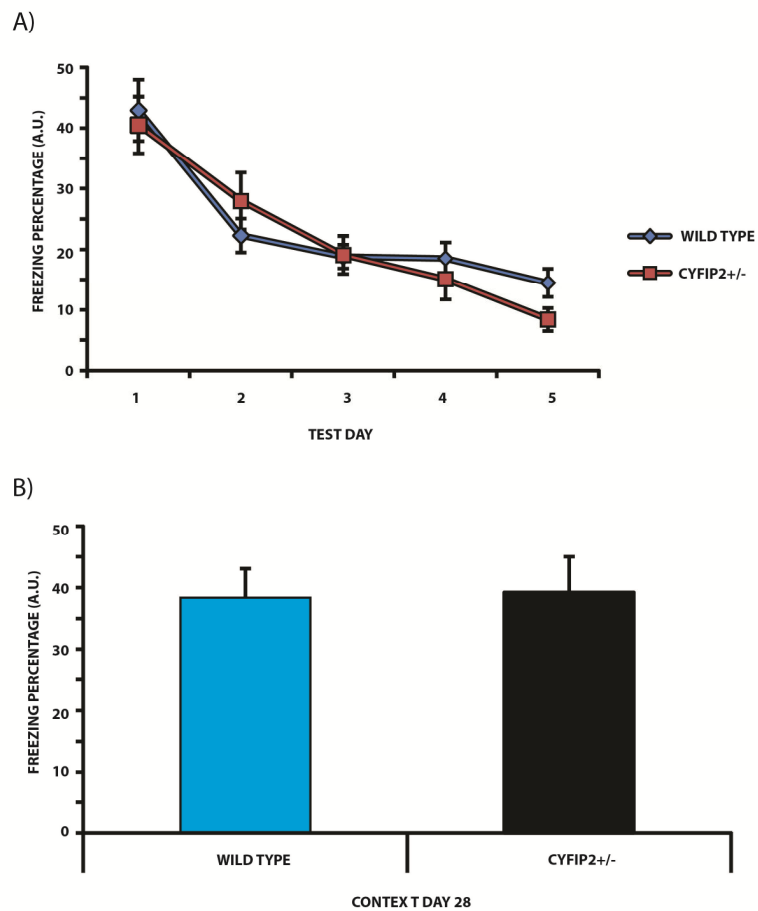
Figure 3.13. CYFIP2$^{+/-}$ animals are not impaired in formation and maintenance of contextual fear memory. (a) Freezing score during the context presentation for wild-type (n=13) and CYFIP2$^{+/-}$ mice (n=15) for 5 subsequent days after training. **(b)** Freezing score 28 days after training on context presentation to wild-type (n=18) and CYFIP2$^{+/-}$ mice (n=12). Means ±s.e.m. are shown.
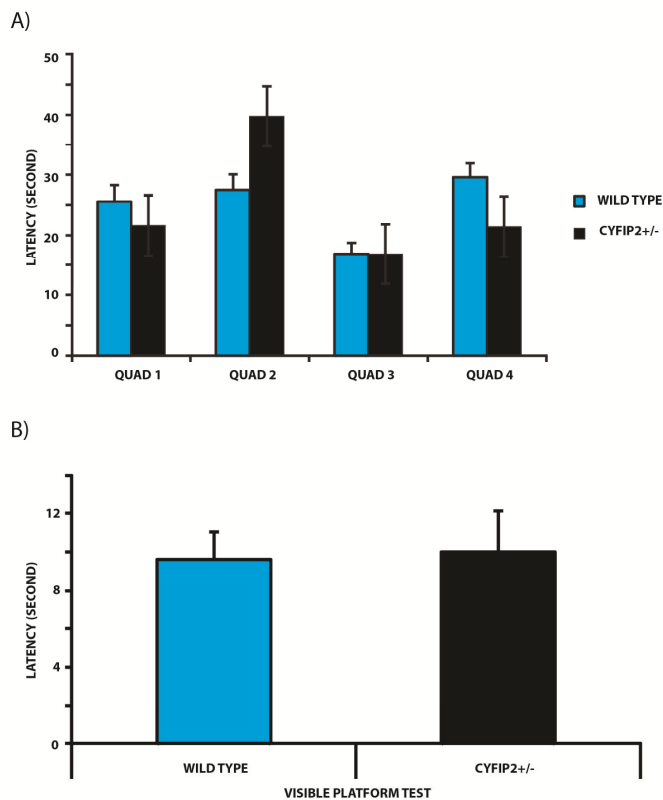
Figure 3.14. CYFIP2$^{+/-}$ animals do not display impairments in visible platform test. (a) Latency (in seconds) test score of wild type (n=7) and CYFIP2 $^{+/-}$ mice (n=6) per quadrant on the probe test day (six days after training). **(b)** Latency (in second) scores of mice from panel **(a)** in a visible platform test paradigm. Means ±s.e.m. are shown.

Figure 3.15. CYIP2 antibody didn't gave any detectable CYFIP2 signal from aged hTau brains in immunoblot study.
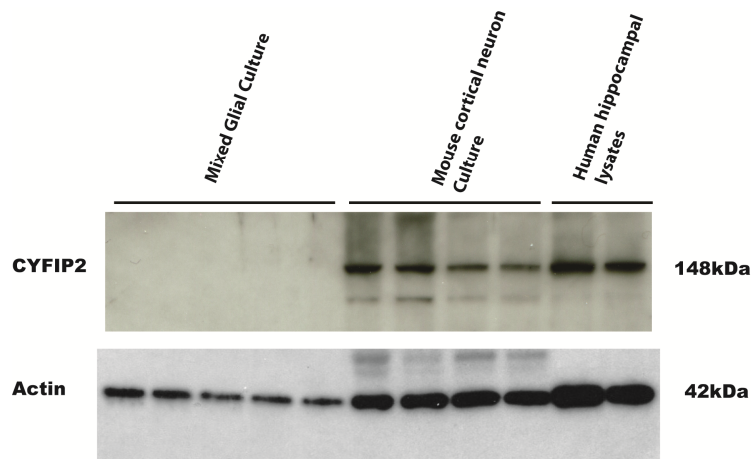
Figure 3.16. CYFIP2 is absent in glial cells. Glial culture (first five lanes from left) along with mouse cortical neuronal culture (lane 6- 9 from left) and human hippocampal lysate (lane 10 and 11 from left) was probed with anti-CYFIP2 antibodies . Actin was used as the loading control. No signal was detected in the lanes with glial cell culture.

2) **Modulation of local translation at synapses** – CYFIP1/2 have been reported to act as modulators of local mRNA translation near dendrites and synapses in an activity dependent manner. Claudia Bagni's group in 2008 reported the existences of a translation repression complex consisting of the core component of a 5'mRNA cap binding protein eIF4E, CYFIP1 and FMRP binding at the 3' end of mRNA (Napoli et al., 2008) **(Fig.3.2.)** This core repressor complex is formed specifically in the brain and suggested to act as a translation repressor for an array of mRNA molecules that are regulated by interaction with FMRP. These include cytoskeletal proteins like MAP1B, p0071(Brown et al., 2001, Nolze et al., 2013), synaptic plasticity molecules like αCaMKII (Zalfa et al., 2003), Amyloid precursor protein (APP) (Westmark and Malter, 2007), and even CYFIP2 (Darnell et al., 2011) .

FMRP bound to a specific mRNA recruits CYFIP1, which in turn binds to eIF4E, leading to repression of translation. Activation of Brain Derived Neurotrophic Factor / NT- 3 growth factor receptor (TrkB) or group 1 metabotrophic glutamate receptors (mGluRs), releases CYFIP1 from eIF4E and translation of the mRNA initiates (Napoli et al., 2008). CYFIP1 and CYFIP2 interact with FMRP, but only CYFIP2 also interacts with the FMRP related proteins- FXR1 and FXR2 (Schenck et al., 2001). Also, the domain of CYFIP binding to FMRP is the site that is used for homo- and heteromerization in FMRP, hence there is a competition for the same site between CYFIP1/2 and FXR proteins (Schenck et al., 2001).

A recent report by Claudia Bagni's group establishes a link between the two aforesaid functions of CYFIP molecules whereby a BDNF driven synaptic signaling mechanism

releases CYFIP1 from the translation inhibition complex , allowing the translation of repressed mRNA and causing the free CYFIP1 to move into WAVE regulatory complex (De Rubeis et al., 2013) . Active Rac1 plays an important role in changing the equilibrium of the two CYFIP1 incorporating complexes – the translation modulating eIF4E-CYFIP1-FMRP complex and the actin cytoskeletal dynamics modulating WAVE regulatory complex (De Rubeis et al., 2013). Also, since a number of mRNAs responsible for actin cytoskeletal modifications including CYFIP2 (which is part of WAVE regulatory complex) are regulated by this translation repression complex, it seems that this complex on its own is also involved in cytoskeletal modification.

3.1.5. What are the diseases in which CYFIP1/2 has been implicated?

Cancer - CYFIP1 knockdown leads to dysregulation in epithelial morphogenesis and along with Ras leads to carcinomas (Silva et al., 2009) . CYFIP2 has also been reported to have a pro-apoptotic effect through its interaction with IMP-1 (Insulin like Growth Factor mRNA binding protein -1) (Mongroo et al., 2011).

Neurodevelopmental disorders - No point mutations in *CYFIP1/2* have been directly implicated in any disease. However, deletions in region 15q11-q13 (which includes *CYFIP1* gene) are associated with Autism spectrum disorders **(Fig.3.3).** The paternal deletion of this region leads to Prader-Willi Syndrome (PWS) , whereas the maternal deletion of this region leads to Angelmann syndrome (Cassidy et al., 2000). The interstitial duplication of maternal region which includes the region relevant to Prader-Willi syndrome and Angelmann syndrome, leads to a behavioral phenotype more variable than Prader-Willi Syndrome and Angelmann Syndrome on their own. This includes developmental delays, seizures, ataxia, autism or atypical autism with minor

features of dysmorphism. On the other hand the duplication in paternal region of the same locus does not lead to any abnormal phenotype (Browne et al., 1997, Abekhoukh and Bardoni, 2014). A microdeletion of a region consisting of 4 genes – *NIPA1,NIPA2,CYFIP1,TUBGCP5*- and a non-coding mRNA WHAMML1 - between the breakpoint1 and breakpoint 2 on 15q11-13 locus, does not lead to Prader-Willi Syndrome but shares common features of learning disabilities, behavioral problems and dysmorphisms (Leblond et al., 2012, Kirov et al., 2009, Madrigal et al., 2012). Hence, the genes between breakpoint 1 and breakpoint 2, of which CYFIP1 is important, play important roles in cognitive and behavioral functions. The importance of CYFIP1 in neurodevelopment is further emphasized by the work of Hagerman's group, which reported a decreased level of CYFIP1mRNA in patients with FragileX syndrome and Prader Willi Syndrome (Nowicki et al., 2007).

CYFIP2 has been reported to be overexpressed in Fragile X patients at the protein level without any change detected in the mRNA level, suggesting an FMRP dependent dysregulation of CYFIP2 expression (Hoeffer et al., 2012). There results were reported in lymphocytes, although a similar result is observed in the postmortem forebrains of Fragile X patients.

Neurodegenerative disorders – To our knowledge, no other group has specifically studied the role of CYFIP1/2 molecules in neurodegenerative disorders. Genome Wide Association studies (GWAS) of Copy Number Variations (CNV's) by Rogaeva's group in Alzheimer's disease using a patient cohort from a Caribbean hispanic population have identified a duplication at the locus 15q11-q13 (consisting of *TUBGCP5,CYFIP1, NIPA2, NIPA1* and *WHAMML1* genes) which showed significant association with Alzheimer's disease. Quantitative PCR confirmed an increase in CYFIP1 levels in this cohort(Ghani et al., 2012). Apart from amyloid precursor protein , widely implicated in

Alzheimer's disease, Bagni's group has also reported an array of other proteins suspected to be involved in Alzheimer's disease onset – Elav protein 3, Elav protein 4, Nck Associated protein 1 and Cholesterol 24- hydroxylase to be regulated by the CYFIP constituting mRNA translation repression complex (De Rubeis et al., 2013).

3.1.6. What kind of animal models have been used until now to study CYFIP1/2functions?

Mandel's group developed a fly knockout model of CYFIP proteins to understand the role of this protein. Drosophila has just one homolog of the CYFIP family – *dCYFIP* and one homolog of FXR family – *dFXR*. The expression of *dCYFIP* is specific to central nervous system and shows interaction with *dFMRP* and *dRac*. It is 67% identical to human *CYFIP1/2*. Loss of function mutations in *dCYFIP* affects axonal growth and branching, establishing the importance of CYFIP in neuronal connectivity (Schenck et al., 2003). There is impaired synapse growth at the neuromuscular junction in *dCYFIP* mutants as indicated by shorter synapse terminals and increased number of buds when compared with wild types (Schenck et al., 2003) . FMRP and CYFIP serve opposite molecular functions as evident by neuro-muscular junction phenotypes in *dFMR1* mutants which are opposite to *dCYFIP* null flies (Zhang et al., 2001). Schenck et al have shown that overexpression of this morphological phenotype (such as short synapses) of *dFMR1* is rescued by co-overexpression of *dCYFIP*. They proposed that *dRac1* controls *dCYFIP* which in turn controls *dFMR1* (Schenck et al., 2003). Zhao et al established in *dCYFIP* fly mutants, that neuro-muscular junction development is regulated by CYFIP (Zhao et al., 2013). They showed that synaptic vesicle size in *dCYFIP* mutants is larger and the numbers of cisternae are elevated, though the number of synaptic vesicles is not changed between wild-type and mutant flies. This shows that

CYFIP may modulate F-actin machinery leading to the regulation of endocytosis and synaptic vesicle recycling (Zhao et al., 2013) . This study helped us to narrow down and screen for the second candidate molecule – Cysteine String Protein α (described in chapter 4) – in our post mortem study. This is a synaptic vesicle protein which showed a significant difference in mass spectrometric analysis of p25 transgenic mice synaptosomes, carried out in lab before this project started (Engmann et al., 2011). Inactivation of *dCYFIP* also results in reduced expression of WAVE complex member proteins like Nckap1 (Kette) and WAVE (Scar) (Bogdan et al., 2004, De Rubeis et al., 2013).

Pittman *et al* , obtained the first mutant in a vertebrate of CYFIP family proteins in 2010 (Pittman et al., 2010). This mutation was in CYFIP2 functions which were specifically investigated in Zebrafish. In a genetic screen to identify the pathways involved in retinotectal axon pathfinding , to understand eye –brain development, a *nev (CYFIP2)* mutant was identified that had impairments in the positional information of dorsonasal axons while projecting through optic tract (Pittman et al., 2010) . This phenotype is similar to the axon branching phenotype reported by Schenck et al in fly mutants of *dCYFIP* gene (Schenck et al., 2001). Pittman et al also showed that CYFIP2 is broadly expressed in central nervous system with a suggested role in protein translation at synapses.

In mice, both the *CYFIP1* and *CYFIP2* null mutations are lethal (Kumar et al., 2013b, Pathania et al., 2014) . Buxbaum's group has studied haploinsufficient CYFIP1 mice and demonstrate that this model mimics the features like more rapid extinction in inhibitory avoidance testing, unaltered LTP in CA1 induced by high frequency stimulation and enhanced mGluR-LTD of *FMR1* knockout mice (Bozdagi et al., 2012). Indeed, mGluR-dependent LTD was significantly increased in CYFIP1 heterozygous

mice as compared to wild type. Similar to FMR1 knockouts, these mice showed an enhanced extinction of inhibitory avoidance. However, there was no difference in learning in the Y-maze and Morris water maze tests (Bozdagi et al., 2012). Bagni's group has shown that siRNA mediated knockdown of CYFIP1 expression in primary cortical neurons leads to a change in dendritic spine morphology (more immature spines) without affecting spine density (De Rubeis et al., 2013). This was also observed in hippocampal neurons of CYFIP1 heterozygous knockout mice (Pathania et al., 2014). Additionally, reduced CYFIP1 expression decreases the complexity of dendrites on CA1 pyramidal neurons (Pathania et al., 2014). As previously demonstrated with *dCYFIP* mutants in the fly , in the case of rat hippocampal neurons ,inactivation of CYFIP1 resulted in impairment of axonal growth , which was similar to the phenotype observed with WAVE inactivation(Kawano et al., 2005).

To study CYFIP2 functions in haploinsufficient state, we have used CYFIP2 heterozygote mice on a C57BL/6 N genetic background. In 2013, Kumar et al studied these mice and proposed cocaine response behavior as one of the functions in which CYFIP2 plays an important role. They also showed that the presence of the S968F mutation in the *CYFIP2* gene within C57BL/6 N train destabilizes the CYFIP2 proteins, reducing the protein half-life to 2.8 hours as compared to 8.5 hours in C57BL/6Jmice(Kumar   et   al.,   2013b).

3.1.7. Previous studies in the lab that lead to the reported study on role of CYFIP1/2

Prior studies in the Giese lab have investigated the role of the CdK5 activator p25 in Alzheimer's disease (Engmann et al., 2011). A mass spectrometric analysis of synaptosomes isolated from hippocampus of p25 transgenic mice (in a C57BL/6J

genetic background) yielded candidate molecules that may be putative downstream molecules to p25. This project began with the aim of investigating synaptic pathology in Alzheimer's disease and dementia. The synaptic proteins from the mass spectrometric study were screened to identify novel molecules responsible for Alzheimer's onset and to investigate their role in Alzheimer's disease. CYFIP2 was one of the synaptic proteins which were identified. Since CYFIP1 belongs to the same protein family, this was also screened. In the subsequent part of the study CSPα (as described chapter 4) –a pre synaptic protein was also studied because Zhao et al's result hinted at the role of synaptic vesicle proteins in Alzheimer's disease (Zhao et al., 2013).

The hypothesis we developed for the project reported in this chapter was – "Local translation modulators of dendritic mRNA (CYFIP1/2) are dysregulated in Alzheimer's disease ".

The aims of the study described in this chapter were –

1) **To investigate the relevance of CYFIP1/2 in Alzheimer's disease by performing a case-control study using post-mortem brain tissues to analyse the levels of CYFIP1/2 in different regions of the brain at different stages of Alzheimer's disease.**

2) **To investigate the relevance of CYFIP1/2 in cognitive decline associated with normal aging by analyzing the levels of CYFIP1/2 in postmortem brain from young and aged subjects.**

3) **To investigate the validity of the hypothesis that functional compensation between CYFIP1 and CYFIP2 may exist by biochemical analysis of brain tissue lysates from CYFIP2 heterozygote mice.**

4) **To understand the role of CYFIP2 in memory as well as dementia associated with Alzheimer's disease by behavioral testing of CYFIP2 heterozygous knockout mice.**

3.2. Results

3.2.1. CYFIP1 is upregulated and CYFIP2 is downregulated in the hippocampus of severe stages of AD

The medial temporal lobe, specifically, the hippocampus is one of the first and most severely affected regions in the brain during the course of AD progression (Braak and Braak, 1991a). Onset of AD is characterized by the loss of memory and defects in cognitive skills. These defects are attributed to the loss of synapses at the onset of AD, particularly in the hippocampus (Arendt, 2009) . This synaptic loss precedes the neuronal death. Therefore, we decided to analyze the changes in the levels of CYFIPs in the hippocampal neurons.

We obtained hippocampus samples of severe (Braak stage 5-6) AD cases and controls in two sets from London Neurodegenerative Brain Bank. For the mild cases, the patient sample was obtained in one set. The patient sample details are listed in **Table 2. 1.** We tried to obtain the samples with lowest possible post-mortem delays available. Since the immunoblots from two sets were analyzed on different scanners, the data obtained from them was pooled together and analyzed using regression statistics described in **Chapter 2**.

We studied first whether in the late stages of AD CYFIP protein expression in hippocampus is affected, comparing post-mortem samples from severe AD patients and control subjects using western blot analysis. The expression of CYFIP was normalized to the neuronal marker NSE (Neuron Specific Enolase) (**Fig.3.5a, 3.9a**). The levels of CYFIP1 were found to be significantly increased in the hippocampus of severe AD patients **($F_{(2,21)}=39.471$, $p<0.01$).** In contrast, CYFIP2 levels were significantly decreased in the hippocampus of severe AD patients **($F_{(2,17)}=3.905, p<0.05$).** To investigate if these dysregulations are already present in the early stages of the disease, we analysed hippocampal lysates from patients with mild AD **(Fig.3.5d, Fig.3.9c).** Western blot analysis revealed no change in the expression of CYFIP1 in these cases **($F_{(2,21)}=1.014, p=0.206$)** . CYFIP2 expression was numerically reduced reduced in these cases, but just failed to reach statistical significance, possible due to the small sample size **($F_{(2,21)}=36.047, p=0.056$)**. This data may suggest that CYFIP2 downregulation is an early event in AD, though CYFIP1 upregulation is not.

3.2.2. CYFIP1 expression is unaltered whilst CYFIP2 is downregulated in the Superior Temporal Gyrus in severe AD

We next studied CYFIP expression in the superior temporal gyrus (STG), which is affected later at moderate stages of AD and to a lesser extent than the hippocampus (Braak and Braak, 1991a).

In a western blot analysis we compared CYFIP expression in post-mortem STG from severe AD to patient controls. The levels of CYFIP expression were normalised with NSE (**Fig. 3.5b, Fig. 3.9b**). In the case of CYFIP1, there were no changes observed between severe AD cases and control subjects **($F_{(2,22)}=81.180; p=0.263$)**.

When CYFIP2 expression was probed, a downregulation was observed in severe AD cases as compared to control **(F(2,22)=10.505,p<0.01)**, replicating our findings in the hippocampus.

3.2.3. CYFIP1 expression is unchanged and CYFIP2 was expressed at undetectable levels in the cerebellum of severe AD cases

The cerebellum is one of the least affected regions in AD pathology (Larner, 1997). Though diffuse amyloid plaques and increased numbers of activated microglia have been reported, there is no neurofibrillary tangle pathology reported in the cerebellum (Larner, 1997) . Furthermore, motor disturbances that can be assigned to cerebellum dysfunction are not observed in AD. These features make it a good internal control in a case-control study of AD patients. To investigate if CYFIP could be one of the factor(s) that play a role in the disease mechanism affecting forebrain but not the cerebellum in AD, we performed case-control studies on cerebellum.

We obtained cerebellum samples from severe AD cases and controls in two sets from the London Neurodegenerative Brain Bank. For the mild cases, the patient sample was obtained in one set. As with hippocampal samples, We tried to obtain the samples with lowest possible post-mortem delays available. Again, since the immunoblots from two sets were analyzed on different scanners, the data obtained from them was pooled together and analyzed using regression statistics described in **Chapter 2**.

We investigated the levels of CYFIP in post-mortem cerebellum from severe AD patients and compared with controls, using western blot analysis. The normalization was carried out with NSE **(Fig. 3.5c).** CYFIP1 expression was not altered in cerebellum of severe AD patients as compared to control **(F(2,17)=4.484,p=0.894)**. We were

unable to detect CYFIP2 expression in our immunoblots from these tissue samples in both cases and controls (Hoeffer et al., 2012).

3.2.4. CYFIP1 expression is unchanged whilst CYFIP2 expression decreases with increasing age in Tg2576 mice

Mouse models are useful to investigate some mechanisms of AD. However, not all aspects of AD can be modelled in mice, possibly due to the short life span of the mice. We studied whether abnormal APP processing in the hippocampus-forebrain is sufficient to induce CYFIP1 upregulation and CYFIP2 downregulation, using Tg2576 mice, a widely used mouse model for abnormal APP processing.

We performed a western blot analysis at two time points, 4 and 12 months of age. At 4 months of age Tg2576 mice do not have amyloid plaques but do show some memory impairment, whereas at 12 months of age, Tg2576 mice memory deficits are more pronounced and amyloid plaques are detectable(Hsiao et al., 1996, Stewart et al., 2011). CYFIP expression at both ages was normalized against NSE. This comparison revealed significant differences between genotypes. CYFIP1 was significantly reduced at the 12 month time point in Tg2576 mice **(Fig.3.6a) (t=-5.487, p<0.01)** but was unchanged at the 4 month time point **(Fig. 3.6b)(t=0.973,p=0.368)**.CYFIP2 was also significantly reduced by 50 % at the 12 month time point **(Fig.3.10a)(t=-6.560,p<0.01)** in Tg2576 mice with no change in 4 month old mice **(Fig.3.10b)(t=-1.031,p=0.343).** Aged Tg2576 mice therefore replicated the same result for CYFIP2 as observed in postmortem human brains, but not for CYFIP1. This suggests that the aged brain of these mice might be modeling the early stages of AD in patient brain.

3.2.5. CYFIP1 is unchanged in ageing cerebellum

The increased CYFIP1 expression in hippocampus in AD indicates that CYFIP1 expression is plastic. This raised the issue whether normal ageing also regulates CYFIP1 expression.

Hence, we analyzed the cerebellar lysates from healthy subjects belonging to two age groups, 15-30 years and 90-105 years **(Fig. 3.8)**. We were unable to obtain the good quality hippocampal tissues from control subjects with short postmortem delays, so, we probed the available cerebellar lysates (with the pre existing limitation of no detectable CYFIP2 signals) in western blots. Western blot analysis and normalization with NSE showed that normal ageing has no effect on CYFIP1 expression in cerebellum **(t=0.581,p=0.573).**

3.2.6. CYFIP2 expression is independent of CYFIP1 expression in CYFIP2+/- mice synapses

To perform further functional studies on the significance of CYFIP2 molecules in AD, we used CYFIP2$^{+/-}$ mouse model. The homozygous knockout of *CYFIP2* gene is lethal at embryonic stage (Kumar et al., 2013b) .

Since CYFIP2 and CYFIP1 molecule have nearly 88 % sequence identity , we wanted to know if the expression of the two molecule are related and if overexpression of one molecule type compensates for the underexpression of another molecule type or *vice versa*. Crude hippocampal synaptosomal preparations (P2) from CYFIP2$^{+/-}$ mice

(as described in chapter 2) were probed for CYFIP2 and CYFIP1 expression in synaptosome **(Fig.3.11a and Fig.3.11b)**. The normalization was carried out with synaptic marker synaptophysin. CYFIP2 expression showed a downregulation of about 50% in the mutant mice **(t=-2.548,p<0.05)** as compared to wild type. Also, these results show that the heterozygotic status at genetic level in these mutant mice is replicated at protein level for CYFIP2, making them a viable model to study the functional effect of CYFIP2 downregulation. The CYFIP1 expression on the other hand was relatively unaffected by the downregulation of CYFIP2 expression, as it was not different in mutant mice **(t=0.090,p=0.929)** synaptosomes as compared to wild type mice.

3.2.7. CYFIP2 $^{+/-}$ mice show deficits in cued fear conditioning memory

Fear conditioning memory tests are fast and precise behavioral tests to analyze the extent of learning and memory impairments in *in vivo* models (Maren, 2001, Maren, 2008). CYFIP2$^{+/-}$ mice were tested in contextual and cued fear conditioning tasks. In the cued fear conditioning tasks the mice were tested at 1 hour **(Fig.3.12a)**, 1 day **(Fig.3.12b)** and 5 day **(Fig.3.12c)** intervals after training (as described in chapter 2). The freezing score of the animal was the readout for learning and memory phenotype. One mouse group was used to score freezing at 1 hour and 1 day after conditioning **(Group 2)** and another mouse group was trained to be used for memory testing 5 days after conditioning **(Group 1)**. The freezing score at each time point was analyzed before (pretone) and during (tone) the presentation of cue while testing. At 1 hour time point, there was no significant difference between wild type and CYFIP2$^{+/-}$ for pretone **(t=-0.707,p=0.485)** and tone **(t=0.335,p=0.740)** scores. At 1 day time point , the same

mouse group was tested and there was no difference between wild type and mutant mice in pretone freezing score **(U=85, Z=-0.976,p=0.329)** and tone freezing score **(t =0.846,p=0.405)**. At 5 day time point, animals from group1 were tested for the first time after training for cued fear conditioning memory. Both mutant and wild type had learned well during the training, since there was a significant difference between freezing pretone and tone score of the group consisting of mutants as well as wild type **(Z= -4.623,p<0.001)**. The pretone freezing score was not different **(U=68.50, Z=-1.614,p=0.107)** between mutant and wild type. However, the freezing tone score was significantly reduced in CYFIP2$^{+/-}$ mice **(t=2.213,p<0.05)** indicating a defect in maintenance of cue dependent memory. The effect of sex on the cue memory impairment at 5 day interval was also tested between 8 male and 20 female in the total sample of group 1 mouse. A 2 way ANOVA test followed by post-hoc test using Tukey revealed that there is no influence of sex on this phenotype **(GenotypeF(1,24)=4.784,p=0.039;SexF(1,24)=0.845,p=0.367;Genotype_SexF(1,24) =0.204,p=0.655) .**

3.2.8. CYFIP2$^{+/-}$ mice do not show any defects in formation and maintenance of context dependent fear memory

In the second part of studying the fear conditioning memory defects in CYFIP2$^{+/-}$ mice, the mice were tested for contextual fear memory. The CYFIP2$^{+/-}$ and wild type mice from Group 1 were tested for 5 subsequent days after training at 24 hours time interval to plot the extinction curve for contextual fear memories **(Fig.3.13a)**. A 2 way repeated measure ANOVA test revealed that the animals have learned during the training but there is no genotype specific difference in contextual fear learning and memory

(Genotype-F(1,12)=0.036,p=0.854; Day-F(4,9)=22.560,p<0.001; Genotype_Day-F(4,9)=1.510,p=0.279). To study contextual fear memory maintenance over a longer period, the mice from Group2 were tested 28 days after training **(Fig.3.13b)**.There was no significant difference between freezing scores of CYFIP2$^{+/-}$ and wild type mice

(t=-0.117,p=0.907).

3.2.9.CYFIP2+/- mice is not impaired in visible platform test and hence suitable for water maze tests

Morris water maze test (D'Hooge and De Deyn, 2001) is the gold standard for studying spatial memories. CYFIP2$^{+/-}$ mice will be tested for defects in hippocampal dependent spatial memories in future studies. To test the suitability of CYFIP2$^{+/-}$ mice for Morris water maze experiments, a preliminary set of experiment were performed which included training on water maze platform for six days followed by probe trial on sixth day (as described in methods) **(Fig.3.14a,)** and visible platform test **(Fig3.14b)**. The training for six days and the probe trial on sixth day didn't reveal any significant difference in wild type and mutant mice as well as showed that there was no learning in the mice group by sixth day **(Target quadrant time spent versus other quadrants, t=1.031,p=0.325 ; Target platform crossing, t=0.590,p=0.567).** The same group of mice were tested on a visible platform test to determine if the CYFIP2 $^{+/-}$ mice have a phenotype apart from spatial memory affecting their suitability for future longer water maze trials (like locomotor defects, visual defects, muscular defects). The visible platform test showed no difference in the performance of CYFIP2$^{+/-}$ mice as compared to wild type mice **(t=1.671, p=0.123).** Hence, these animals are suitable for future spatial memory tests utilizing Morris water maze paradigm.

3.4. Discussion

Our investigation of CYFIP1/2 molecules have revealed the following important findings (1) CYFIP2 is downregulated in the milder stages of AD in forebrain and this downregulation may be responsible for dementia in AD as shown by memory impairment in behavioral studies of CYFIP2 $^{+/-}$ mice (2) CYFIP1 is upregulated in the later stages of AD in hippocampus (3) CYFIP1 and CYFIP2 expression regulations are not dependent on each other (4) CYFIP1 expression in human cerebellum does not change with normal aging (5) CYFIP2 downregulation, but not CYFIP1 upregulation, is modeled in Tg2576 mice, an AD model that has abnormal APP processing .

With respect to the first finding, we have shown that CYFIP2 downregulation is an early event in AD hippocampus and STG as seen in the case control postmortem studies. In the milder stages of disease, hippocampus is one of the first brain regions to be affected, where we observed a trend ($p < 0.10$; though not significant perhaps due to a smaller sample size) for downregulation of CYFIP2. In the later stages of disease CYFIP2 expression in the hippocampus and STG showed a very significant downregulation.

From the previous studies with p25 transgenic mouse as described previously (Engmann et al., 2011) , a downregulation of CYFIP2 expression was expected. In cultured hippocampal neurons CYFIP2 is expressed in dendritic spines and its expression is enriched at excitatory synapses (Pathania et al., 2014). Further CYFIP2 overexpression in these hippocampal neurons tend to increase dendritic branching similar to CYFIP1 overexpression (Pathania et al., 2014). Thus, a downregulation of

CYFIP2 expression may reduce dendritic branching as was shown for a downregulation of CYFIP1 expression (Pathania et al., 2014). Further, CYFIP1 downregulation affects spine morphology in cortical and hippocampal neurons (De Rubeis et al., 2013, Pathania et al., 2014) in that mushroom spines are increased whereas long thin spines are reduced. We have modeled the CYFIP2 downregulation in AD in CYFIP2^{+/-} mice. We found that CYFIP2^{+/-} mice have normal memory formation of cued fear memory, but the maintenance of this memory is impaired, as it declines within 5 days after training. Interestingly, in the CYFIP2^{+/-} mice memory loss was not found for contextual fear memory, which requires the amygdala and the hippocampus. Cued fear memory requires the amygdala and it is a matter of debate whether the hippocampus is also needed (Maren, 2008) . Our data suggest that downregulation of CYFIP2 expression in the hippocampus can lead to memory loss. A recent BSc project in our lab showed that spine morphology is affected in CA1 pyramidal neurons in CYFIP2^{+/-} mice, in that there are more immature and less mature spines. This spine phenotype may have accounted for the observed memory loss in our fear conditioning studies. Moreover, Kumar et al have reported the presence of S968F mutation in C57BL/6 N strain of mice that we have used in the present study (Kumar et al., 2013b). This mutation reduces the half life of CYFIP2 to 2 hours as opposed to 8 hours in the C57BL/6 J strain. This data by Kumar et al was published by the end of this PhD study, hence we continued with the use of C57BL/6 N strain. If there are any minimal phenotypic differences as a result of the reduced half life of CYFIP2 molecules, they must have been accounted during the data analysis, since the test and control mice in our study have the same genetic background from the same strain.

One of the mechanisms by which CYFIP2 reduced expression might be leading to synaptic losses could be an altered activity of the WRC complex. As reported by Pathania et al group , CYFIP1^{+/-} mice with reduced expression of CYFIP1, has been

known to be leading to altered WRC mediated actin cytoskeletal dynamics affecting cell viability and altered spine morphology (Pathania et al., 2014). This hints towards a similar phenotype for CYFIP2$^{+/-}$ mice.

An alternative mechanism by which CYFIP2 haploinsufficieny could be leading to synaptic losses causing dementia in AD patients is by its involvement as a translation modulator of dendritically localized mRNA. Bagni's group has shown the existence of a eIF4E-CYFIP-FMRP complex acting as a modulator of dendritically or synaptically localized mRNA s in a synaptic activity dependent manner by inhibiting the translation of target mRNA (Zalfa et al., 2003, De Rubeis et al., 2013). Thus, downregulation of CYFIP2 may reduce FMRP-mediated translation repression of mRNAs, which could results in overexpression of synaptic proteins that may become toxic for the synapse.

Our study with Tg2576 mice leads to two conclusions. First, the CYFIP2 downregulation observed in postmortem studies of AD brain was modeled in Tg2576 mice. This was at a developmental time point when the cognitive deficits in these mice have started to emerge like reduced spine density and impaired contextual fear conditioning (D'Amelio et al., 2011). The CYFIP2 downregulation in Tg2576 mice is in agreement with a trend of a downregulation in postmortem hippocampus of mild AD. In contrast, Tg2576 mice do not model the CYFIP1 overexpression in hippocampus which occurs in postmortem hippocampus of late AD. Taken together, this suggests that Tg2576 mice model only the early stages of AD. Second, since CYFIP2 downregulation was detected in Tg2576 mice, it shows that abnormal APP processing is sufficient to cause this downregulation in AD.

CYFIP2 mRNA is itself one of the mRNAs regulated by FMRP based translation repression complex, leading us to speculate about a probable self correcting loop

(Darnell et al., 2011). But, at the same time APP mRNA translation may be regulated by FMRP and CYFIP2. This is suggested as a recent BSc project in our lab has shown that synaptic APP protein expression is increased in CYFIP2$^{+/-}$ mice. Taken together, the following model can be proposed: abnormal APP processing leads to a downregulation of CYFIP2 expression which in turn may lead to over expression of synaptic APP expression **(Fig.5.1)**. This model suggests a feed-forward loop for amyloid toxicity at synapses; it was already proposed on the grounds that FMRP regulates APP mRNA translation, (Westmark, 2013). *Fmr1* knockout mice have elevated levels of APP. The synaptic and behavior impairments in *Fmr1* knockout mice could be rescued by rescue of APP overexpression in them. At the same time, in primary cultured neurons, amyloid β leads to elevation of APP levels in dendrites (Westmark et al., 2011).These studies suggest a role of CYFIP2 both as modulator of local translation mechanism as well as in actin cytoskeletal dynamics. As demonstrated by Bagni's group (De Rubeis et al., 2013) for CYFIP1 involvement in both these respective process, as well as by Kittler's group showing a dysregulation of CYFIP1 expression levels that leads to pathological changes in CNS maturation and neuronal connectivity((Pathania et al., 2014), it is very likely that CYFIP2 also plays a similar role especially in context with AD .

However, to what extent does could CYFIP1 overexpression compensate for CYFIP2 haploinsufficiency? A survey of literature shows that apart from similarities in sequences, there are considerable parallels in terms of functional role. However, there are differences as stated in introduction - CYFIP2 but not CYFIP1 can interact with the other members of FMRP family – FXR1 and FXR2 , as well as being one of the molecules regulated by p53 , which is not the case with CYFIP1 . Also, the results from Pathania et al have shown that CYFIP1 overexpression impairs dendritic branching and spine morphology in hippocampal neurons (*in vivo*). Thus, only if

CYFIP1 and CYFIP2 would have exactly the same functions in the hippocampus, then the CYFIP1 upregulation in late AD may compensate for the CYFIP2 downregulation. However, CYFIP1 upregulation was not found in postmortem STG. Further, CYFIP2$^{+/-}$ mice do not upregulate CYFIP1 in the hippocampus. Thus, it is more likely that the CYFIP1 upregulation in late AD hippocampus represents a further wave of neurodegenerative mechanisms.

3.5. Conclusion

Our studies with CYFIP1/2 molecules have suggested a relevance for both CYFIPs in synaptic degeneration in AD , using postmortem human brain analyses and mouse models. Our work has confirmed the hypothesis that CYFIP family proteins are dysregulated in AD. Further functional studies with CYFIP2$^{+/-}$ mice as well using Adeno Associated Virus mediated CYFIP2 **(Fig.2.2)** knockdown studies are planned to firmly establish the mechanistic impact of this dysregulation.

Chapter 4: CSPα may be neuroprotective in AD

4.1 Introduction

4.1.1. What is CSPα?

CSPα (Cysteine String Protein α) is a 34 kDa synaptically located J- Domain containing protein (Zhao et al., 2008). It contains an N-Terminal J domain and a string of 13-15 cysteine residues in the middle region (Braun and Scheller, 1995). The cysteine string residues undergo palmitoylation which anchors CSPα to synaptic vesicles (Ohyama et al., 2007). There are two other protein variants homologous to CSPα sequence in the mammalian genome – CSPβ and CSPγ (Evans et al., 2003). However, these proteins are not expressed in brain (Fernandez-Chacon et al., 2004)

4.1.2. Discovery of CSPα

CSP was discovered in Eric Buchner's lab in *Drosophila* using an 'antibody to gene approach' (Zinsmaier et al., 1990) . This approach was pioneered by Seymour Benzer and approximately 200 *Drosophila* brain-specific monoclonal antibodies were isolated from the mice immunized with *Drosophila* head protein homogenates (Buchner et al., 1988). One of these antibodies, mAb ab49, bound to the neuropils and synaptic boutons in the neuro-muscular junction. The recovery of antigen cDNA was carried out by screening a cDNA expression library from the same group. Sequencing of the cDNA identified a string of 11 cysteine residues leading to the name Cysteine String Protein (Zinsmaier et al., 1990). The first CSP protein in vertebrates was discovered by Umbach's group in *Torpedo* fish (Gundersen and Umbach, 1992).

Subsequently, three isoforms of CSP were identified in the mammalian genome – CSPα, CSPβ and CSPγ. CSPα and CSPβ sequences share a high degree of sequence homology with each other and with the CSP sequence identified in *Drosophila* (Evans et al., 2003).In *Drosophila* there is only CSP isoform in contrast to mammals.

4.1.3. What is the expression pattern of CSPα ?

CSPα is present in synaptic vesicles and constitutes about 1 % of total synaptic vesicle protein (Eybalin et al., 2002) . It is expressed in most synapses including the neuromuscular junctions and is abundant in central nervous system neuropils (Evans and Morgan, 2005). CSPα is also present in secretory vesicles of endocrine, neuroendocrine and exocrine cells (Redecker et al., 1998, Zhang et al., 1998) The presence of CSPα has been detected in non-neuronal tissues like liver, pancreas and adrenal glands (Chamberlain et al., 1996)

4.1.4. What are the functions of CSPα at the synapse?

CSPα has been shown to be involved in the following functions in neurons- (1) Exocytosis - It acts as a co-chaperone of a trimeric complex by interacting with two other proteins : Heat Shock Protein 70 (Hsc 70) and small glutamine –rich TPR-containing protein(SGT) **(Figure 4.1 a)** . Thomas Sudhof's group has shown that this trimeric complex is involved in exocytosis by interacting and co-chaperoning the SNARE proteins leading to synaptic vesicle fusion in presynaptic terminals (Sharma et al., 2011). SNARE proteins are a set of synaptically localized membrane fusion proteins involved in the vesicle recycling mechanism in presynaptic terminals. In CSPα knockout mice, the levels of SNARE proteins (like α Synuclein, SNAP-25) are

reduced (Chandra et al., 2005). (2) Endocytosis – CSPα interacts with dynamin 1 and facilitates the polymerization of dynamin, which is important for endocytotic vesicle fission (Zhang et al., 2012) **(Figure 4.1b)** (3) Modulation of calcium dependent K $^+$ channels (BK channels) - CSPα is important for modulation of presynaptic BK channel expression (Kyle et al., 2013) **(Figure 4.2)**. CSPα knockout mouse models show upregulation of BK channels and hence aberrant synaptic activity and (4) Modulation of presynaptic calcium levels by regulating calcium channels (Ranjan et al., 1998).

4.1.5. What are the diseases in which CSPα has been implicated?

Alzheimer's disease, Parkinson's disease and polyglutamine diseases (such as Huntington's disease), are all characterized by mis-folded protein aggregates. These protein aggregates have been shown to be a result of a defect in chaperone machinery and, indeed, molecular chaperones can act as neuroprotective factors (Muchowski and Wacker, 2005). A number of abnormally folded proteins show co-localization with identical molecules of a molecular chaperone system. CSPα interacting partner Hsc70 has been shown to be important for regulating the accumulation and toxicity of misfolded protein aggregates in models of Alzheimer's,   Parkinson's and polyglutamine diseases (Muchowski and Wacker, 2005, Bonini and Fortini, 2003). Moreover, a recent study has shown that loss of function CSPα mutations are responsible for a hereditary neurodegenerative condition known as adult onset autosomal dominant neuronal ceroid lipofuscinosis (ANCL) (Benitez et al., 2011).
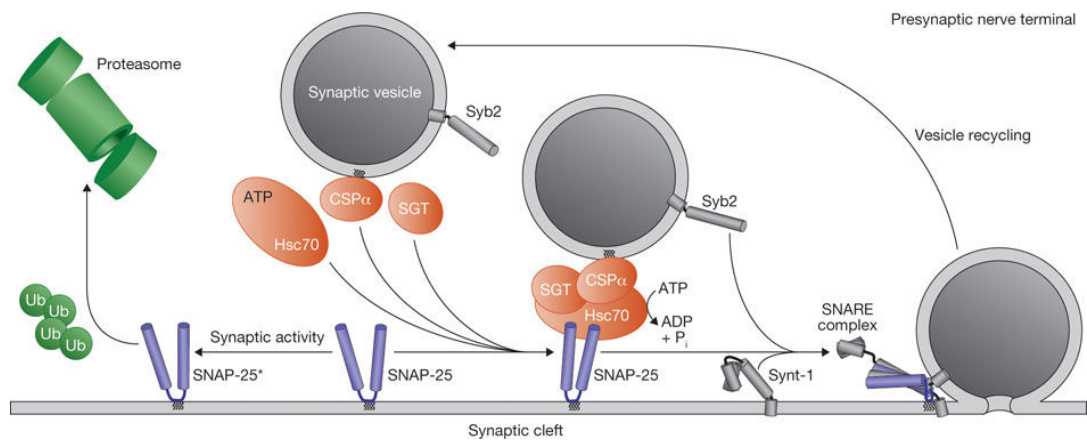
Figure 4.1 a. **CSPα-Hsc-70-SGT complex at the presynapse .** CSPα forms a trimeric complex by interacting with two other proteins: Heat Shock Protein 70 (Hsc 70) and small glutamine –rich TPR-containing protein (SGT). This complex mediates the exocytosis of synaptic vesicles at pre-synaptic terminals. Taken from (Sharma et al., 2011).
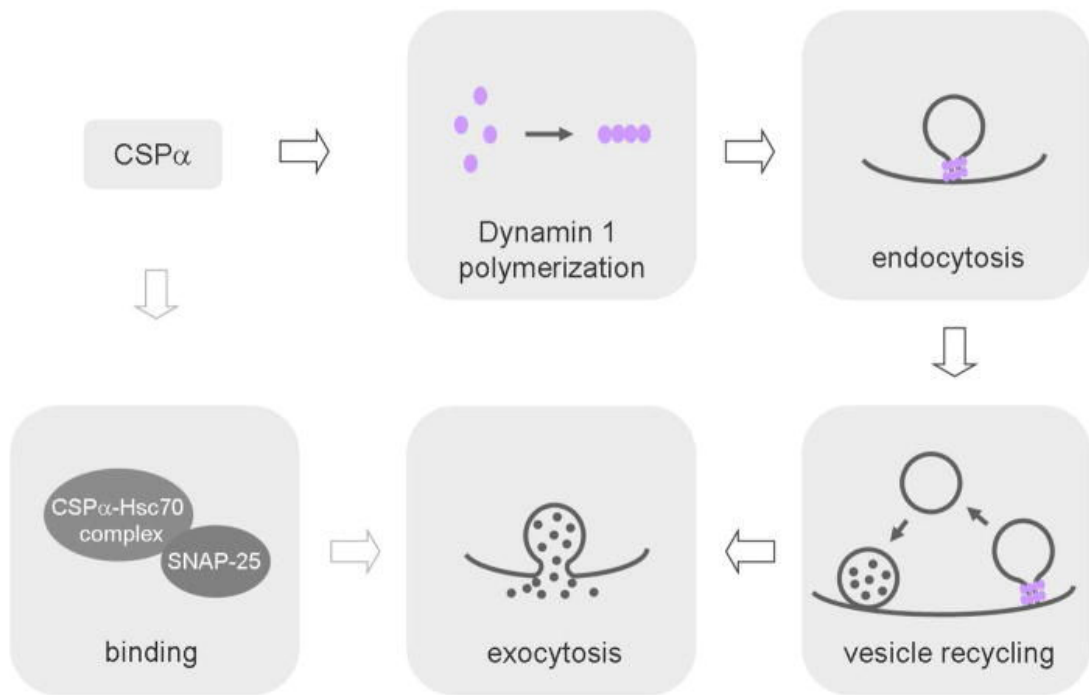
Figure 4.1 b. **CSPα regulates vesicle recycling.** CSPα binds dynamin 1 leading to dynamin polymerization, which is important for endocytosis. CSPα forms a trimeric complex with SGT and Hsc70 , and this complex interacts with SNAP-25 leading to exocytosis .Taken from (Sheng and Wu, 2012).
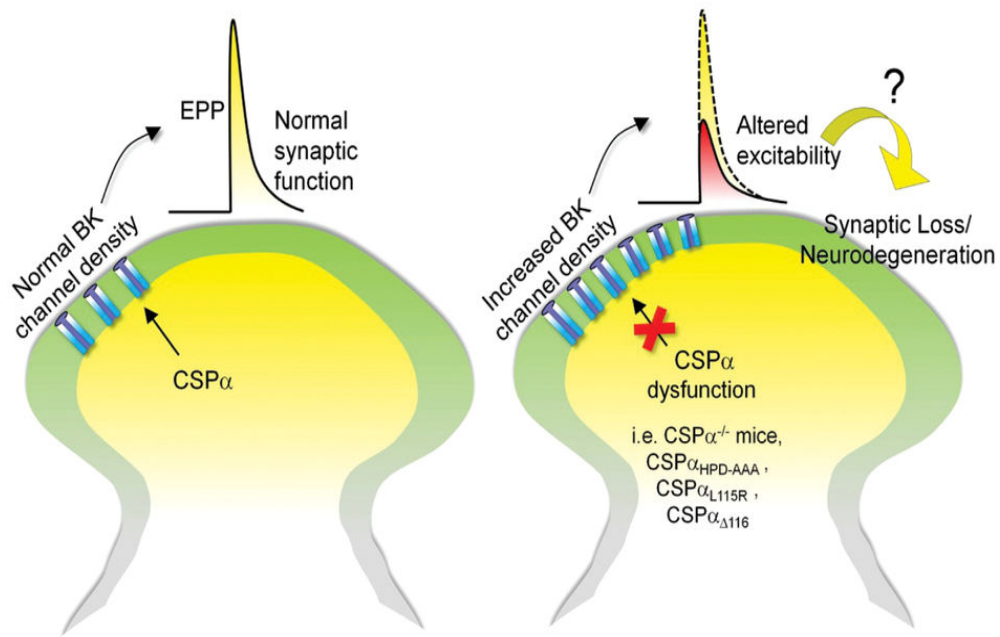
Figure 4.2 CSPα is involved in BK channel density regulation. CSPα modulates BK channel density at pre-synapse and the defect in CSPα levels impact BK channel density thereby regulating the excitability at synapse. CSPα knockout leads to increased membrane excitability possibly leading to neurodegeneration. Taken from (Kyle et al., 2013).
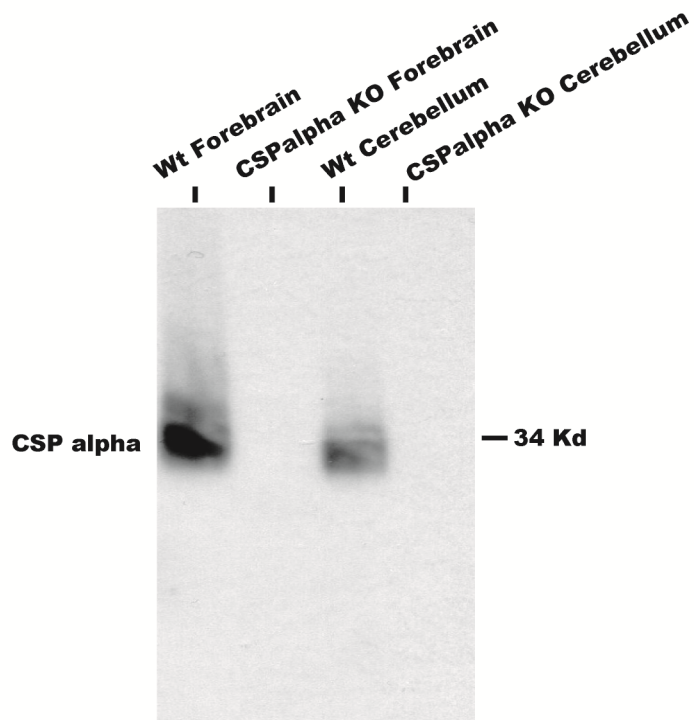
Figure 4.3. Specific binding of anti-CSPα antibodies. CSPα knockout mouse (Fernandez-Chacon et al., 2004) forebrain and cerebellum lysate was probed alongside forebrain and cerebellum from wild type mice with anti-CSPα antibody in an immunoblot study. There was no signal detected from the lanes containing CSPα knockout brain lysates.

Figure 4.4. CSPα expression is increased in Alzheimer's disease cerebellum. (A) CSPα expression in post-mortem cerebellum from patients with severe Alzheimer's disease (n = 10; average age at death = 74.7 ± 4.0 years) and control subjects (n = 10; average age at death = 76.3 ± 4.2 years). CSPα expression was normalized against NSE. **(B)** CSPα expression in post-mortem cerebellum from patients with mild Alzheimer's disease (n = 10; average age at death = 81.3 ± 4.1 years) and control subjects (n = 10). CSPα expression was normalized against NSE. **(C)** The same samples as in panel **(A)** were used but CSPα expression was normalized against synaptophysin. **(D)** The same samples as in panel **(B)** were used but CSPα expression was normalized against synaptophysin. Panel **(E)** shows the representative western blots. Means ± s.e.m. are shown. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 4.5. Increased CSPαimmunostaining in Alzheimer's disease cerebellum. Fixed cerebellar cortex sections from a patient with severe Alzheimer's disease **(A,B)** and a control subject **(C,D)** were probed with anti-CSPα antibodies for analysis of CSPα expression. CSPα expression in the cerebellar cortex of the Alzheimer's disease patient appeared higher than in the control subject. In **A** and **C** ,the granule cells of the cerebellum show increased cytoplasmic and neuropil labelling in AD as compared to an

age-matched control case . In **B** and **D**, cerebellar cortex with the Purkinje cell layer and granule cells are visible in the lower right corner . There is increased immunoreactivity in the neuropil in AD as compared to an age-matched control case. Negative control images are shown in **E** and **F** (immunohistochemistry with omission of the primary antibody) and confirm the specificity of labelling in Figure 4.5, 4.6 and S1 .Haematoxylin counterstain. Original magnification: 400x _Scale bars represent 20 µm.

A

CSP Control

B
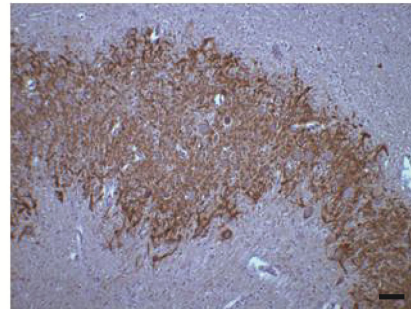
Synaptophysin Control

Figure 4.6. CSPα immunostaining in cerebellum is typical for synaptic expression. Immunohistochemical sections of cerebellar dentate nucleus region from a control patient were immunostained with anti-CSPα antibodies **(A)** and with antibodies against the synaptic marker synaptophysin **(B)**. This comparison indicated that the CSPα immunostaining is synaptic as obtained for synaptophysin immunostaining. Scale bars represent 200 µm.

Figure 4.7. CSPα protein expression is reduced in Alzheimer's disease hippocampus when normalized against synaptophysin. (A) CSPα expression in post-mortem hippocampus from patients with severe Alzheimer's disease (Braak stages V and VI; n = 12; average age at death = 75.2 ± 2.0 years) and control subjects (n = 12; average age at death = 76.5 ± 2.9 years). CSPα expression was normalized against the neuron specific house keeping marker protein NSE. **(B)** CSPα expression in post-mortem hippocampus from patients with mild Alzheimer's disease (Braak stages I and

II; n = 12; average age at death = 80.3 ± 3.2 years) and control subjects (n = 12). CSPα expression was normalized against NSE. **(C)** The same samples as in panel **(A)** were used but CSPα expression was normalized against the synaptic marker protein synaptophysin (severe Alzheimer's disease, n = 11; control, n = 11). **(D)** The same samples as in panel **(B)** were used but CSPα expression was normalized against synaptophysin (mild Alzheimer's disease, n = 12; control, n =

11). Panel **(E)** shows the representative western blots. Means ± s.e.m. are shown. *, $p < 0.05$; **, $p < 0.01$.

Figure 4.8. CSPα protein expression is reduced in Alzheimer's disease superior temporal gyrus. (A) CSPα expression in post-mortem STG from patients with severe Alzheimer's disease (n = 13; average age at death = 73.2 ± 3.4 years) and control subjects (n = 12; average age at death = 76.9 ± 2.1 years). CSPα expression was normalized against NSE. **(B)** The same samples as in panel **(A)** were used but CSPα expression was normalized against synaptophysin (severe Alzheimer's disease, n = 13; control, n = 12). Panel **(C)** shows the representative western blots. Means ± s.e.m. are shown. **, $p < 0.01$; ***, $p < 0.001$.

Figure 4.9. **Normal ageing reduces CSPα expression in healthy human cerebellum. (A)** CSPα expression in post-mortem cerebellum from healthy young (n = 6; average age at death = 21.3 ± 1.6 years) and aged (n = 7; average age at death = 96.1 ± 1.4 years) subjects. CSPα expression was normalized against NSE. **(B)** The same samples as in panel **(A)** were used but CSPα expression was normalized against synaptophysin. Panel **(C)** shows the representative western blots. Means ± s.e.m. are shown. *, $p < 0.05$.
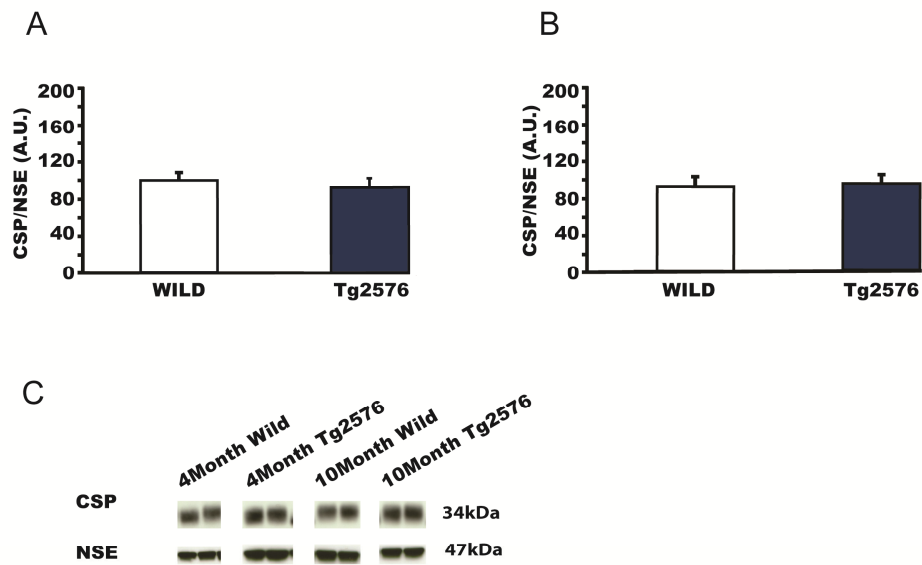
Figure 4.10. **Abnormal APP processing is not sufficient to cause CSPα**

upregulation in cerebellum. (A) CSPα expression in cerebellum of 4 month-old wild-

type (n = 4) and Tg2576 mice (n = 4). **(B)** CSPα expression in cerebellum of 10 month-

old wild-type (n = 3) and Tg2576 mice (n = 3). CSPα expression was normalized

against NSE. Panel **(C)** shows the representative western blots. Means ± s.e.m. are

shown.
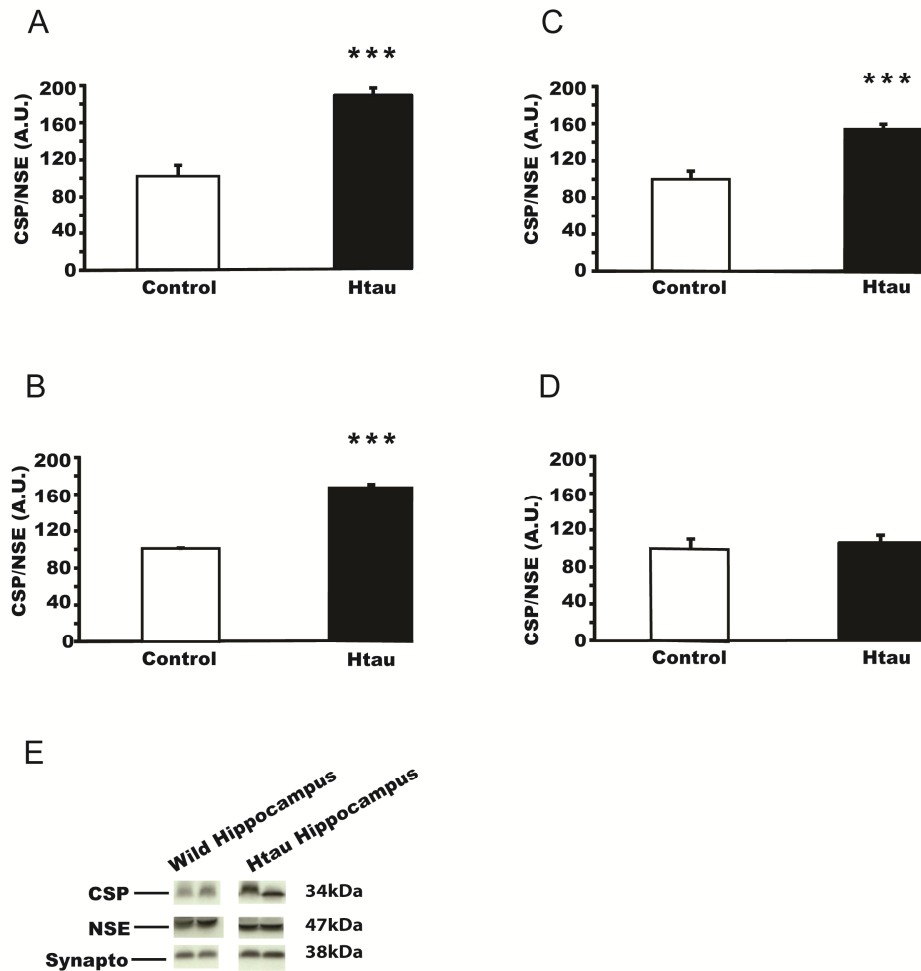
Figure 4.11. Mice expressing human tau have an age-dependent CSPα overexpression that correlates with neuroprotection. **(A)** CSPα expression in hippocampus of young (3-4 months) wild type (n = 6) and htau mice (n = 6). **(B)** CSPα expression in cortex of young (3-4 months) wild type (n = 6) and htau mice (n = 6). **(C)** CSPα expression in cerebellum of the same mouse sample as in panel **(A)** and **(B)**. **(D)** CSPα expression in old (24 months) wild type (n = 4) and htau mice (n = 7). CSPα expression was normalized against NSE in all panels. Panel **(E)** shows the representative western blots. Means ± s.e.m are shown. ***, p<0.001.

Figure 4.12. CSPα expression in cerebellum is not changed in FTLD. CSPα expression in post-mortem cerebellum from patients with FTLD (n = 5; average age at death = 73.0 ± 3.0 years) and control subjects (n = 5; average age at death = 70.2 ± 7.2 years). CSPα expression was normalized against NSE **(A)**. Panel **(B)** shows the representative western blots. Mean ± s.e.m. are shown.

4.1.6. What kind of animal models have been used to date to study CSPα functions?

Genetic studies carried out on mice and flies have provided critical insights into the synaptic role of CSP. Deletion of the CSP gene in *Drosophila* causes paralysis and decreases life expectancy (Ranjan et al., 1998, Umbach et al., 1994). At elevated temperatures, there is a complete loss of evoked neurotransmitter release and the flies paralyze within minutes. The loss of neurotransmitter release at the CSP mutant neuromuscular junction can be overcome by increased extracellular Ca^{2+} or accumulated intracellular Ca^{2+} level during high-frequency stimulation (Nie et al., 1999, Bronk et al., 2005) .

CSPα knockout mice also develop progressive motor and sensory impairments leading to paralysis, blindness and premature death by postnatal day 40-60 (Chandra et al., 2005, Fernandez-Chacon et al., 2004). Synapses of CSPα knockout mice display an age dependent deterioration of synaptic function that correlates well with breakdown of synaptic structure and the progressing neurological symptoms. However, as opposed to *Drosophila*, the evoked neurotransmitter release is normal in young CSPα knockout mice and it progressively becomes asynchronous as well as deteriorates with age (Fernandez-Chacon et al., 2004, Ruiz et al., 2008). By age of P30 the level of evoked release at mutant mice neuromuscular junctions and Calyx of Held synapses is quantitatively equivalent to the levels in mutant Drosophila neuromuscular junctions. Similar to Drosophila , in the knockout mice as well the evoked neurotransmitter

release can be restored by high extracellular Ca^{2+} or with high frequency stimulation (Ruiz et al., 2008).

4.1.7. Previous studies in the lab that lead to identification of CSPα

A previous study in the lab showed that the truncated cyclin-dependent kinase 5 activator p25 is reduced in AD (Engmann et al., 2011). Normally, p25 generation is linked to the synthesis of particular synaptic proteins, synaptogenesis and memory formation (Engmann et al., 2011). Thus, impaired p25 generation may cause early synaptic dysfunction in AD. Furthermore, the mass spectrometric study of synaptosomal fractions from P25 transgenic mice demonstrated that p25 generation regulates expression of the synaptic chaperone protein cysteine string protein (CSP)α (Engmann et al., 2011).

Based on the studies of neuroprotective mechanism of CSPα published in literature, we developed and began to explore the hypothesis that: **CSPα might be involved in neuroprotective mechanisms in AD**

To investigate this hypothesis we performed case-control studies with post mortem brain tissues and with brain tissues from animal models of AD as described previously in chapter 2 (Methods).

Our objectives for this study were –

1) **To investigate the relevance of CSPα in Alzheimer's disease by performing a case-control study using post-mortem brain tissues to analyse the levels of CSPα in different regions of the brain at different stages of Alzheimer's disease**

2) **To investigate the relevance of CSPα in cognitive decline associated with normal aging by analyzing the levels of CSPα in postmortem brain from young and aged subjects**

3) **To find a suitable mice model of AD that replicates the CSPα results observed in postmortem human brain tissue for future studies**

4.2. Results

4.2.1. CSPα is upregulated in Cerebellum

Cerebellar regions are one of the least affected regions in AD pathology (Larner, 1997). Though diffused amyloid plaques and increased microglia have been reported, there is no neurofibrillary tangle pathology reported in the cerebellum (Larner, 1997) . Further, motor disturbances that can be assigned to cerebellar dysfunction are not observed in AD. These features suggest that there are mechanisms in cerebellar regions that provide neuroprotection against AD and hence make it a good internal control in case-control study of AD patients. To investigate if CSPα could be one of the factors that might be playing a role in the neuroprotective mechanism in cerebellar regions in AD, we performed case-control studies on cerebellum.

We obtained cerebellar samples of severe AD cases and controls in two sets from the London Neurodegenerative Diseases brain bank. For the mild cases, the patient samples were obtained in one set. The details of the patient samples have been provided in **Table.2.1**. We tried to obtain the samples with lowest possible post-mortem delays. Since the immunoblots from two sets were analyzed on different scanners, the data

obtained from them was pooled together and analyzed using regression statistics described in Chapter 2.

We investigated the levels of CSPα in post-mortem cerebellum from severe AD patients and compared with controls, using western blot analysis. The normalization was carried out with NSE and synaptophysin **(Fig. 4.4. A, C).** In both cases CSPα levels were significantly increased by about 50% (referring to NSE expression: **F(2,17)=2.761**; $p<0.05$; referring to synaptophysin expression: **F(2,17)=5.281**, $p<0.01$). This surprising finding suggested that already in early AD there could be an upregulation of CSPα expression in cerebellum. Therefore, we also analysed CSPα expression in mild AD **(Fig. 4.4.B, D).** We found that the levels of CSPα were significantly increased in the cerebellum when normalized with both NSE and synaptophysin (referring to NSE expression:**F(2,17)=7.245**; $p<0.05$; referring to synaptophysin expression: **F(2,17)=4.179**, $p<0.05$)**(Fig.4.7).** This finding was opposite to the changes observed in hippocampus of mild AD patients.

4.2.2. Immunohistochemical studies

To further investigate cellular localization of CSPα, qualitative immunohistochemical studies were performed on cerebellar sections from an AD case and control. To confirm the cerebellar expression changes, we carried out a qualitative immunohistochemical analysis with post-mortem cerebellar cortical tissues from one severe AD patient (n=1) and one control subject (n=1) **(Fig. 4.5)**. A neuropathologist blind to the disease state of the tissue performed a qualitative comparison of CSPα signal. A similar comparison was carried out with hippocampal and STG sections of control (n=1) and AD patients

(n=1) **(Fig.S1).** This qualitative comparison confirmed an increase in CSPα levels in cerebellum in severe AD compared to a healthy control case as well as a decrease of CSPα in forebrain of severe AD patients.

4.2.3. CSPα has synaptic expression

To validate the synaptic specificity of the anti-CSPα antibodies, the immunohistochemistry was compared with synaptophysin expression patterns **(Fig.4. 6)**. Staining of cerebellar dentate nucleus from a control subject showed similar results with both antibodies, confirming that the CSPα immunostaining was typical for synaptic staining and is increased in AD.

4.2.4. CSPα is downregulated in the hippocampus

The hippocampus is one of the first and most severely affected regions in the brain during the course of AD progression (Braak and Braak, 1991a). AD onset is characterized by the loss of memory and defects in cognitive skills. These defects are attributed to the loss of synapses at the onset of AD. Synaptic loss precedes the neuronal loss. Based on this, we decided to analyze the changes in the levels of CSPα in hippocampal neurons.

We obtained hippocampal samples of severe AD cases and controls in two sets from the London Neurodegenerative Diseases brain bank. For the mild cases, the patient samples were obtained in one set. The details of the patient samples have been provided in **Table.2.1**. We tried to obtain the samples with lowest possible post mortem

delays. Since the immunoblots from two sets were analyzed on different scanners, the data obtained from them was pooled together and analyzed using regression statistics described in Chapter 2.

We first studied whether, in the late stages of AD, CSPα protein expression in the hippocampus is affected, comparing post-mortem samples from severe AD patients and control subjects in a western analysis. As with the Cerebellum, the CSPα expression was normalized to either the neuronal marker NSE or the synaptic marker synaptophysin **(Fig. 4.7. A, C)**. In both cases CSPα levels were significantly decreased in the hippocampus of severe AD patients (referring to NSE expression: **$F_{(2,21)}=21.287$**; $p<0.01$; referring to synaptophysin expression: **$F_{(2,19)}=14.622$**, $p<0.05$). This result suggests that in severe AD CSPα expression is not simply reduced due to neuronal or synaptic loss, and it suggests that reduced CSPα may precede synaptic loss during AD progression. Therefore, we also analysed CSPα expression in mild AD **(Fig. 4.7. B, D)**. Western analysis revealed a significant decrease in CSPα levels in the hippocampus of patients when normalized with synaptophysin (**$F_{(2,20}=4.257$**; $P<0.05$), but showed no difference when normalized with NSE (**$F_{(2,21}=0.366$**, $p=0.427$), which indicates that the drop in CSPα level in hippocampus is an early event in AD.

4.2.5. CSPα is downregulated in the Superior Temporal Gyrus

After investigating the expression of CSPα in hippocampus, which is the earliest as well as most severely affected in AD, we studied CSPα expression in the superior temporal gyrus (STG), which is affected later at moderate stages of the disease and less severely than the hippocampus (Braak and Braak, 1991a) .

In a western blot analysis we compared CSPα expression in post-mortem STG from severe AD patients and controls. The level of CSPα expression was normalised with NSE or synaptophysin **(Fig. 4.8)**. In both cases there was a significant reduction in CSPα expression (referring to NSE expression: **F (2,22)=14.827**; p<0.01; referring to synaptophysin expression: **F(2,22)=7.481**, p<0.001), similar to the result seen in the hippocampus **(Fig. 4.8. A, C).**

4.2.6. CSPα decreases during ageing in the cerebellum

The increased CSPα expression in the cerebellum in AD patient tissue indicates that CSPα expression is plastic. This raised the question as to whether normal ageing also regulates CSPα expression in the cerebellum.

To investigate this, we analysed the cerebellar lysates from healthy subjects belonging to two age groups, 15-30 years and 90-105 years **(Fig. 4.9)**.Normalization with NSE showed that normal ageing significantly downregulates CSPα expression by approx. 50% in the cerebellum (t =2.443 ; p<0.05). When normalized to synaptophysin levels the average CSPα expression in the aged cerebellum was much smaller than in the young cerebellum, although this did not reach significance (t=1.351 ; p=0.234), due to large variability in young cerebellum . Taken together, we found an age-dependent decrease of CSPα expression in healthy cerebellum samples. Interestingly, the CSPα expression level in AD cerebellum is similar to the expression level in young healthy cerebellum.

4.2.7. CSPα is unchanged in Tg2576 cerebellum and forebrain

Mouse models are useful to investigate some mechanisms of AD. However, not all aspects of AD can be modelled in mice, possibly due to the short life span of the mice. We studied whether abnormal APP processing in the cerebellum is sufficient to induce CSPα upregulation, using Tg2576 mice, a widely used mouse model for abnormal APP processing.

We performed a western blot analysis at two age points, 4 and 10 months **(Fig.4.10)**.

At 4 months of age Tg2576 mice do not have amyloid plaques but have some memory impairment, whereas at 10 months of age Tg2576 mice memory deficits are more pronounced and amyloid plaques are detectable(Hsiao et al., 1996, Stewart et al., 2011). CSPα expression in cerebellum at both ages was normalized against NSE as well as synaptophysin. This comparison did not reveal any significant difference between genotypes (p=0.169). Therefore, abnormal APP processing does not appear to be sufficient to cause CSPα upregulation in cerebellum.

A similar study was carried out with the forebrain of 4 month and 10-12 month old mice forebrain lysates **(Fig S2)**. There was no change in the forebrain CSPα level at 4 month **(t=1.935 , p =0.101)** or 12 month **(t= -0.449 , p= 0.672)** stage of Tg2576 mice.

4.2.8. CSPα shows age dependent downregulation in htau mouse forebrain

Neurofibrillary tangles (NFT) and hyperphosphorylated tau are the other hallmarks of Alzheimer's disease apart from amyloid plaques. Formation of NFTs leads to neuronal loss. We investigated the levels of CSPα in a mouse model overexpressing human tau (Andorfer et al., 2005)- the htau mice. These mice progressively develop

hyperphosphorylated tau and neurofibrillary tangles, predominantly in the cortex and hippocampus and widespread neuronal loss is apparent in aged mice (Andorfer et al., 2003, Kelleher et al., 2007). The tau pathology is visible from 3 weeks of age, but the neuronal loss starts from 17 months onwards.

We looked at CSPα levels in the hippocampus **(Fig. 4.11 A)**, frontal cortex **(Fig. 4.11 B)** and cerebellum **(Fig.4.11 C)** of these mice at 3- 4 months of age. At the same time we also looked at CSPα levels at 24 months age in hippocampal-cortical lysates of these mice **(Fig.4.11 D)**. We were unable to procure the cerebellar tissues of these mice at the 24 month age time point.

The samples were normalised with NSE levels. We saw a significant upregulation of CSPα levels in hippocampus **(Fig.4.11 A, t=6.539 , p<0.001)** , frontal cortex **(Fig.4.11 B , t=8.005, p<0.001)** and cerebellum **(Fig. 4.11 C, t=5.200 , p < 0.001)** in the mice at the 3-4 month time point. At this stage there is no neuronal loss in these mice and the result replicates the result observed in the cerebellum of AD patients. At 24 months of age, there is no difference in the levels of CSPα between hippocampal –cortical regions from wild type and from htau mice **(Fig.4.11 D. t = -0.220, p =0.831)**. This result suggests that a CSPα based neuroprotective mechanism that was preventing neuronal loss in the hippocampal-cortical region in the younger age (inspite of the presence of tau pathology), is eliminated in the old age, possibly making the neurons more prone to tau based toxicity. The cerebellum at the young age is protected by elevated CSPα level as in humans.

To study the levels of previously studied CYFIP protein in Htau mouse, all the mouse tissue samples in figure 4.11 were probed with anti CYFIP2 and anti CYFIP1 antibodies. The CYFIP2 levels in the membranes with htau forebrain lysates were

undetectable **(Fig.3.15**). CYFIP1 levels did not change in htau mice forebrain or cerebellum at any age point **(Fig.3.7)**

4.2.9. CSPα is unchanged in FTLD cerebellum

Frontotemporal lobar degeneration (FTLD) is a prominent form of dementia that is characterized by neurodegeneration of the frontotemporal region(Cruts et al., 2013). Additionally, as opposed to AD, the cerebellum may be affected (King et al., 2009).

We performed a western blot analysis to study whether altered CSPα expression is linked to neuropathology of the cerebellum in FTLD **(Fig. 4.12).** CSPα levels were normalized with NSE, and no significant difference was found (**t= - 0.373, p=0.719**). Hence, increased CSPα expression in the cerebellum appears specific for AD.

4.3. Discussion

The main findings of our study are that 1) expression of CSPα is reduced in degenerating forebrain in mild and severe AD, and 2) CSPα expression is upregulated in AD cerebellum, a brain region not affected in AD. Taken together, these findings link CSPα expression with neurodegeneration, and suggest that CSPα upregulation may be neuroprotective.

CSPα is a p25-regulated protein, and we have previously shown that p25 expression is downregulated in AD forebrain (Engmann et al., 2011). In addition, CSPα mutations cause a hereditary neurodegenerative condition (Benitez et al., 2011). We therefore hypothesized that CSPα expression could be altered in AD. Here, we confirm

this hypothesis. We found that CSPα expression is reduced in hippocampus and STG in severe AD when normalized to expression of a neuronal or synaptic marker. This finding is consistent with another study, which was published after we started our project, showing that in Brodmann area 9 of severe AD CSPα expression is reduced by about 40% (Zhang et al., 2012). Furthermore, we also detected a downregulation of CSPα expression in hippocampus in mild AD when CSPα amounts were normalized to the synaptic marker synaptophysin. Traditionally, synaptophysin is used as a marker of synaptic degeneration in AD (Arendt, 2009). However, our finding that CSPα levels are reduced prior to noticeable changes in synaptophysin expression, and considering the importance of CSPα for synaptic function (Fernandez-Chacon et al., 2004), indicates that CSPα is likely to be a better marker of synaptic degeneration than synaptophysin.

We obtained double bands of CSPα in our immunoblot studies and for quantification and analysis of the toal CSPα amount both the bands were included. The presence of double bands in our CSPα blots have been reported by and explained by studies from other groups. The specificity study of our antibodies with CSP α knockout mouse have shown that both the bands are from CSP α isoform and not the beta or gamma isoform of CSP α **(Fig.4.3)**. The double bands in our CSP α blot could be explained by two posttranslational event that CSP α goes through. Palmitoylation in CSP α has been described in great detail in previous reports and this posttranslational modification is important for intracellular functions of CSP α (Greaves et al., 2008). CSP undergoes extensive palmitoylation on 14 cysteine residues present within central cysteine rich domain(Umbach et al., 1994). The palmitoylated form is essential for sorting of CSP α, though the mechanism is not yet well understood (Greaves and Chamberlain, 2007, Greaves et al., 2008). To determine, which band to study, at the start of the project, I analyzed the level of difference in either palmitoylated or unpalmitoylated protein states between cases and control. However, the ratio of playmitoylated versus

unpalmitoylated protein varies with the individual cases having a degree of standard deviation which made the difference between patient versus control insignificant. Hence, I analyzed the total CSP α level, irrespective of their palmitoylation status.

The second posttranslational event is oligomerization of CSP α which might be important for its role in synapses (Swayne et al., 2003). In my studies, I have only encountered double bands and the higher band was always less than 70 kD (a CSP α dimer molecular weight), so the oligomeric CSP α was either present in undetectable amount in my samples or was cleaved to the monomeric form during sample preparation.

CSPα, along with its interacting partners Hsc-70 and SGT, is involved in exocytotic mechanisms in pre-synaptic terminals that are mediated by its interactions with SNARE complexes (Evans et al., 2003). Downregulation of CSPα may therefore lead to reductions in the number of synaptic vesicles binding at presynaptic membranes, thereby affecting synaptic activity. Further, CSP α is also important for endocytosis of synaptic vesicles. CSPα interacts with dynamin to facilitate dynamin polymerization which is important for endocytotic vesicle fission (Rozas et al., 2012, Zhang et al., 2012). This is important for normal synaptic function since the number of synaptic vesicles readily available for exocytosis is reduced when there are defects in endocytotic fission (Wu et al., 2009). This suggests that defects observed in exocytotic mechanisms in CSPα knockout mice could be explained by deficits in CSPα-dependent endocytotic mechanisms. Hence, CSPα downregulation could lead to loss of function at different stages of synaptic vesicular recycling to contribute to synaptic loss. Additionally, reduced CSPα expression is expected to increase BK channel density at synapses, which reduces excitability at presynaptic terminals (Kyle et al., 2013). Loss

of synaptic activity is thought to be lethal for synapses, therefore, the downregulation in CSPα expression we observe in AD hippocampus could be closely associated with synaptic degeneration and the resulting impaired memory formation in early AD.

CSPα modulates the calcium dependent K^+ (BK) channel expression and current density (Kyle et al., 2013). CSPα knockout mice show aberrant activity due to increased BK channel expression at synapses. This contributes to synaptic degeneration and neuronal loss. Animal models of AD display epileptic seizures and network dysfunctions, (Palop and Mucke, 2010, Palop et al., 2007) and, based on our results, it is highly likely that the decrease in CSPα levels in cortical regions may lead to aberrant network functions of cortical circuits by dysregulation of BK channel expression at synapses.

The second major finding from our study is the identification of CSPα overexpression in AD cerebellum. The cerebellum is relatively protected from neurodegeneration in AD. For example, there is no synaptic and neuronal loss in this area, although there are some diffuse amyloid plaques present in the cerebellum (Larner, 1997). The molecular mechanisms that impart neuroprotection to the cerebellum in AD are not known. Our results suggest that CSPα may be one of the factors contributing to this neuroprotection. We observed an overexpression of CSPα in cerebellum in mild and severe AD. Importantly, we found that the level of overexpression in this region is comparable to the amount of CSPα expression detected in young, healthy cerebellum, in contrast to an age-dependent decrease in CSPα expression in normal cerebellum. Additional experimental support for the suggestion that CSPα overexpression could be neuroprotective comes from our analysis of CSPα expression in cerebellum from patients with FTLD. These patients have neuropathology in the cerebellum unlike AD patients (Al-Sarraj et al., 2011; King et

al., 2013). This report mentions the FTLD cases with C9orf72 hexanucleotide repeat. However, this suggests that FTLD effects cerebellum as opposed to AD. We found that CSPα expression is unchanged in degenerating FTLD cerebellum, thereby supporting our hypothesis. Additional support for our hypothesis comes from analysis of htau mutant mice, where we found that CSPα overexpression occurs only at times when no neuronal loss is observed. Taken together, the evidence we present here suggests that CSPα overexpression in AD cerebellum may be neuroprotective and additional functional studies are needed to investigate this hypothesis.

How could CSPα overexpression protect synapses and consequently neurons in AD cerebellum? In AD forebrain, amyloid-induced aberrations in synaptic activity are one of the causes of synaptic toxicity (Westmark, 2013). In particular, dysfunctional synaptic machinery could be an after-effect of impaired synaptic vesicle trafficking. Chang's group has shown that amyloid β oligomers impair synaptic vesicle recycling by hindering endocytosis as well as the formation of fusion-competent vesicles (Park et al., 2013). Considering the role of CSPα in endocytosis and vesicle recycling, an upregulation of CSPα in AD cerebellum could be a compensatory mechanism that prevents impairments in synaptic vesicle recycling that are induced by factors causing AD. This would result in protection of synapses and neurons from degeneration. Also, the effect of CSPα in modulating BK channel activity could also be important for protection from neurodegeneration. CSPα knockout mice have an increase in BK channel density which causes synaptic and neuronal loss (Kyle et al., 2013), therefore, an upregulation of CSPα protein at synapses in AD cerebellum may lead to maintenance of normal BK channel density, hence avoiding any damage to the synaptic and network structure of AD cerebellum.

4.4. Conclusion

Our studies have shown that CSPα is an early marker for synaptic degeneration in AD forebrain, and suggest that CSPα overexpression is neuroprotective in AD cerebellum. CSPα is a synapse-specific protein that has multiple functions at the presynapse. Thus, our results are in agreement with the idea that presynaptic degeneration is pivotal in AD, as suggested by studies using transgenic mouse models of AD (Zhang et al., 2009). Further functional studies with CSPα knockout and overexpression models will provide further insights into the mechanistic basis of our observations.

Chapter 5: Discussion

5.1. Revisiting the questions

AD is a devastating neurodegenerative condition and an increasing number of reports have shown that synaptotoxicity is one the first pathological changes apparent in AD, preceding neuronal loss and leading to the emergence of cognitive as well as memory impairments (Arendt, 2009). However, the molecular mechanisms leading to this synaptotoxicity are not yet fully known. The broad theme of this PhD study was to investigate novel molecular mechanisms leading to degeneration of synapses in AD. P25 is a Cdk5 activator that has been shown to be an important molecule in AD onset by several groups (Tandon et al., 2003, Engmann et al., 2011, Yoo and Lubec, 2001). Our lab has previously shown that p25 levels are downregulated in the post-mortem early-stage AD brain (Engmann et al., 2011). Furthermore, p25 is generated during memory formation and it is linked with synaptogenesis (Engmann et al., 2011). A mass spectrometric study of synaptosomes isolated from p25 transgenic mice identified proteins that are regulated by p25 and that may also be dysregulated in AD and therefore could contribute to synaptic degeneration in AD (Giese, 2014).

With the broad aim of identifying the novel molecular mechanisms of synaptic pathology in AD, we screened molecules from the aforementioned mass spectrometric study and began to focus on CYFIP2, since amounts of this protein were significantly altered in synaptosomes prepared from male p25 transgenic mice relative to wild-types (Engmann et al., 2011). CYFIP2, and the structurally related protein CYFIP1, bind to the mRNA binding protein FMRP that regulates local translation of dendritic/synaptic mRNA, including APP encoding mRNA (Westmark and Malter, 2007). This suggested

the hypothesis that dysregulation of local translation may lead to synaptotoxicity in AD. CYFIP1 being a member of the same family as CYFIP2 was also a molecule for interest for us. Within the framework of this broad hypothesis, we decided the following objectives for our project –

1) To investigate the relevance of CYFIP1/2 in Alzheimer's disease by performing a case-control study using post-mortem brain tissues to analyze the levels of CYFIP1/2 in different regions of the brain at different stages of Alzheimer's disease.

2) To investigate the relevance of CYFIP1/2 in cognitive decline associated with normal aging by analyzing the levels of CYFIP1/2 in postmortem brain from young and aged subjects.

3) To investigate the validity of the hypothesis that functional compensation between CYFIP1 and CYFIP2 may exist by biochemical analysis of brain tissue lysates from CYFIP2 heterozygote mice.

4) To understand the role of CYFIP2 in memory as well as dementia associated with Alzheimer's disease by behavioral testing of CYFIP2 heterozygous knockout mice.

In the same mass spectrometric study with p25 transgenic mice mentioned before, another molecule that showed a marked difference in female p25 transgenic mouse versus wild-type mice was CSPα. Since, both CYFIP and CSPα have no reported linkage to sex chromosomes and have never been reported to have an association with the sex of the animal, we concluded that this sex associated difference (with synaptosomes from male p25 mice showing the largest increase in CYFIP2 and synaptosomes from female p25 mice showing the largest increase in CSPα) might be a result of noise from the

experiment. Mouse model studies have implicated CSPα in synaptic degeneration but it was unknown whether CSPα expression is altered in Alzheimer's disease. We not only found that CSPα expression is downregulated in post-mortem AD forebrain, we also identified an upregulation of CSPα in post-mortem cerebellum of AD patients – a relatively protected brain region in AD. Hence, we hypothesized that CSPα might be neuroprotective in AD. To study this second protein, we added the following objectives for this project –

1) **To investigate the relevance of CSPα in Alzheimer's disease by performing a case-control study using post-mortem brain tissues to analyze the levels of CSPα in different regions of the brain at different stages of Alzheimer's disease**

2) **To investigate the relevance of CSPα in cognitive decline associated with normal aging by analyzing the levels of CSPα in postmortem brain from young and aged subjects**

3) **To find a suitable mice model of AD that replicates the CSPα results observed in postmortem human brain tissue for future studies**

Hence, we have tried to look for answers to these questions in this study and in doing so, we discovered some novel molecular mechanisms that likely contribute to dysregulation of synapses in AD.

5.2. Answers for discussion

5.2.1. Dysregulation of p25 regulated molecules was observed in AD postmortem brain tissues

Our screening of CYFIP1/2 and CSPα molecules through western blot analysis of post-mortem brain (case-control studies) has yielded several interesting results about these synaptic molecules in AD. In the hippocampus, there is a trend of downregulation of CYFIP2 in the early stages of AD and this downregulation becomes significant in the severe stages of the disease. CYFIP1 expression, on the other hand, does not change in the early stages of disease but it is significantly decreased in the severe stages. CSPα , which is a pre-synaptic molecule as opposed to post-synaptically reported CYFIP , also shows a significant downregulation (with respect to a synaptic marker –synaptophysin) in the hippocampus in the early stages of disease and stays reduced up to and including the severe stages of disease (with respect to a synaptic marker – synaptophysin and a neuronal marker- NSE). This finding shows that CSPα could be a more sensitive marker for synaptic degeneration in the early stages of AD than synaptophysin.

We also analysed the expression of these molecules in the STG, a brain region that is affected later in the progression of AD. CYFIP2 expression is downregulated in severe AD STG, whereas CYFIP1 amounts do not change in this brain area at any stage of disease. Similar to CYFIP2 expression, CSPα expression is downregulated in the STG at severe stages of AD.

The downregulation of the p25-regulated proteins CSPα and CYFIP2 in post-mortem AD forebrain that was identified here suggests that these proteins could contribute to synaptic degeneration in the disease. If this assumption is correct, then this would suggest that synapses in AD forebrain undergo differential degeneration

processes – which means that the mechanism that are impaired and lead to the amyloid induced synaptic toxicity on the pre and post synaptic side are different. Pre- and post-synaptic degeneration in AD is consistent with the observation that oligomeric amyloid peptide binds to both sides of the synapse (Dinamarca et al., 2012, Russell et al., 2012).

The cerebellum is a relatively protected region in AD, hence it was used as a control tissue in our case-control studies. We studied all three molecules in post-mortem AD cerebellum. Surprisingly, CSPα expression was significantly upregulated in cerebellum in the mild and severe stages of the disease. This finding was confirmed with qualitative immunohistochemical analysis. No changes in the expression levels of CYFIP1 were detected in control, mild or severe AD cerebellum relative to control brain. As reported by some other authors in literature (Hoeffer et al., 2012), CYFIP2 expression level was undetectable in cerebellum and hence couldn't be quantified in this tissue.

CSPα expression was also studied in the cerebellar region from FTLD patients where cerebellar pathology has been observed, as an additional control. CSPα levels were not changed in FTLD tissue relative to control brain.

Another important question was to look for the effect of ageing on the expression of these molecules. Since only cerebellar tissues were available from young control patients, we screened CYFIP1 and CSPα in cerebellar postmortem tissues from young (<30 years) and old (> 90 years) patients. There were no changes in CYFIP1 amounts with ageing in healthy cerebellum. However, CSPα levels in healthy cerebellum were found to decrease with ageing.

The differential expression of CSPα in AD versus control cerebellum, and in the cerebellum during normal aging suggests the novel hypothesis that CSPα may be neuroprotective for synapses.

An important question that can be raised here is about the impact of postmortem delay (PMD), age of patients, gender of patients and pH of the tissues, on our results. In this study, we have tried to acquire the samples with lowest PMD that were available to us and as far as possible; we used the samples with PMD of less than 24 hours. Since the data used in this project involved pooling the data from two different sets, and hence utilized the regression analysis model for statistical studies, it was not suitable to perform a co-relation study between different aforesaid parameters and the protein levels observed in post-mortem tissues. Hence, I adopted the strategy of comparing the significant difference between the age, PMD and Gender (nonparametric analysis) of patient samples grouped into different pathological state of disease– control, mild, severe AD. The analysis for pH couldn't be performed as some of the samples I used in my study were from a previous published study in lab where the pH data was not recorded or analysed. Statistical analysis discussed in Chapter 2 **(section 2.9)** shows that the impact of PMD, age and gender of the patient is statistically insignificant and these parameters have not affected our results.

Since the differential results we have obtained for CYFIP1 and CYFIP2 are from the same patient samples and same tissues region, and CYFIP2 levels were downregulated in forebrain but at the same time CYFIP1 was upregulated in forebrain, therefore it is highly unlikely that PMD is skewing our results in any particular direction. Also, CSPα from the same patient samples showed downregulation in cerebellar regions whereas it was upregulated in the forebrain. Furthermore, the results from analysis of CYFIP (1/2) and CSPα amounts show that wide-spread proteolytic degradation due to PMD have not influenced our results since in the same tissue regions from same patients we observed upregulation of one protein and downregulation of the other. One of the experiments that could have been ideally performed was to analyze the impact of PMD on CYFIP and CSP degradation using

simulated post mortem mouse brain assay at different PMD intervals and checking the protein levels.

5.2.2. Different mouse models of AD confirmed the results observed obtained from postmortem brain

We next investigated if the expression changes observed in post-mortem AD brain could be replicated in mouse models of AD. We first studied CYFIP1, CYFIP2 and CSPα expression in the Tg2576 mouse model of AD that has abnormal APP processing and deposits Aβ in senile plaques with age (Hsiao et al., 1996) . The CYFIP1/2 molecules showed an age-dependent decline in expression in the hippocampus and associated cortex of Tg2576 mice relative to background controls, with no changes detectable at 4 months of age, but significant reductions in CYFIP1/2 expression at 12 months of age, an age at which cognitive decline as well as amyloid plaque pathology has been reported in this mouse model. Thus, Tg2576 mice model the CYFIP2 downregulation observed in post-mortem AD brain, but they do not model the upregulation of CYFIP1 expression observed in post-mortem AD hippocampus. Furthermore, analysis of CSPα expression in Tg2576 cerebellum (both at 4 months and 10 months of age) did not show any changes in protein expression when compared to age-matched wild-type mice. Thus, it seems that abnormal APP processing on its own is insufficient to cause upregulation of CYFIP1 in the hippocampus or to upregulate CSPα expression in cerebellum, whereas it appears to be sufficient for modelling the CYFIP2 downregulation observed in AD brain. Since CYFIP2 downregulation is an early event in AD, 10 month-old Tg2576 mice could be a good model for examining

the early stages of AD. It is conceivable that Tg2576 mice do not model AD accurately since they still express endogenous mouse APP. Thus, transgene expression will not only result in abnormal APP processing but also in an overexpression of C-terminal fragments. A very recent paper has shown that knock-in of the Swedish APP mutations results in a different phenotype from that observed in Tg2576 mice (Saito et al., 2014). However, these knock-in mutants do not develop memory impairment at 18 months of age, suggesting that the life span of mice is too short to fully model AD.

Since the CSPα upregulation observed in AD cerebellum was not modelled in Tg2576 mice, we studied another mouse model of AD, which does not develop amyloid pathology, but instead develops AD-like tau pathology- the htau mouse model. CSPα expression was analyzed at two time points in forebrain tissue from these mice – at 3/4 months of age when there is no neuronal loss but visible tau pathology, and at 24 months of age, a time point when there is cognitive deficits, neuronal loss and tau pathology (Andorfer et al., 2003, Polydoro et al., 2009). There was an upregulation of CSPα in the forebrain of young htau mice, but at older ages when neuronal loss occurs, this upregulation is absent. In the cerebellum of young htau mice this upregulation was also observed. Hence, htau mice model the CSPα upregulation observed in AD cerebellum and this upregulation again correlates with a stage of disease which precedes substantial neuronal loss. This again suggests that CSPα may play a role in protecting tissues from neuronal loss.

5.2.3. CYFIP2 functional study revealed its significance for synaptic functions and AD

To understand the mechanistic relevance of the p25 downregulated molecules in AD, amongst the three candidate molecules identified (CYFIP1, CYFIP2 and CSPα), we focused on CYFIP2 – the least studied of them yet. We performed functional studies with CYFIP2$^{+/-}$ mutant mice that are heterozygous for a null mutation. Homozygous CYFIP2 knockout mice are lethal at embryonic stage. We showed that the heterozygotes express reduced CYFIP2 amounts in crude synaptosome fractions, without showing any changes in CYFIP1 expression. Fear conditioning studies have demonstrated that these mice are significantly impaired in retaining memory after cued fear conditioning. Hence, downregulation of CYFIP2 by about 50 % (as observed in the AD postmortem brain in mild and severe stages as well as in aged Tg2576 animal model) is likely to lead to memory loss. Further follow-up studies in this lab have shown that this behavioural readout correlates with altered synapse morphology (reduction in stable, mushroom dendritic spines and increase in 'immature' long thin spines) and protein expression (upregulation of APP protein expression in synaptosomes). Hence, these results suggest that reduced levels of CYFIP2 expression contribute to memory impairments in AD.

5.3. CYFIP2 and CSPα downregulation follows p25 downregulation in the forebrain of mild AD

In a previously published study from the Giese group, it was shown that the p25 is downregulated in AD at the mild stage of disease (Engmann et al., 2011). CYFIP2 and CSPα were shown to be candidate p25-regulated molecules from a mass spectrometric analysis of brain from p25 transgenic mice. In this PhD project it has been shown that CYFIP2 as well as CSPα are downregulated in forebrain regions in the early stages of

AD, and this downregulation persists until the severe stages of disease. Hence, it can be inferred that downregulation of p25 in the forebrain in the mild stages of AD leads to the downregulation of CSPα in pre-synaptic sites and CYFIP2 in the post-synaptic side. The p25 dowregulation itself could be an amyloid-induced mechanism but this has yet to be investigated.

A question that could be raised here is why CYFIP2 but not CSPα downregulation is modelled in Tg2576 mice? Tg2576 mice model abnormal APP processing, which is only part of the AD pathophysiology seen in human disease. For example, these mice do not have substantial changes in tau processing, develop NFTs or show marked neuronal loss. In contrast to Tg2576 mice, htau mice, which recapitulate many tau aspects of human AD, model the CSPα downregulation observed in AD forebrain. Hence, it is likely that abnormal APP processing is not sufficient for CSPα downregulation and that modulation of CSPα amounts may involve some mechanism downstream of APP processing that is more relevant to the development of tau pathology.

5.4. CYFIP1 upregulation may be a second wave of neurodegeneration

CYFIP1 expression is upregulated in the severe stages of AD and this upregulation was found only in the post-mortem hippocampus. Since CYFIP1 and CYFIP2 are very similar proteins, it is likely that these proteins can compensate for each others function, although CYFIP2 and not CYFIP1 can bind to FXR1 and FXR2 (Schenck et al., 2001, Napoli et al., 2008). Furthermore, CYFIP1 downregulation and overexpression have similar, not opposite, effects on dendritic branching (Pathania et al., 2014). We

therefore tested whether a reduction of CYFIP2 expression can induce an upregulation of CYFIP1 expression, using CYFIP2$^{+/-}$ mice. In hippocampal synaptosomes of the mutant mice there was no upregulation of CYFIP1, demonstrating that reduced CYFIP2 does not cause CYFIP1 upregulation. A collaborative study conducted in parallel to this project has shown that overexpression of CYFIP1 appears to be toxic *in vitro*. Since the most neuronal loss occurs in the hippocampus in end-stage AD, we can infer that CYFIP1 upregulation may be a factor contributing to neurodegeneration and it might represent a second wave of neurotoxic processes that emerge in the severe stages of AD. This conclusion is strengthened by the results obtained from analysis of brain tissues from aged Tg2576 mice. As discussed before, aged Tg2586 mice display CYFIP2 downregulation similar to that observed in the mild stages of AD. Furthermore, there is no CYFIP1 upregulation in this mouse model and no neuronal loss. It is conceivable that upregulation of CYFIP1 expression is needed to cause neuronal cytotoxicity and that this is lacking in Tg2576 mice. However, to what extent CYFIP1 upregulation is p25-dependent and what factors are downstream of CYFIP1 upregulation is a question to be addressed in future studies.

5.5. A p25-CYFIP2-CSPα feed-forward model of AD synaptotoxicity

How can we mechanistically explain all the results from this PhD study? The results from our studies have led us to propose a feed-forward model similar to the one proposed by Westmark (Westmark, 2013) **(Fig.1.3)** but including novel insights from this study. According to this p25-CYFIP2-CSPα model - in the milder stages of AD, there is a downregulation of p25 molecules in the forebrain. The amyloid oligomers

could be leading to decreased calpain-mediated p25 formation as suggested by the reports of amyloid-induced reduction of calcium signaling due to increased internalization and desensitization of NMDA receptors (Palop and Mucke, 2010, Paula-Lima et al., 2013, Giese, 2014). However, the exact upstream factors of this p25 downregulation are not yet identified but may have a parallel affect on other pathways as well, leading to AD pathology.

P25 is a cleavage product of p35 which is membrane bound and is not present in the nucleus. P25 on the other hand is not membrane bound and has been reported in both the nucleus and cytoplasm (O'Hare et al., 2005). Thus, p25 could be acting as a signaling molecule from the synapse to the nucleus (O'Hare et al., 2005).

P25 downregulation may lead to a decrease in the concentration of p25 in the nucleus. It is possible that p25 is essential for the transcriptional expression of many genes and among them *CSPα* on the pre-synaptic side and *CYFIP2* on the postsynaptic side. In the future, it will be important to study whether CYFIP2 and CSPα expression are reduced in AD brain at the transcriptional level to support this model.

Acetylation of lysine residues in histone protein , relaxes the chromatin structure, exposing the genes to the transcription factors(Brownell and Allis, 1996) *(Brownell, Allis, Curr Opinio Gen Development, 1996)* . Histone acetylation is regulated by opposing activities of Histone Deacetylase (HDAC) and Histone Acetyl Tranferase enzyme (Berger et al., 2009) . A segment in N-terminal region of HDAC1 within its catalytic site for histone deacetylation, binds directly with p25 and this interaction impairs HDAC1 activity (Kim et al., 2008). P25 down regulation will lead to increased HDAC activity. Reduced HDAC may lead to reduced transcription of genes as less of those genes will be accessible to the transcription factors. CYFIP and CSP α could be

one of those genes which might be regulated by this mechanism, causing the reduction in their levels, though this hasn't been studied by any group yet.

Ubiquitin-Proteasomal machinery could be another pathway that might be effected by decreased p25-CDK5 activity. P25-CDK5 in nucleus might be involved in regulation of genes that inhibit the proteasomal activity. Also, It has been shown that p25/CDK5 activity on its own is sufficient for the stability of cyclin B1 proteins in cortical neurons(Maestre et al., 2008). A decrease in P25 levels could lead to increase in proteasomal degradation of synaptic proteins (which may include CYFIP and CSP α proteins) either by reduced availability of P25 for target protein stabilization or by reduced transcription of proteasomal inhibitor genes . However, many studies have shown that proteasomal mechanism is inhibited in AD, leading to increased amyloid beta(Kumar et al., 2007, Kaneko et al., 2010, Gong et al., 2010). So, the impact of p25 mediated proteasomal degradation of CYFIP and CSPα in AD is an open question.

The downregulation of CSPα on the presynaptic side will lead to impaired synaptic vesicular trafficking as well as reduced clearance of amyloid species from the pre-synaptic side, thereby causing synaptic impairments. On the post synaptic side, the downregulation of CYFIP2 can have two types of effect. First , it might impair the cytoskeletal machinery by affecting the working of WAVE complexes, since CYFIP2 is an essential component of this complex (Derivery et al., 2009). This is evident from the results obtained in a recent B.Sc project on dendritic spine morphology in the hippocampal CA1 region in CYFIP2$^{+/-}$ mice, which showed a reduction of stubby/mushroom spines, the type needed for long-term memory retention. The reduction in mushroom/ stubby spines observed in this work suggests there may be a defect in cytoskeletal structure. Second, the CYFIP2 downregulation might lead to a

toxic, unrepressed translation of dendritically localized mRNA since CYFIP molecules are part of a FMRP based local translation repression complex (Schenck et al., 2001, Napoli et al., 2008). These locally regulated mRNA molecules consist of cytoskeletal proteins like MAP1B, which will again affect dendritic or synaptic structure (Brown et al., 2001, Nolze et al., 2013). Also, APP mRNA is regulated by this translation repression complex (Westmark and Malter, 2007). Downregulation of CYFIP2 will lead to overexpression of APP proteins at synapses. Towards the end of this PhD study, this overexpression was demonstrated by a recent B.Sc project in our lab following analysis of crude hippocampal synaptosomes isolated from the CYFIP2$^{+/-}$ mice. Overexpression of APP may lead to increased release of toxic amyloid species at the synapse. Toxic amyloid oligomers may further interact on both presynaptic and post synaptic sides, leading to synaptic loss as well as aggravating the p25-dependent pathway. The proposed model is explained in **Figure 5.1**.

(A) Control subjects

(B) Alzheimer's Disease
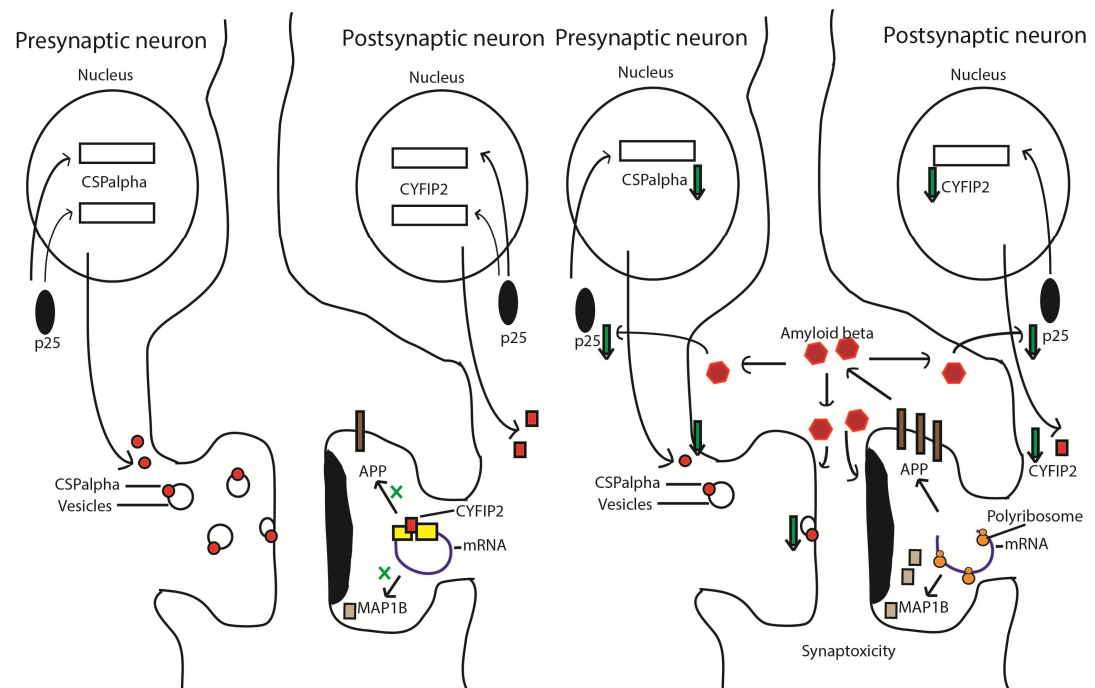
Figure 5.1. p25-CYFIP2-CSPα feed-forward model of AD synaptotoxicity. *(A) In normal individuals, p25 may act as a signal from the synapse to the nucleus leading to transcriptional expression of presynaptic genes (e.g. CSPα) or postsynaptic genes (e.g. CYFIP2). CSPα is involved in synaptic vesicule trafficking and clearance of toxic species. CYFIP2 is a component of local translation repression complex which regulates expression of many mRNAs at the synapse in an activity-dependent manner.These mRNAs encode APP as well as many cytoskeltal proteins like MAP1B. (B) In patients with Alzheimer's disease (mild stage) , there is a downregulation of p25 molecules, possibly due to the effect of amyloid oligomers on NMDA receptor function. This downregulation leads to reductions in CSPα and CYFIP2 mRNA expression. Downregulation of CSPα is synaptoxic. At the same time downregulation of CYFIP2 leads to removal of local translation repression and toxic overexpression of many*

mRNAs. Overexpression of MAP1B mRNA would affect the cytoskeletal mechanisms influencing spine structure. At the other end, overexpression of APP would lead to more amyloidogenic processing releasing amyloid β monomers and oligomers into the synapse. This toxic release of amyloid species would further impair pre- and post-synaptic mechanisms leading to synaptotoxicity. Also, the amyloid species may complete this feed-forward loop by causing further downregulation of p25 molecules.

5.6. Future studies

To test the above model, as well as to develop better mechanistic insights about how CYFIP1/2 and CSPα contribute to synaptic impairments, a number of studies can be performed in the future. Specifically, it is of interest to address the following questions:

1) *Is CYFIP2 important for spatial memory*? Our studies have shown that CYFIP2$^{+/-}$ mice are not impaired in visible platform tests, which makes them suitable for performing Morris water maze tests – the gold standard for testing spatial memory. Impaired spatial memory formation in CYFIP2$^{+/-}$ mice would point to hippocampal impairments and would provide a functional link with the synapse morphology phenotype which was detected in area CA1.

2) *What are the molecules downstream of CYFIP2 at synapses*? More biochemical studies using CYFIP2$^{+/-}$ synaptosomes will have to be performed to understand the downstream impact of CYFIP2 expression at synapses. A mass spectrometric study could be performed to identify alterations in synaptic protein expression caused by CYFIP2 downregulation.

3) *What is the synaptic phenotype of CYFIP2$^{+/-}$ mice and is there an effect of age*? This could be ascertained by performing electrophysiological studies at different age points to assess whether synaptic plasticity, such as long-term potentiation, is affected by reduced CYFIP2 expression.

4) *What kind of changes occurs in the synaptic biochemistry, dendritic morphology, synaptic electrophysiology and behaviour of models if CYFIP2 is knocked-down conditionally at different ages rather than globally*? During my PhD project, I have developed CYFIP2 shRNA packaged in an adeno-associated viral delivery system. This could be used to perform conditional knock-down of CYFIP2 expression, followed by a multi-level analysis of the phenotype.

5) *Could overexpression of CYFIP2 rescue synaptic and behavioural impairments*? This could be studied by virus-mediated overexpression of CYFIP2 in the CYFIP2$^{+/-}$ mouse model and determining if the phenotypes have been rescued.

6) *Is local protein translation impaired in AD mouse models*? This could be tested by incorporating radioactive lysine and monitoring the level and distribution of different proteins at synapse in mouse models of AD.

7) *Is CSPα overexpression neuroprotective as suggested from observations in cerebellum of AD patients?* I have cloned a CSPα overexpressing plasmid (a gift from Prof. Sudhof's lab), which could be packaged in a lentiviral delivery system to be injected in the hippocampus of mouse models of AD. This approach could analyze if CSPα overexpression rescues synaptic impairments or deficits in memory formation.

5.7. Conclusion

Synaptotoxicity in AD is an important event that precedes neurodegeneration, however the factors leading to it are not well known. This PhD study was aimed at uncovering novel candidate molecular pathways that could be involved in synaptotoxicity (or synaptic protection) in AD. From this study, CYFIP2 and CSPα have emerged as two novel p25-regulated synaptic molecules at the opposite sides of synaptic clefts, which might play an important role in synaptotoxicity from the onset of AD. Furthermore, a new role for CYFIP2 in memory retention has been established. CYFIP1 has emerged as one of the late markers of AD, possibly contributing to synaptotoxicity in severe stages. At the same time, CSPα upregulation has emerged as a possible neuroprotective factor in AD. Further detailed functional analysis is proposed to be carried out in future to build on this study as well as to establish the validity of the **p25-CYFIP2-CSPα feed-forward model** of AD proposed in this project. If the proposed model were correct, it would lead to novel diagnostic and therapeutic targets for addressing the challenge of AD in future.

Supplementary Data

Table S1. The details of mice used in this study. Cb- Cerebellum, Hc-Hippocampus, Fx- Frontal cortex.

A. Young hTau mice

S.No	Genotype	Age	Sex	Tissue
1	hTau	3-4 Months	F	Cb,Hc,Fx
2	hTau	3-4 Months	M	Cb,Hc,Fx
3	hTau	3-4 Months	M	Cb,Hc,Fx
4	hTau	3-4 Months	M	Cb,Hc,Fx
5	hTau	3-4 Months	M	Cb,Hc,Fx
6	hTau	3-4 Months	M	Cb,Hc,Fx
7	Wild	3-4 Months	F	Cb,Hc,Fx
8	Wild	3-4 Months	M	Cb,Hc,Fx
9	Wild	3-4 Months	M	Cb,Hc,Fx
10	Wild	3-4 Months	M	Cb,Hc,Fx
11	Wild	3-4 Months	M	Cb,Hc,Fx
12	Wild	3-4 Months	F	Cb,Hc,Fx

B. Aged hTau mice

S.No	Genotype	Age	Sex	Tissue
1	Wild	24 Months	F	Hc,Fx
2	Wild	24 Months	F	Hc,Fx
3	Wild	24 Months	M	Hc,Fx
4	Wild	24 Months	M	Hc,Fx
5	hTau	24 Months	M	Hc,Fx
6	hTau	24 Months	M	Hc,Fx
7	hTau	24 Months	F	Hc,Fx
8	hTau	24 Months	F	Hc,Fx
9	hTau	24 Months	M	Hc,Fx
10	hTau	24 Months	F	Hc,Fx
11	hTau	24 Months	F	Hc,Fx

C. Tg276 mice

S.No	Genotype	Age	Sex	Tissue
1	Tg2576	10-12 Months	M	Cb,Fx,Hc
2	Tg2576	10-12 Months	M	Cb,Fx,Hc
3	Tg2576	10-12 Months	F	Cb,Fx,Hc
4	Wild	10-12 Months	M	Cb,Fx,Hc
5	Wild	10-12 Months	M	Cb,Fx,Hc
6	Wild	10-12 Months	M	Cb,Fx,Hc
7	Wild	10-12 Months	F	Cb,Fx,Hc
8	Tg2576	4 Months	F	Cb,Fx,Hc
9	Tg2576	4 Months	M	Cb,Fx,Hc
10	Tg2576	4 Months	F	Cb,Fx,Hc
11	Tg2576	4 Months	M	Cb,Fx,Hc
12	Wild	4 Months	M	Cb,Fx,Hc
13	Wild	4 Months	M	Cb,Fx,Hc
14	Wild	4 Months	F	Cb,Fx,Hc
15	Wild	4 Months	F	Cb,Fx,Hc
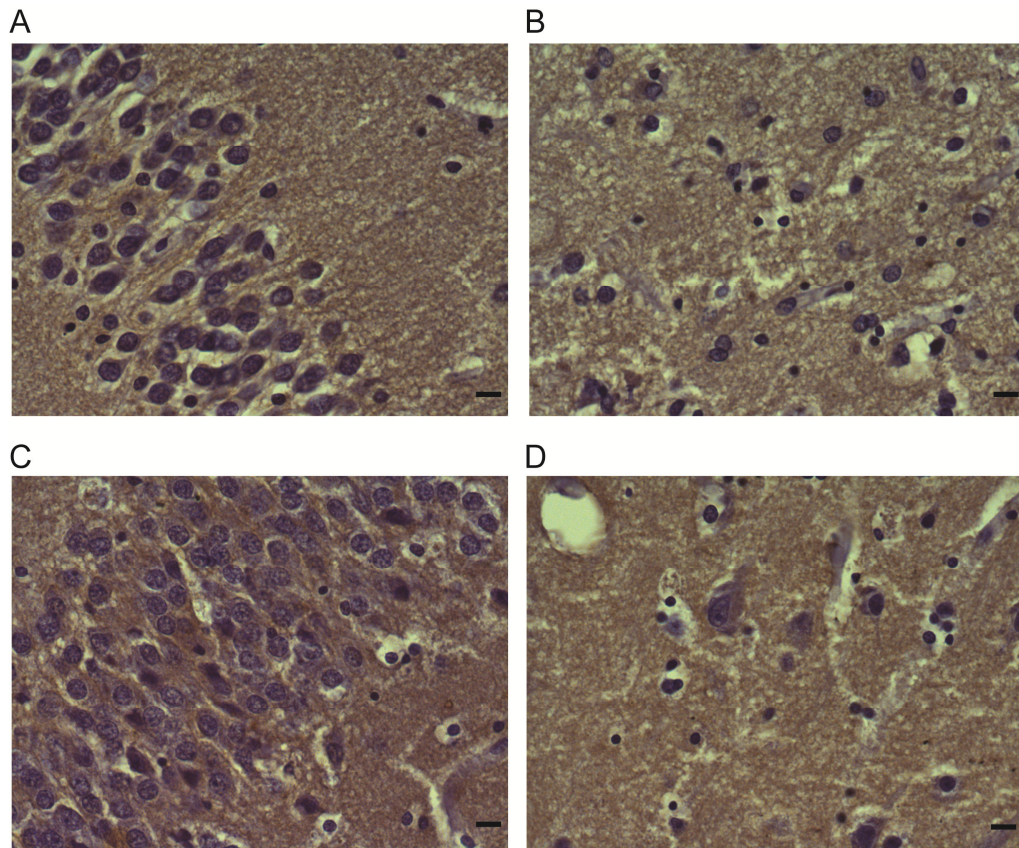
Figure S1. Decreased CSPα immunostaining in Alzheimer's disease hippocampus and STG. Fixed hippocampal sections from a patient with severe Alzheimer's disease **(A)** and a control subject**(C)** were probed with anti-CSPα antibodies for analysis of CSPα expression. Sections from the hippocampus with the granular cell layer show decreased immunoreactivity with a synaptic staining pattern in AD as compared to age-matched control case. A similar analysis was carried out with STG from a patient with severe Alzheimer's Disease **(B)** and a control subject **(D)**. In the STG there is decreased immunoreactivity in AD as compared to age-matched controls. CSPα expression in both the regions from the AD patient appeared lower than in the control subject. Haematoxylin counterstain. Original magnification**: x400**. Scale bars represent **20 μm**.
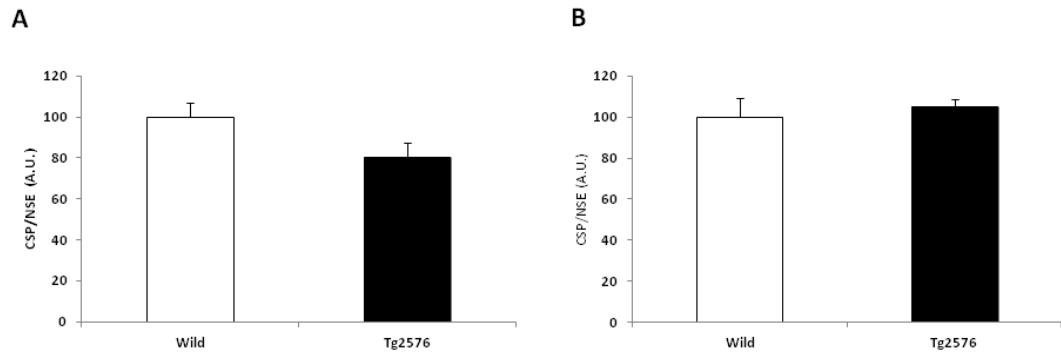
Figure S2. CSPα levels are not changed in Tg2576 mouse forebrain. (A) CSPα levels in 4 month old Tg2576 mouse forebrain, (B) CSPα levels in 12 month old Tg2576 mouse forebrain. The statistics from forebrain of Tg2576 , 4 month **(n=4)** showed no change compared to wild type **(n=4)** in CSP levels when normalized with NSE **(t=1.935 , p =0.101).** There was also no change in the CSP levels at 12 month **(wild,n=4;Tg2576,n=3)** stage in Tg2576 forebrain compared to control **(t= - 0.449 , p= 0.672).**

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Molecular Brain

Evidence that the presynaptic vesicle protein CSPalpha is a key player in synaptic degeneration and protection in Alzheimer's disease

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Abstract

Background: In Alzheimer's disease synapse loss precedes neuronal loss and correlates best with impaired memory formation. However, the mechanisms underlying synaptic degeneration in Alzheimer's disease are not well known. Further, it is unclear why synapses in AD cerebellum are protected from degeneration. Our recent work on the cyclin-dependent kinase 5 activator p25 suggested that expression of the multifunctional presynaptic molecule cysteine string protein alpha (CSPalpha) may be affected in Alzheimer's disease.

Results: Using western blots and immunohistochemistry, we found that CSPalpha expression is reduced in hippocampus and superior temporal gyrus in Alzheimer's disease. Reduced CSPalpha expression occurred before synaptophysin levels drop, suggesting that it contributes to the initial stages of synaptic degeneration. Surprisingly, we also found that CSPalpha expression is upregulated in cerebellum in Alzheimer's disease. This CSPalpha upregulation reached the same level as in young, healthy cerebellum. We tested the idea whether CSPalpha upregulation might be neuroprotective, using htau mice, a model of tauopathy that expresses the entire wild-type human tau gene in the absence of mouse tau. In htau mice CSPalpha expression was found to be elevated at times when neuronal loss did not occur.

Conclusion: Our findings provide evidence that the presynaptic vesicle protein CSPalpha is a key player in synaptic degeneration and protection in Alzheimer's disease. In the forebrain CSPalpha expression is reduced early in the disease and this may contribute to the initial stages of synaptic degeneration. In the cerebellum CSPalpha expression is upregulated to young, healthy levels and this may protect cerebellar synapses and neurons to survive. Accordingly, CSPalpha upregulation also occurs in a mouse model of tauopathy only at time when neuronal loss does not take place.

Keywords: Alzheimer's disease, Cerebellum, Cysteine string protein, Hippocampus, Synapses, Neuroprotection

Introduction

Alzheimer's disease is a devastating neurodegenerative condition and the most prominent cause of dementia. The neuropathological features of Alzheimer's disease are substantial neuronal death in the forebrain, but almost no neurodegeneration in the cerebellum [1,2]. In the forebrain extracellular amyloid plaques and intracellular neurofibrillary tangles are characteristic of Alzheimer's disease. Further, synaptic loss precedes neuronal loss and the former correlates best with early deficits in memory formation [3,4]. Our recent research provided a novel window into the mechanisms underlying synaptic degeneration in Alzheimer's disease [5]. We found that the truncated cyclin-dependent kinase 5 activator p25 is reduced in Alzheimer's disease [6]. Normally, p25 generation is linked to the synthesis of particular synaptic proteins, synaptogenesis and memory formation [6]. Thus, impaired p25 generation may cause early synaptic dysfunction in Alzheimer's disease. Furthermore, we demonstrated that p25 generation regulates expression of the

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Tiwari *et al. Molecular Brain* (2015) 8:6

Page 2 of 12

synaptic chaperone protein cysteine string protein (CSP) alpha [6]. CSPalpha is a synaptic vesicle protein that belongs to a conserved gene family [7,8] that includes CSPalpha, CSPbeta and CSPgamma of which only CSPalpha is expressed in the brain [9,10]. CSPalpha function is essential for synaptic survival as indicated in mouse knockout studies [10]. Furthermore, loss-of-function CSPalpha mutations are responsible for autosomal dominant Kufs disease, an adult-onset neurodegenerative disorder with associated dementia [11,12]. CSPalpha is proposed to serve various functions at the presynapse, including: 1) Formation of a trimeric complex with SGT and Hsc 70, resulting in a CSP/SGT/Hsc70 chaperone complex that is localised at synaptic vesicles [13] and interacts with SNARE proteins leading to calcium-triggered synaptic vesicle exocytosis [14,15]. 2) Modulation of presynaptic calcium levels by regulating the activity of presynaptic calcium channels [8,16]. 3) Regulation of endocytosis by facilitating dynamin 1 polymerization [17]. 4) Regulation of the density of calcium-dependent K$^+$ (BK) channel at the presynaptic terminal, controlling the excitability there [18,19].

Our finding that CSPalpha is a p25-regulated protein [6] suggested that CSPalpha expression may be impaired in Alzheimer's disease. Here we tested this hypothesis by examination of post-mortem human tissues. As expected, we found that CSPalpha expression is reduced in forebrain of early and late Alzheimer's disease. Interestingly, CSPalpha expression was reduced before synaptophysin levels drop, suggesting that it contributes to the initial stages of synaptic degeneration. Surprisingly, we discovered an upregulation of CSPalpha expression in Alzheimer's disease cerebellum, a brain area that is protected from synaptic degeneration. Further post-mortem investigations and work with a mouse model of tauopathy established a novel correlation between CSPalpha upregulation and neuroprotection.

Results

Specificity of anti-CSPalpha antibody

To study CSPalpha protein expression we performed western blots and immunohistochemistry with an anti-CSPalpha antibody, which does not react with other protein in CSPalpha knockout mice [10] (Figure 1).

CSPalpha expression is reduced in post-mortem Alzheimer's disease hippocampus

The hippocampus is one of the earliest and one of the most severely affected brain regions in Alzheimer's disease [20]. We studied whether CSPalpha protein expression is affected in this brain region, comparing post-mortem samples from severe Alzheimer's disease patients (Braak stages 5 and 6) and age-matched control subjects by western blot analysis. CSPalpha expression was normalized to either the neuronal marker NSE or the synaptic marker
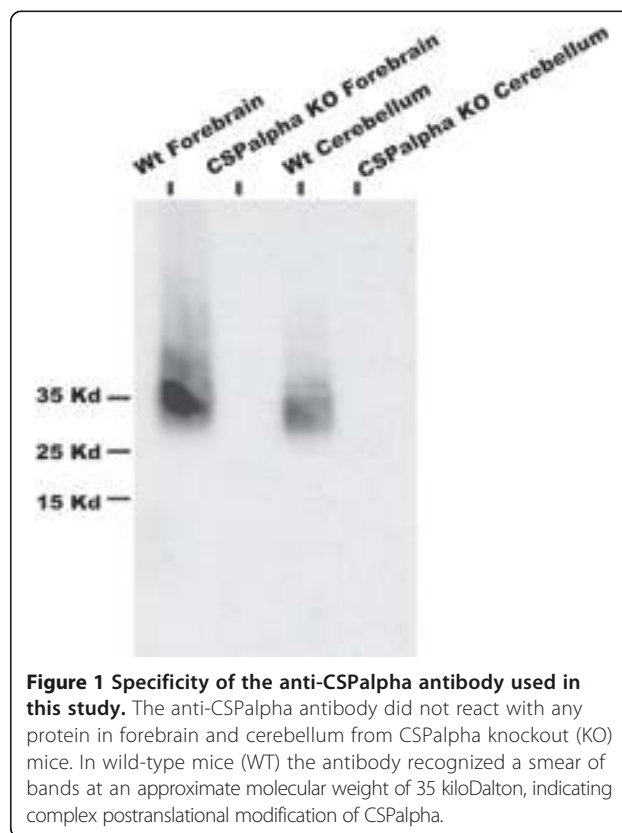

Figure 1 Specificity of the anti-CSPalpha antibody used in this study. The anti-CSPalpha antibody did not react with any protein in forebrain and cerebellum from CSPalpha knockout (KO) mice. In wild-type mice (WT) the antibody recognized a smear of bands at an approximate molecular weight of 35 kiloDalton, indicating complex postranslational modification of CSPalpha.

synaptophysin (Figure 2A, C). In both cases CSPalpha levels were significantly decreased in the hippocampus of severe Alzheimer's disease patients (referring to NSE expression: $F_{(2,21)} = 21.3$; $p < 0.01$; referring to synaptophysin expression: $F_{(2,19)} = 14.6$, $p < 0.05$). This result suggests that in severe Alzheimer's disease CSPalpha expression is not simply reduced as a result of neuronal or synaptic loss, and that reduced CSPalpha expression may precede synaptic loss during the progression of Alzheimer's disease.

Interestingly, we did not find that synaptopysin levels are reduced in Alzheimer's disease when normalized to NSE expression (Additional file 1: Figure S1). In contrast synaptophysin levels are reduced when absolute expression levels are considered (e.g., [21-23]). Therefore, neuronal loss in Alzheimer's disease appears to mainly contribute to reduced expression of synaptophysin, which can be corrected for when expression is normalized to NSE.

We also analysed CSPalpha expression in mild Alzheimer's disease (Braak stages 1 and 2) (Figure 2B, D). Analysis of western blot results revealed a significant decrease in CSPalpha levels in the mild Alzheimer's disease hippocampus when normalized with synaptophysin ($F_{(2,20} = 4.26$; $P < 0.05$), but showed no difference when normalized with NSE ($F_{(2,21} = 0.366$, $p = 0.427$). These results indicate that decreases in CSPalpha expression in the hippocampus is an early event in Alzheimer's disease.
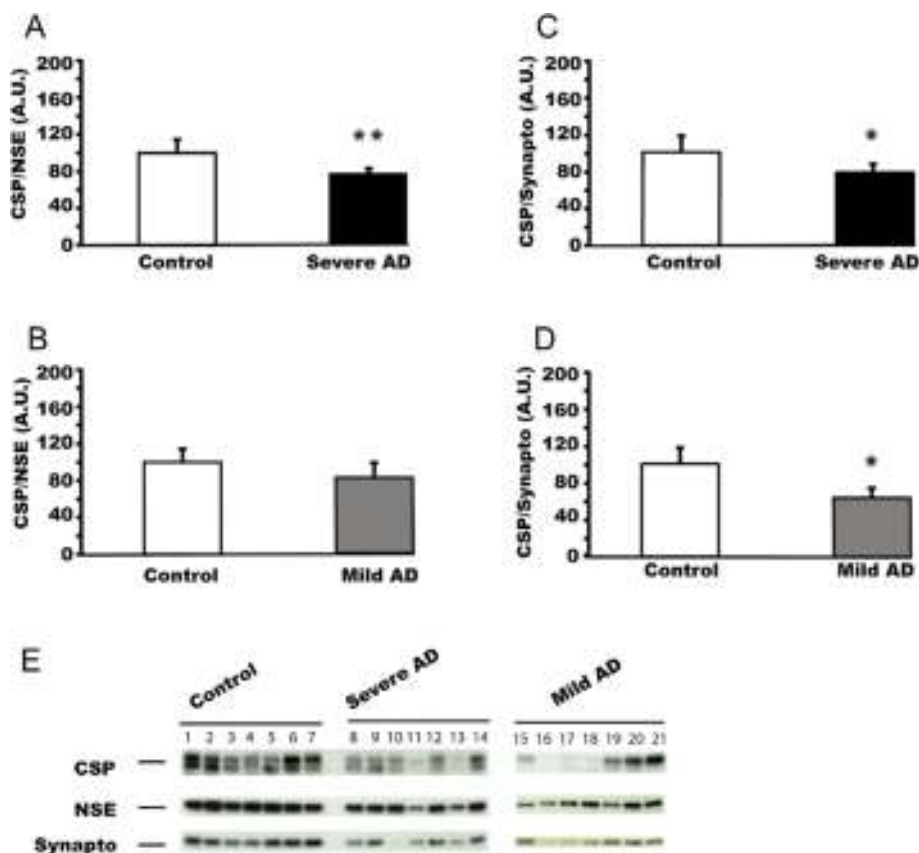
Tiwari *et al. Molecular Brain* (2015) 8:6

Page 3 of 12

Figure 2 CSPalpha protein expression is reduced in Alzheimer's disease hippocampus. (A) CSPalpha expression in post-mortem hippocampus from patients with severe Alzheimer's disease (Braak stages 5 and 6; n = 12; average age at death = 75.2 ± 2.0 years) and control subjects (n = 12; average age at death = 76.5 ± 2.9 years). CSPalpha expression was normalized against the neuron-specific house keeping marker protein NSE. **(B)** CSPalpha expression in post-mortem hippocampus from patients with mild Alzheimer's disease (Braak stages 1 and 2; n = 12; average age at death = 80.3 ± 3.2 years) and control subjects (n = 12). CSPalpha expression was normalized against NSE. **(C)** The same samples as in panel **(A)** were used but CSPalpha expression was normalized against the synaptic marker protein synaptophysin (severe Alzheimer's disease, n = 11; control, n = 11). **(D)** The same samples as in panel **(B)** were used but CSPalpha expression was normalized against synaptophysin (mild Alzheimer's disease, n = 12; control, n = 11). Panel **(E)** shows the representative western blots for 7 controls, 7 patients with severe AD and 7 patients with mild AD. Note that the anti-CSPalpha antibody recognizes two bands at an approximate molecular weight range of 35 kiloDalton, which are likely to represent distinct post-translational modifications of CSPalpha. Means ± s.e.m. are shown. *, p < 0.05; **, p < 0.01.

CSPalpha expression is reduced in Alzheimer's disease superior temporal gyrus

We next studied CSPalpha expression in the superior temporal gyrus (STG), which is affected later and less severely than the hippocampus in Alzheimer's disease. In a western blot analysis we compared CSPalpha expression in severe Alzheimer's disease and control STG. The level of CSPalpha expression was again normalised to NSE or synaptophysin (Figure 3). In both cases, there was a significant reduction in CSPalpha expression (referring to NSE expression: $F_{(2,22)}$ = 14.8; p < 0.01; referring to synaptophysin expression: $F_{(2,22)}$ = 7.48, p < 0.001), similar to that found in the hippocampus (Figure 2A, C). These results show that changes in CSP levels are not limited to the hippocampus in Alzheimer's disease brain, but are also found in other degenerating forebrain regions.

CSPalpha expression is increased in Alzheimer's disease cerebellum

The cerebellum is the least affected brain structure in Alzheimer's disease [1,2]. There is no synapse and neuronal loss in this brain region in this disease. We investigated the levels of CSPalpha expression in cerebellum from severe Alzheimer's disease and controls, using western blot analysis. As in the case of hippocampus, CSPalpha amounts were normalized against NSE and synaptophysin (Figure 4A, C). In both cases CSPalpha levels were significantly increased by about 50% (referring to NSE expression: $F_{(2,17)}$ = 2.76; p < 0.05; referring to synaptophysin expression: $F_{(2,17)}$ = 5.28, p < 0.01). We also analysed CSPalpha expression in mild Alzheimer's diseaseand control cerebellum (Figure 4B, D). We found that the level of CSPalpha expression was significantly increased in mild Alzheimer's disease cerebellum when
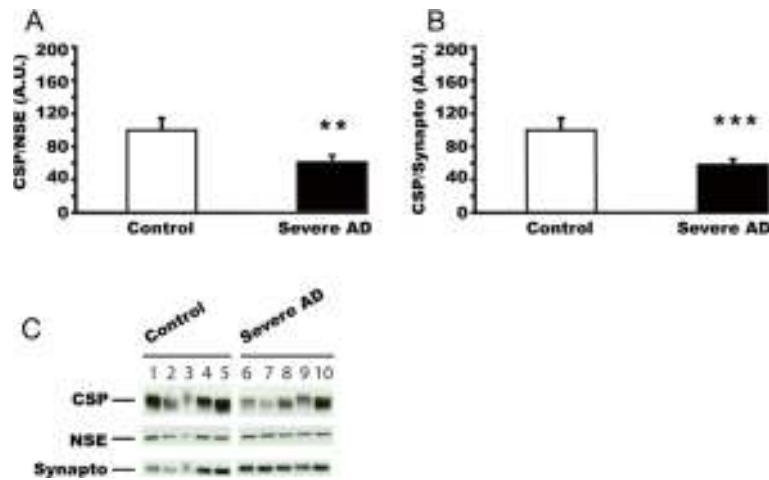
Tiwari *et al. Molecular Brain* (2015) 8:6

Page 4 of 12

Figure 3 CSPalpha protein expression is reduced in Alzheimer's disease superior temporal gyrus. (A) CSPalpha expression in post-mortem STG from patients with severe Alzheimer's disease (n = 13; average age at death = 73.2 ± 3.4 years) and control subjects (n = 12; average age at death = 76.9 ± 2.1 years). CSPalpha expression was normalized against NSE. **(B)** The same samples as in panel **(A)** were used but CSPalpha expression was normalized against synaptophysin (severe Alzheimer's disease, n = 13; control, n = 12). Panel **(C)** shows the representative western blots for 5 controls and 5 patients with severe AD. Means ± s.e.m. are shown. **, p < 0.01; ***, p < 0.001.
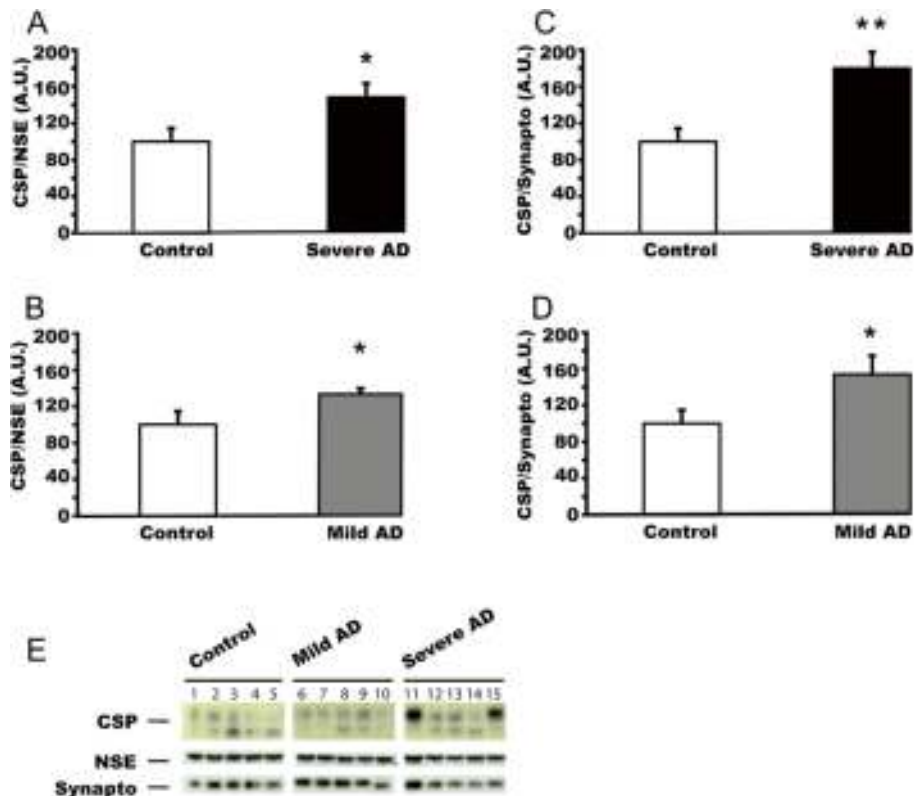

Figure 4 CSPalpha expression is increased in Alzheimer's disease cerebellum. (A) CSPalpha expression in post-mortem cerebellum from patients with severe Alzheimer's disease (n = 10; average age at death = 74.7 ± 4.0 years) and control subjects (n = 10; average age at death = 76.3 ± 4.2 years). CSPalpha expression was normalized against NSE. **(B)** CSPalpha expression in post-mortem cerebellum from patients with mild Alzheimer's disease (n = 10; average age at death = 81.3 ± 4.1 years) and control subjects (n = 10). CSPalpha expression was normalized against NSE. **(C)** The same samples as in panel **(A)** were used but CSPalpha expression was normalized against synaptophysin. **(D)** The same samples as in panel **(B)** were used but CSPalpha expression was normalized against synaptophysin. Panel **(E)** shows the representative western blots for 5 controls, 5 patients with mild AD and 5 patients with severe AD. Means ± s.e.m. are shown. *, p < 0.05; **, p < 0.01.

Tiwari *et al. Molecular Brain* (2015) 8:6

Page 5 of 12

normalized with both NSE and synaptophysin (referring to NSE expression: $F_{(2,17)} = 7.25$; $p < 0.05$; referring to synaptophysin expression: $F_{(2,17)} = 4.18$, $p < 0.05$). Therefore, we have observed reduced CSPalpha amounts in degenerating regions of early and severe Alzheimer's disease brain, and increased levels of CSPalpha in areas of Alzheimer's disease brain that are relatively spared from degeneration. These findings suggest that there may be a mechanistic link between CSPalpha expression levels and neuroprotection in Alzheimer's disease.

Immunohistochemical analysis confirms CSPalpha downregulation in forebrain and upregulation in cerebellum in Alzheimer's disease

To confirm the changes in CSPalpha protein amounts determined by western blotting, we carried out a qualitative immunohistochemical analysis with post-mortem cerebellum, hippocampus and STG from a severe Alzheimer's disease patients and a control subject (Figure 5; Additional file 2: Figure S4). A neuropathologist blinded to the disease state of the tissue performed a qualitative
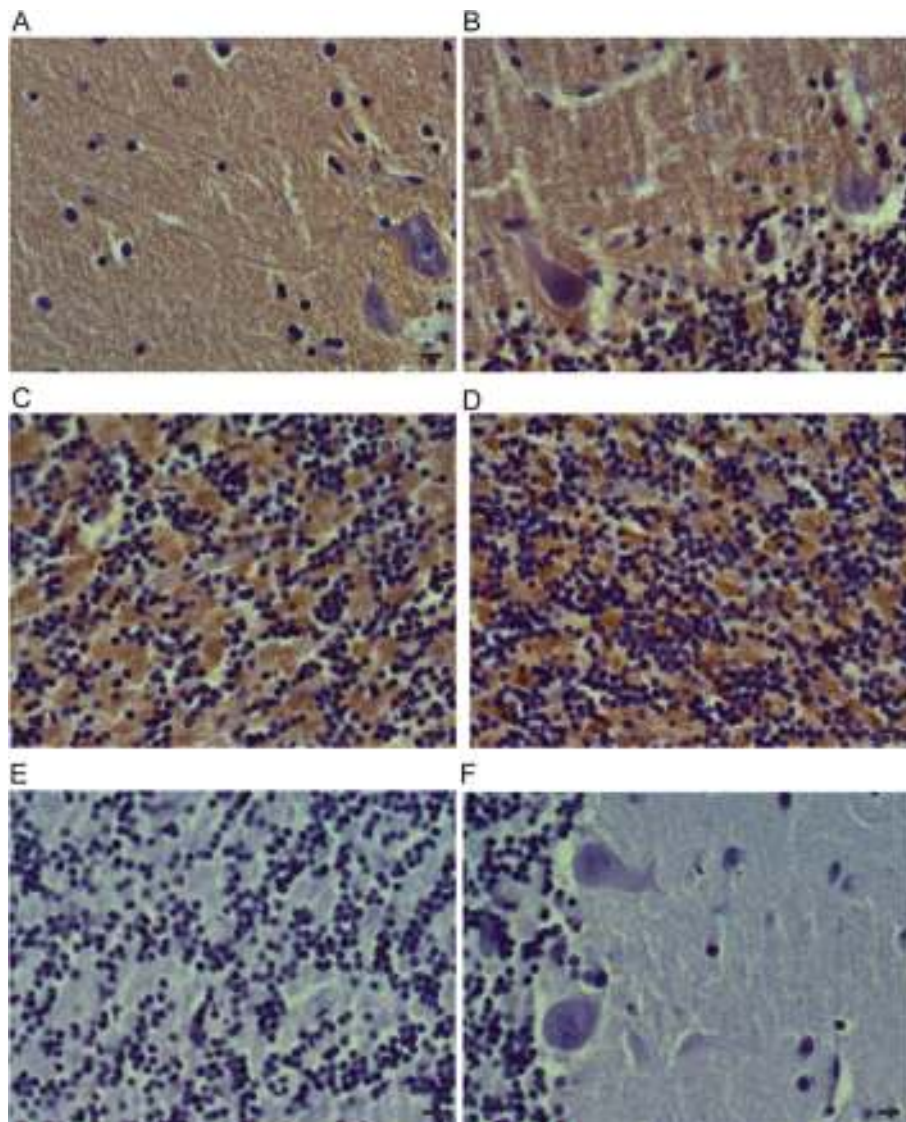

Figure 5 Increased CSPalpha immunostaining in Alzheimer's disease cerebellum. Fixed cerebellar cortex sections from a patient with severe Alzheimer's disease **(B, D)** and a control subject **(A, C)** were probed with anti-CSPalpha antibodies for analysis of CSPalpha expression. CSPalpha expression in the cerebellar cortex of the Alzheimer's disease patient appeared higher than in the control subject. In **A** and **B** cerebellar cortex with the Purkinje cell layer and granule cells are visible in the lower right corner. There is increased immunoreactivity in the neuropil in Alzheimer's disease as compared to an age-matched control case. In **C** and **D** the granule cells of the cerebellum show increased cytoplasmic and neuropil labeling in Alzheimer's disease as compared to an age-matched control. Negative control images are shown in **E** and **F** (immunohistochemistry with omission of the primary antibody) and confirm the specificity of labeling in Figure 5 and Additional file 1: Figure S1. Haematoxylin counterstaining is also shown. Original magnification: 400×. Scale bars represent 20 μm.

Tiwari *et al. Molecular Brain* (2015) 8:6

Page 6 of 12

comparison of CSPalpha expession. This comparison confirmed an increase in CSPalpha levels in cerebellar regions (Figure 5) and a decrease in hippocampus and STG in severe Alzheimer's disease compared to control (Additional file 2: Figure S4). To validate the synaptic specificity of the anti-CSPalpha antibodies, the immunohistochemistry was compared with synaptophysin expression patterns (Additional file 3: Figure S2). Staining of cerebellar dentate nucleus from a control subject showed similar results with both antibodies, confirming that the CSPalpha immunostaining was typical for synaptic staining.

CSPalpha expression is not changed in frontotemporal lobar degeneration (FTLD) cerebellum

Frontotemporal lobar degeneration (FTLD) is a prominent form of dementia that is characterized by neurodegeneration of the frontotemporal region [24]. In addition to frontal lobe degeneration, the cerebellum is also reported to be affected in FTLD cases [25]. This disparity provided an opportunity to test the specificity of our observation that CSPalpha is upregulated in Alzheimer's disease cerebellum. We performed a western blot analysis to study whether altered CSPalpha expression is linked to cerebellar neuropathology in FTLD (Figure 6). CSPalpha expression levels did not differ between FTLD

Figure 6 CSPalpha expression in cerebellum is not changed in FTLD. CSPalpha expression in post-mortem cerebellum from patients with FTLD (n = 5; average age at death = 73.0 ± 3.0 years) and control subjects (n = 5; average age at death = 70.2 ± 7.2 years).CSPalpha expression was normalized against NSE **(A)**. Panel **(B)** shows the representative western blots for 5 controls and 5 patients with FTLD. Means ± s.e.m. are shown.

and control cerebellum (t = − 0.373, p = 0.72). Hence, increased CSPalpha expression in the cerebellum is not a common feature of neurodegenerative disease, but appears to be specific for Alzheimer's disease.

Normal ageing reduces CSPalpha expression in human cerebellum

The difference in CSPalpha expression in the hippocampus and cerebellum of Alzheimer's disease brain suggests that CSPalpha expression may be differentially regulated under physiological and/or pathological conditions. Therefore, we studied whether normal ageing also regulates CSPalpha expression in cerebellum. Using western blots, we analysed CSPalpha protein expression in cerebellum from healthy subjects belonging to two age groups, 15–30 years (21.3 ± 1.6 years) and 90–105 years (96.1 ± 1.4 years) (Figure 7). CSPalpha expression was reduced by approximately 50% in the aged cerebellum when expression was normalized to NSE expression (t = 2.443; p < 0.05). Similarly, when normalized to synaptophysin levels the average CSPalpha expression in the aged cerebellum appeared lower than in young cerebellum, although this did not reach significance (t = 1.351; p = 0.23), most likely due to a large variability of CSPalpha amounts in young cerebellum. Taken together, our results suggest that CSPalpha amounts are subject to age-dependent decreases in healthy cerebellum. Interestingly, the CSPalpha expression level in Alzheimer's disease cerebellum is similar to the expression level found in young healthy cerebellum.
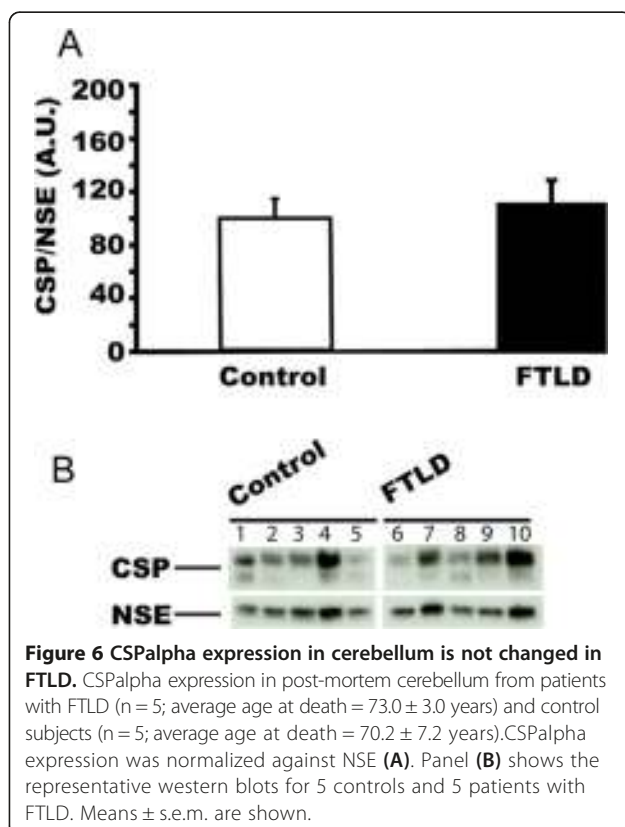
Overexpression of human tau leads to an age-dependent decline in hippocampal-cortical CSPalpha levels

Together with amyloid plaques, neurofibrillary tangles containing hyperphosphorylated tau aggregates are a pathological hallmark of Alzheimer's disease. We investigated the levels of CSPalpha in a mouse model of tauopathy that which expresses the entire wild-type human tau gene in the absence of mouse tau [26]. These mutants progressively develop hyperphosphorylated tau and form neurofibrillary tangles predominantly in the cortex and hippocampus. This model also has deficits in basal synaptic transmission, long-term potentiation and memory, and a widespread neuronal loss in old age [27,28]. The tau pathology is visible with biochemical analysis from 3 weeks of age, but neuronal loss is only apparent from 17 months of age onwards. We studied CSPalpha expression in the hippocampus/overlying cortex (Figure 8A, Additional file 4: Figure S3), frontal cortex (Figure 8B) and cerebellum (Figure 8C) of htau mutants and wild-type mice at 3–4 months of age. We found a significant upregulation of CSPalpha expression in hippocampus (Figure 8A, t = 6.539, p < 0.001), frontal cortex (Figure 8B, t = 8.005, p < 0.001) and cerebellum (Figure 8C, t = 5.200, p < 0.001) in 3–4 month-old htau

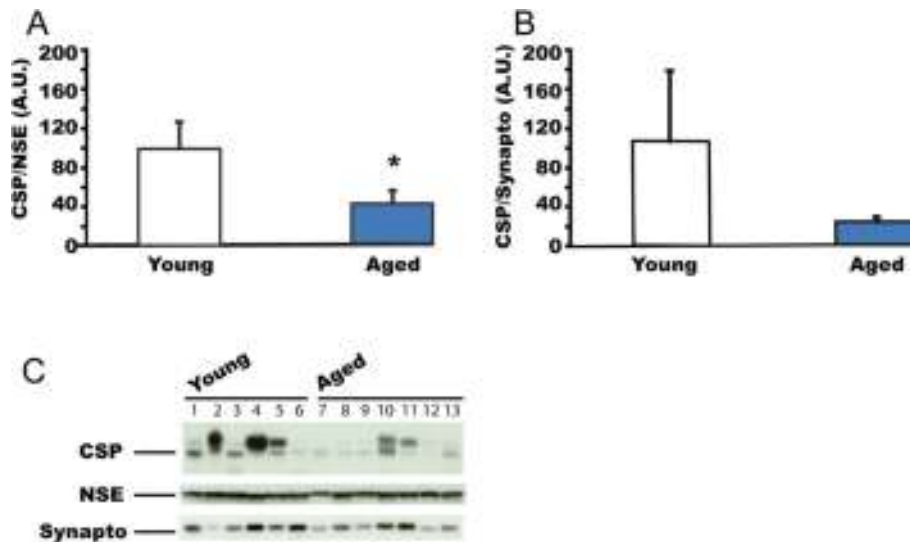Tiwari *et al. Molecular Brain* (2015) 8:6

Page 7 of 12

Figure 7 Normal ageing reduces CSPalpha expression in healthy human cerebellum. (A) CSPalpha expression in post-mortem cerebellum from healthy young (n = 6; average age at death = 21.3 ± 1.6 years) and aged (n = 7; average age at death = 96.1 ± 1.4 years) subjects. CSPalpha expression was normalized against NSE. **(B)** The same samples as in panel **(A)** were used but CSPalpha expression was normalized against synaptophysin. Panel **(C)** shows the representative western blot for 6 young subjects and 7 aged subjects. Means ± s.e.m. are shown. *, p < 0.05.
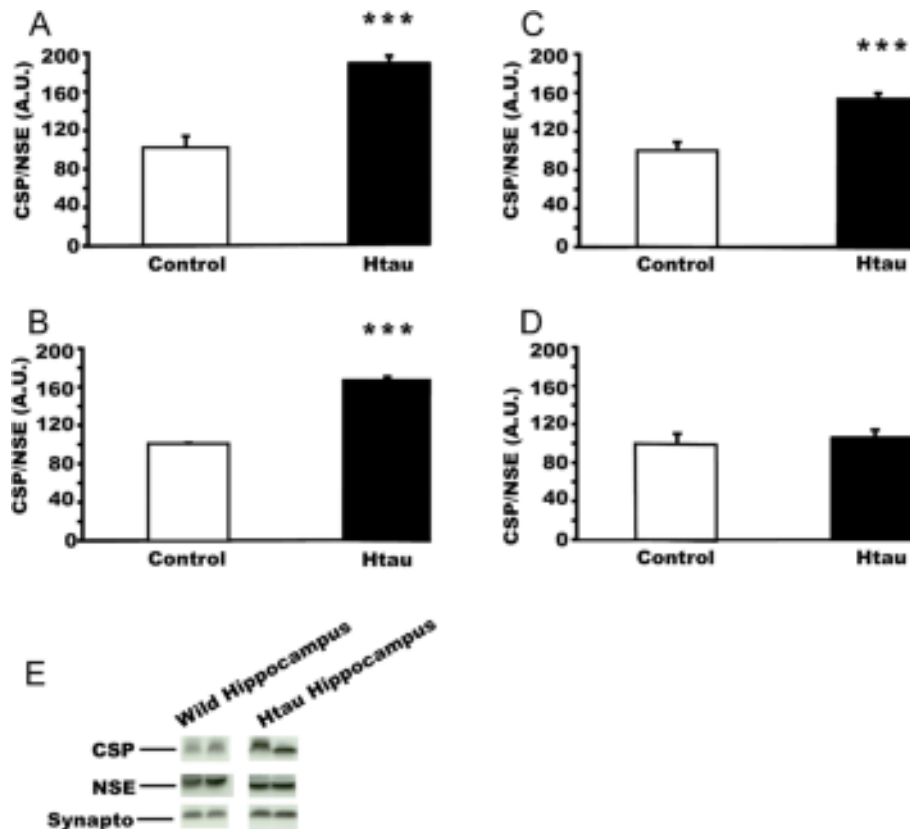

Figure 8 Mice expressing human tau have an age-dependent CSPalpha upregulation that correlates with neuroprotection. (A) CSPalpha expression in hippocampus of young (3–4 months) wild type (n = 6) and htau mice (n = 6). **(B)** CSPalpha expression in cortex of young (3–4 months) wild type (n = 6) and htau mice (n = 6). **(C)** CSPalpha expression in cerebellum of the same mouse sample as in panel **(A)** and **(B)**. **(D)** CSP alpha expression in old (24 months) wild type (n = 4) and htau mice (n = 7). CSPalpha expression was normalized against NSE in all panels. Panel **(E)** shows the representative western blots. Means ± s.e.m are shown. ***, p < 0.001.

Tiwari *et al. Molecular Brain* (2015) 8:6

Page 8 of 12

mice relative to controls. At this age there is no neuronal loss in htau mice, and therefore this findings is in agreement with the idea that increased CSPalpha expression may be a neuroprotective mechanism. We also investigated CSPalpha expression at 24 months of age in hippocampal/overlying cortex lysates prepared from htau and wild type mice (Figure 8D). At this age point no differences in the levels of CSPalpha in the hippocampus from wild type and htau mice was apparent (Figure 8D, t = −0.220, p =0.83). Thus, at a time point when neuronal loss is observed, CSPalpha expression is no longer elevated.

Discussion

The main findings of our study are that 1) expression of CSPalpha is reduced in degenerating forebrain in mild and severe Alzheimer's disease. This downregulation occurs before synaptophysin levels drop. 2) CSPalpha expression is upregulated in Alzheimer's disease cerebellum, a brain region protected from synaptic and neuronal loss in Alzheimer's disease. This upregulation is at a level that occurs in young healthy cerebellum. 3) CSPalpha expression is not upregulated in FTLD cerebellum where neuropathology occurs. 4) In a mouse model of tauopathy CSPalpha upregulation inversely correlates with neurodegeneration. Taken together, these findings provide evidence that CSPalpha is a critical player of synaptic degeneration and synaptic survival in Alzheimer's disease.

CSPalpha is a p25-regulated protein, and we have previously shown that p25 expression is downregulated in Alzheimer's disease forebrain [6]. In addition, loss-of-function CSPalpha mutations cause adult-onset Kufs disease that is associated with dementia [11,12]. We therefore speculated that CSPalpha expression could be altered in Alzheimer's disease. Here we confirm this idea. We found that CSPalpha expression is reduced in hippocampus and STG in severe Alzheimer's disease. In western blots we detected CSPalpha as two bands due to posttranslational modifications. The posttranslational modifications and the levels of CSPalpha appear variable within a given group. However, when normalized to NSE or synaptophysin and when outliers were excluded (see, Material and methods) significant differences in expression between groups were identified. Our finding that CSPalpha expression is reduced in AD hippocampus and STG is consistent with another study, which was published after we started our project, showing that in Brodmann area 9 of severe Alzheimer's disease CSPalpha expression is reduced by about 40% [29]. Furthermore, we also detected a downregulation of CSPalpha expression in hippocampus in mild Alzheimer's disease when CSPalpha amounts were normalized to the synaptic marker synaptophysin. Traditionally, synaptophysin is used as a neuropathological marker of synaptic degeneration in Alzheimer's disease [3]. However, our finding that CSPalpha levels are reduced

without noticeable changes in synaptophysin expression, when relative neuronal expression rather than absolute protein expression is analyzed. When considering the importance of CSPalpha for synaptic function [10], our findings suggest that reduced CSPalpha expression is likely to be involved in the initial stages of synaptic degeneration. Further, for investigating synaptic degeneration in Alzheimer's disease analysis CSPalpha expression appears more suitable than assessing synaptophysin expression.

CSPalpha, along with its interacting partners Hsc-70 and SGT, is involved in exocytotic mechanisms in presynaptic terminals that are mediated by its interactions with SNARE complexes [9]. Downregulation of CSPalpha may therefore lead to reductions in the number of synaptic vesicles binding at presynaptic membranes, thereby affecting synaptic activity. Further, CSPalpha is also important for endocytosis of synaptic vesicles. CSPalpha interacts with dynamin to facilitate the of dynamin polymerization which is important for endocytotic vesicle fission [17,29]. This is important for normal synaptic function since the number of synaptic vesicles readily available for exocytosis is reduced when there are defects in endocytotic fission [30]. This suggests that defects observed in exocytotic mechanisms in CSPalpha knockout mice could be explained by deficits in CSPalpha-dependent endocytotic mechanisms. Hence, CSPalpha downregulation could lead to loss of function at different stages of synaptic vesicular recycling to contribute to synaptic loss. Additionally, reduced CSPalpha expression is expected to increase BK channel density at synapses, which reduces excitability at presynaptic terminals [18,19]. BK channel activation has been reported to decrease basal synaptic transmission in hippocampal CA1 region of a mouse model of Alzheimer's disease [31]. Loss of synaptic activity is thought to be lethal for synapses, therefore, the downregulation in CSPalpha expression we observe in Alzheimer's disease hippocampus could be closely associated with synaptic degeneration and the resulting impaired memory formation in early Alzheimer's disease.

The second major finding from our study is the identification of CSPalpha upregulation in Alzheimer's disease cerebellum. The cerebellum is relatively protected from neurodegeneration in Alzheimer's disease. For example, there is no synaptic and neuronal loss in this area, although there are some diffuse amyloid plaques [1]. The molecular mechanisms that impart neuroprotection to the cerebellum in Alzheimer's disease are not known. Our results suggest that CSPalpha may be a factor contributing to this neuroprotection. We observed an upregulation of CSPalpha in cerebellum both in mild and severe Alzheimer's disease. Importantly, we found that the level of upregulation in this region is comparable to the amounts of CSPalpha expression detected in young,

Tiwari *et al. Molecular Brain* (2015) 8:6

Page 9 of 12

healthy cerebellum, in contrast to an age-dependent decrease in CSPalpha expression in normal cerebellum. Additional experimental support for the suggestion that CSPalpha upregulation could be neuroprotective in Alzheimer's disease comes from our finding that CSPalpha expression is not altered in cerebellum from patients with FTLD, although there is neuropathology in the cerebellum in this disease [25,32,33]. Additional support for our hypothesis comes from analysis of htau mutant mice, where we found that CSPalpha upregulation occurs only at times when no neuronal loss is observed. Taken together, the evidence we present here suggests that CSPalpha upregulation in Alzheimer's disease cerebellum might be neuroprotective, although in future functional studies in model systems are needed to support this idea.

How could CSPalpha upregulation protect synapses and consequently neurons in Alzheimer's disease cerebellum? In Alzheimer's disease forebrain, amyloid-induced aberrations in synaptic activity are one of the causes of synaptic toxicity [34]. In particular, dysfunctional synaptic machinery could be an after-effect of impaired synaptic vesicle trafficking. Aβ oligomers impair synaptic vesicle recycling by hindering endocytosis as well as the formation of fusion-competent vesicles [35]. Furthermore, transgenic mouse studies have suggested that presynaptic degeneration is pivotal in Alzheimer's disease [36]. Considering the role of CSPalpha in endocytosis and vesicle recycling, an up regulation of CSPalpha in Alzheimer's disease cerebellum could be a compensatory mechanism that prevents impairments in synaptic vesicle recycling that are induced by factors causing Alzheimer's disease. This might result in protection of synapses and neurons from degeneration. Functional studies with CSPalpha knockout and upregulation models will provide further insights into the mechanistic basis of our observations.

Conclusion

Synapse loss in forebrain, but not cerebellum, is a key hallmark of in Alzheimer's disease. However, the mechanisms causing brain region-dependent synapse loss and protection are unknown. Here we provide evidence that the presynaptic vesicle protein CSPalpha is a critical player in Alzheimer's disease. In the forebrain CSPalpha expression reduces in the initial stages of synaptic degeneration before synaptophysin levels drop. In cerebellum CSPalpha expression is upregulated both in mild and severe Alzheimer's disease. This upregulation of CSPalpha is to a level that occurs in young health cerebellum. In a mouse model of tauopathy we confirmed a lack of neuronal loss when CSPalpha expression is elevated. Taken together, these findings point to critical role for CSPalpha in synaptic degeneration and protection in Alzheimer's disease.

Material and methods

Post-mortem human brain samples

Brain tissues in 10% (v/v) formalin-fixed, paraffin-embedded tissue blocks and as frozen tissues were available from the Medical Research Council (MRC) London Neurodegenerative Diseases Brain Bank (Institute of Psychiatry, King's College London, UK). All tissue collection and processing was carried out under the regulations and licensing of the Human Tissue Authority and in accordance with the Human Tissue Act, 2004. Frozen samples were received in two sets for western blot analysis. The first set contained hippocampal tissue from control subjects, mild Alzheimer's disease (Braak stages 1–2) and severe Alzheimer's disease (Braak stages 5–6) [n = 7 for each group], as well as superior temporal gyrus (STG) samples from controls and severe Alzheimer's disease [n = 7 and n = 9, respectively]. The second set comprised hippocampus, STG and cerebellum samples from control, mild and severe Alzheimer's disease patients (n = 5 for each group). To increase the sample size of cerebellum, a new cohort (n = 5 per group) was later added to the analysis. Cerebellum samples (n = 5) were obtained from frontotemporal lobar degeneration (FTLD) patients. Cerebellum tissues were also obtained from healthy subjects less than 30 years old (n = 6) and older than 90 years (n = 7). Additional file 5: Table S1 summarizes the details.

Lysate preparation from human brain samples

Frozen brain samples were lysed at 4°C in RIPA lysis buffer (Santa Cruz Biotechnology, Inc., USA) consisting of 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.004% sodium azide in TBS (pH 7.5). Protease inhibitors cocktail, sodium orthovanadate and α-toluenesulphonyl fluoride in DMSO (all Santa Cruz Biotechnology, Inc., USA) were added to the buffer, diluted to 0.01%. The SDS concentration was increased to 0.25%. Approximately 100 mg of brain tissue was lysed in 300 μl buffer. Samples were homogenized using a dounce homogenizer (12 up and down strokes, 700 rotations per minute) at 4°C, and centrifuged at 3,000 rpm for 10 minutes at 4°C. Supernatants were immunoblotted and the bands from protein of interest were normalized with housekeeping proteins.

Mouse brain samples

Frontal cortex, cerebellum and hippocampus/overlying cortex was isolated from 3–4 month old human tau (htau) mice in the C57BL/6 J background (Jackson Laboratories, Bar Harbor, Maine USA; Stock number: 005 491). Mice were genotyped by PCR to confirm the presence of the human *MAPT* transgene and the mouse *Mapt null* background using primers for the *MAPT* gene (forward 5′-ACTTTGAACCAGGATGGCTGAGCCC-3′, reverse 5′-CTGTGCATGGCTGTCCCTACCTT-3′), and the mouse *Mapt* gene (forward 5′-CTCAGCATCCCACCTGTAAC-3′,

Tiwari *et al. Molecular Brain* (2015) 8:6

Page 10 of 12

reverse 5′-CCAGTTGTGTATGTCCACCC-3′), as described in [20]. The primers for the disrupted *Mapt* gene were: forward 5′-AAGTTCATCTGCACCACC G-3′, reverse 5′-TCCTTGAAGAAGATGGTG CG-3′.

Mice were housed on 12 h light:12 h dark cycles with food and water available *ad libitum*. Mice were killed by cervical dislocation and brain regions snap frozen on dry ice. All animal procedures were conducted in accordance with the UK Home Office, Animals Scientific Procedures Act 1986.

Lysate preparation from mouse brain samples
Frozen tissue was homogenised at 100 mg/ml in 2× sample buffer (0.5 M Tris–HCl, pH 6.8, 4.4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.01% bromophenol blue, and complete mini-protease inhibitor cocktail (Roche Products Ltd., UK). Following brief sonication, homogenates were centrifuged at 25,000 g for 20 minutes at 4°C, and the supernatant was collected.

Western blot analysis
The same protein amounts were separated on criterion TGX precast gels (4-15%) gels (BioRad) and the protein was transferred onto a methanol activated PVDF membrane (BioRad), using standard protocols. Non-specific binding was blocked by 5% non-fat dried milk in TBST for 1 hour at room temperature. Subsequently, membranes were incubated overnight at 4°C in primary antibody solution prepared in blocking buffer. After three ten minute washes in TBST at room temperature, membranes were incubated for two hours at room temperature with horse-radish peroxidase conjugated secondary antibodies in blocking buffer. After three ten minute washes with TBST, the membrane was incubated for 3 minutes in ECL reagent (Thermo Scientific) and then exposed to an X-ray film (Amersham) in the linear range. To probe the membranes with other primary antibodies, membranes were treated with a stripping buffer (Santa Cruz Biotechnology) for one hour at room temperature, followed by three washes with TBST of 10 minutes each and subsequent labelling as described above. Primary antibodies against CSPalpha (1:50,000, AB1576 Merck Millipore), synaptophysin (1:1000, 4329 Cell Signalling Technology) and neuron specific enolase (NSE) (1:60,000, AB 951 Merck Millipore) were used. Signals were analyzed using ImageJ software (NIH). With the antibodies against CSPalpha sometimes two bands were detected in human postmortem brain. These bands are not CSPbeta and CSPgamma since these proteins are not expressed in brain [10]. For standardization CSPalpha was normalized against NSE or synaptophysin.

Immunohistochemistry
Sections of human brain of 7 μm thickness were cut from paraffin-embedded tissue blocks. Sections were deparaffinised in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation of sections with 2.5% H_2O_2 in methanol. To enhance antigen retrieval sections were exposed to citrate buffer (2.94 g/L, pH 6.0) for 16 minutes microwave treatment (6 minutes high, two 5 minutes simmer). After blocking in normal swine serum (DAKO Ltd), primary antibodies against CSPalpha (1:500, AB1576 Merck Millipore), and synaptophysin (1:100, SY38 DAKO Ltd) were applied overnight at 4°C. Following rinsing and two five minutes washes in TBS, sections were incubated with appropriate biotinylated secondary antibodies (1:100, Swine anti-rabbit immunoglobulin/biotinylated, E0353 DAKO Ltd), followed by incubation with avidin:biotin enzyme complex (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK). Following washing, sections were incubated for 10–15 min with 0.5 mg/ml 3,3′-diaminobenzidine chromogen (Sigma-Aldrich Company Ltd, Dorset UK) in Tris-buffered saline (pH 7.6) containing 0.05% H_2O_2. Sections were counterstained with Harris's haematoxylin.

Statistical analysis
Un-paired t tests were used for comparison of data from samples tested in one western blot. – In some cases, the sample size was so large so that data from more than one western blot needed to be pooled. To allow pooling of data by linear regression, the following equation was used -

$$\left(\frac{CSP}{\underset{(\text{NSE, Synaptophysin})}{\text{Normalization Marker}}} \right)_i = \beta_0 + \beta_1 \times X_{1i} + \beta_2 \times X_{2i} + \varepsilon_i$$

Where X_{1i} is the categorical predictor coding for the group difference (e.g. Control versus Severe), and X_{2i} is the categorical predictor coding for the different experiments ("1^{st}cohort" versus "2^{nd}cohort").

This regression model allowed us to pool the CSPalpha score from two different set of samples by eliminating the contribution made by the difference in experimental conditions. This analysis was performed using SPSS (version 20), which provides the output as an ANOVA score. The contribution and the significance of the factor of interest (e.g. the disease pathology) to the overall significance is subsequently determined by the score from this output. The level of significance for the analysis was 0.05 and outliers were decided by using mean ± 4*SD as threshold. See Additional file 6 in supplementary information for analysis of post-mortem brain tissue.

Additional files

Additional file 1: Figure S1. Synaptophysin protein expression in reference to NSE expression is unchanged in Alzheimer's disease

Tiwari *et al. Molecular Brain* (2015) 8:6

Page 11 of 12

hippocampus. **(A)** Synaptophysin expression in post-mortem hippocampus from patients with severe AD (n = 12) and control subjects (n = 12) was normalized against the neuron specific house keeping marker protein NSE. **(B)** Synaptophysin expression in post-mortem hippocampus from patients with mild AD (n = 12) and control subjects (n = 12) was normalized against NSE. Means ± s.e.m. are shown. *, p < 0.05; **, p < 0.01.

Additional file 2: Figure S4. Decreased CSPalpha immunostaining in Alzheimer's disease hippocampus and STG. Fixed hippocampal sections from a patient with severe Alzheimer's disease **(D)** and an age-matched control subject **(C)** were probed with anti-CSPalpha antibodies for analysis of CSPalpha expression.Sections from the hippocampus with the granule cell layer show decreased immunoreactivity with a synaptic staining pattern in Alzheimer's disease as compared to the control case. A similar analysis was carried out for expression in superior temporal gyrus (STG) from a patient with severe Alzheimer's disease **(B)** and an age-matched control subject **(A)**. In the STG there is decreased immunoreactivity in Alzheimer's disease as compared to control. Haematoxylin counterstain is included in the analysis. Original magnification: ×400. Scale bars represent 20 μm.

Additional file 3: Figure S2. CSPalpha immunostaining in cerebellum is typical for synaptic expression. Immunohistochemical sections of cerebellar dentate nucleus region from a control patient were immunostained with anti-CSPalpha antibodies **(A)** and with antibodies against the synaptic marker synaptophysin **(B)**. This comparison indicated that the CSPalphaimmunostaining is synaptic as obtained for synaptophysin immunostaining. Scale bars represent 200 μm.

Additional file 4: Figure S3. Synaptophysin levels relative to NSE expression are unchanged in hTau mutant mouse hippocampus. Synaptophysin expression in hippocampus of young (3–4 months) wild type (n = 6) and hTau mice (n = 6) was normalized against NSE in all panels. Means ± s.e.m are shown.

Additional file 5: Table S1. Details of post-mortem brain tissues for western blots. PMD refers to post-mortem delay.

Additional file 6: Statistical analysis of the effect of age, gender and post mortem delay on post-mortem brain tissues used in this study.

Abbreviations
CSP: Cysteine String Protein; STG: Superior Temporal Gyrus; FTLD: Fronto Temporal Lobar Degeneration; NSE: Neuronal Specific Enolase.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SST, MD, CT, BNS, OE and WN performed the experiments. SST, MD, TH and KPG analyzed the data. SST, TH and KPG wrote the manuscript. SST and KPG designed and coordinated the study. SST, MD, CT, BNS, OE, WN, TH and KPG reviewed, edited and approved the manuscript.

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Tiwari *et al. Molecular Brain* (2015) 8:6

Page 12 of 12

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