The Amyloid-beta-dependent phosphorylation of CRMP-2 and its contribution in Alzheimer's disease

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DEDICATION

I dedicate my dissertation work to my brother, Ibrahim, who has left his studies in Saudi Arabia to help me complete this achievement. Ibrahim has been a constant source of support during all the challenges I faced in this degree

and in my life as a whole. I am sincerely thankful for having you in my life you are my best cheerleader. This work is also dedicated to my parents, Hussain and Susan, who have been a source of unconditional love and whose amazing examples have taught me to work hard for all things I hope to achieve.

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GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy (PhD) regulation the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degrees or diplomas at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer-reviewed journals. The core theme of the thesis is "The elucidation of how the amyloid-beta protein induced phosphorylation of CRMP-2 contributes to the dysfunction of neurites in Alzheimer's disease". The writing up of all the papers in the thesis was the principal responsibility of myself, the candidate, working within the Central Clinical School under the supervision of Dr. Steven Petratos

In the case of Chapter 1, 2, 3, 4, 5 and 6 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
1	The Beta-Amyloid Protein of Alzheimer's Disease: Communication Breakdown by Modifying the Neuronal Cytoskeleton	Published	Key ideas and writing of article
2, 3,4,5 and 6	The Beta-Amyloid Dependent Phosphorylation of CRMP-2 Dissociates Kinesin in Alzheimer's Disease	Submitted	Conducting experiments and writing of manuscript

Signed: 

Date: 19/10/2015

ABBREVIATIONS

Aβ	Amyloid beta
oAβ	Oligomeric amyloid beta
fAβ	Fibrillar amyloid beta
AD	Alzheimer's disease
ADF	Actin-depolymerising factor
AICD	APP intracellular domain
Aph-1	Anterior pharynx-defective-1
APP	Amyloid precursor protein
βCTF	β C-terminal fragment
BACE1	Beta-site AβPP-cleaving enzyme
BDNF	Brain-derived neurotrophic factor
CAA	Cerebral amyloid angiopathy
Cdk5	Cyclin-dependent kinase-5
ChEIs	Cholinesterase inhibitors
CK2	Casein kinase 2
CNS	Central nervous system
CRMP	Collapsin reponse mediator protein
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
DTI	Diffusion tensor imaging

EEG	Electroencephalography
EOFAD	Early-onset familial AD
FA	Fractional anisotropy
FAT	Fast axonal transport
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDD	Familial Danish dementia
FTD	Fronto-Temporal Dementia
GSK-3β	Glycogen synthase kinase-3β
HD	Huntington's disease
IDE	Insulin degrading enzyme
IVIGs	Intravenous Igs
JNK3	Jun amino-terminal kinase 3
KHC	Kinesin heavy chain
KI	Knockin
KLC	Kinesin light chain
KLC1vE	Kinesin light chain-1 splice variant E
KO	Knockout
LIMK	LIM kinase
LOFAD	Late-onset familial AD
LPA	Lysophosphatidic acid
LRP1	Lipoprotein receptor-related protein
mAbs	Monoclonal antibodies

MAG	Myelin-associated glycoprotein
MAPs	Microtubule-associated proteins
MBOs	Membrane-bound organelles
MCI	Mild cognitive impairment
MEG	Magnetoencephalography
MLCK	Myosin light chain kinase
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MT	Microtubules
MTC	Methylene blue chloride
NCT	Nicastrin
NF	Neurofilament
NFTs	Neurofibrillary tangles
NgR	Nogo receptor
NMDAR	N-methyl-D-aspartate receptor
NNDC	Non neurological disease control
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Okadaic acid
OCT	Optimal Cutting Temperature compound
PAD	Phosphatase-activating domain
PAK	p21-activated kinase
PBS	Phosphate-buffered saline
PD	Parkinson's disease

PEN-2	Presenilin enhancer-2
PFA	Paraformaldehyde
PHFs	Paired helical filaments
PiB-PET	PET Pittsburgh compound B
PKC	Protein Kinase C
PME-1	Phosphatase methylesterase-1
PP1	Protein phosphatase 1
PrP	Prion protein
PS	Presenilin
pS518 CRMP-2	Phospho-Serine518 Collapsin reponse mediator protein
pS522 CRMP-2	Phospho-Serine522 Collapsin reponse mediator protein
pT509 CRMP-2	Phospho-Threonine509 Collapsin reponse mediator protein
pT555 CRMP-2	Phospho-Threonine555 Collapsin reponse mediator protein
pT514 CRMP-2	Phospho-Threonine514 Collapsin reponse mediator protein
PVDF	Polyvinylidene fluoride membranes
RA	Retinoic acid
RGMa	Repulsive guidance molecule A
RIPA	Radio-immunoprecipitation
ROCK	Rho kinase
RTN	Reticulon family
SCAs	Spinal Cerebellar Ataxias
scFv	Single-chain variable fragment
Sema3A	Semaphorin-3A

SVZ	Subventricular zone
TBST	Tris-Buffered Saline and Tween
TPRs	Tetratricopeptide repeats
TrkB	Tyrosine kinase
TTBK1	Tau-tubulin kinase-1
VZ	Ventricular zone

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LIST OF PUBLICATIONS AND PRESENTATIONS

Publications

1. The Beta-Amyloid Protein of Alzheimer's Disease: Communication Breakdown by Modifying the Neuronal Cytoskeleton, Sara H. Mokhtar, Maha M. Bakhuraysah, David S. Cram, and Steven Petratos, 2013, *Internationl Journal of Alzheimer's Disease*

Abstract: Alzheimer's disease (AD) is one of the most prevalent severe neurological disorders afflicting our aged population. Cognitive decline, a major symptom exhibited by AD patients, is associated with neuritic dystrophy, a degenerative growth state of neurites. The molecular mechanisms governing neuritic dystrophy remain unclear. Mounting evidence indicates that the AD-causative agent, β-amyloid protein (Aβ), induces neuritic dystrophy. Indeed, neuritic dystrophy is commonly found decorating Aβ-rich amyloid plaques (APs) in the AD brain. Furthermore, disruption and degeneration of the neuronal microtubule system in neurons forming dystrophic neurites may occur as a consequence of Aβ-mediated downstream signaling. This review defines potential molecular pathways, which may be modulated subsequent to Aβ-dependent interactions with the neuronal membrane as a consequence of increasing amyloid burden in the brain.

2. The Amyloid-beta-dependent phosphorylation of CRMP-2 dissociates kinesin in Alzheimer's disease, Sara H Mokhtar, Maha M Bakhuraysah, Pei Mun Aui, Kylie A Magee, Amani A Alrehaili, David L Steer, Rachel Kenny, Steven Petratos, 2015, *Journal of Neurochemistry*

Abstract: Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the accumulation of amyloid plaques and neurofibrillary tangles. Before the development of these two characteristic features, impairments in anterograde axonal transport develop. However, molecules that initiate these impairments are still unknown. Collapsin response mediator protein-2 (CRMP-2) plays an integral role in kinesin-1-dependent axonal transport and there is evidence that phosphorylation of CRMP-2 releases kinesin-1. However, the molecular trigger regulating CRMP-2 phosphorylation is not known. Here, we tested the hypothesis that amyloid beta (Ab)-dependent phosphorylation of CRMP-2 regulates disruption from the kinesin-1 axonal transport motor protein in Alzheimer's disease. We found enhanced Ab-dependent phosphorylation of CRMP-2 at the T555 site and reduced CRMP-2 association with kinesin-1, while the overexpression of an unphosphorylatable form of CRMP-2 in neurons promoted the re-establishment of CRMP-2-kinesin association and axon elongation. Additionally, in the transgenic Tg2576 mouse model of familial AD (FAD), that carries the Swedish mutation in amyloid precursor protein (APP) enhancing Ab overproduction, we found substantial staining with pT555CRMP-2 and axonal dystrophy. Consistent with these findings, brain lysates from AD patients demonstrated phosphorylation of CRMP-2 at T555 site and dissociation of CRMP-2 from kinesin-1. These data suggest that Aβ-dependent phosphorylation of CRMP-2 at the T555 site may directly impair anterograde axonal transport and is sufficient to lead to axonal defects.

3. Nogo-receptor 1 expression on B-cell populations in the central nervous system during experimental autoimmune encephalomyelitis, Maha Bakhuraysah, Amani Alrehaili, Sara Mokhtar, Jae Lee, Pei Mun Aui, Steven Petratos, *Journal of Neurochemistry*

Abstract: Although the fact that deletion of Nogo receptor 1 (NgR1) can protect against axonal degeneration and thus progression of disease, in the animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), the immunological role of this receptor is unclear. To further understand the function of NgR1 in regulating immune cells, flow cytometry-based phenotypic analysis was performed on isolates from spleens, lymph nodes and spinal cords at different clinically defined stages of EAE disease. The Central nervous system (CNS)-infiltrating blood cells revealed an augmented response in the B-cell population, which expressed NgR, seeing in ngr1+/+ mice with the onset and progression of the disease. This population of cells could not be demonstrated within the spinal cords of EAE-induced ngr1-/- mice or during the chronic stage of disease in ngr1+/+ mice. At the onset of disease onset, there was a significant increased in IgM-B-cells-expressing NgR in the spinal cord, when compared with the IgD populations. Remarkably, there was a cluster of B-cells expressing NgR present at the meninges of the spinal cords of ngr1+/+ EAE-induced mice at clinical score 1.5 and these cells localised within small follicles in submeningeal regions. Furthermore, there was clustering of B-cell activating factor (BAFF) and NgR-positive immune cell infiltrates within the spinal cords of EAE-induced ngr1+/+ mice at disease onset. Collectively, these data indicate that there exists the inducible expression of NgR1 in specific immune lineage cells upon the induction of EAE, as well as, a strong correlation between the expression profiles of NgR1 and BAFF on neighbouring B-cells within spinal cord follicular structures.

4. Does nogo-receptor 1 (NGR1) play a role in microglial activity within neuroinflammatory lesions? Amani A Alrehaili, Maha M Bakhuraysah, Jae Lee, Sara H Mokhtar, Pei Mun Aui, Kylie A Magee, Steven Petratos, 2015, *Journal of Neurochemistry*

Abstract: Multiple sclerosis (MS) is a neurodegenerative disease comprising of axonal damage and demyelination as the main pathological hallmarks. As the disease progresses, substantial central nervous system (CNS) degeneration can be correlated histopathologically with amoeboid microglia and numerous studies report this activity as central to the disease course of MS. In this study we focused on identifying whether there exists a role for nogo-receptor (NgR) in regulating microglial activity during experimental autoimmune encephalomyelitis (EAE). Immunohistochemistry was initially utilised to identify and enumerate microglial cells, which populate lesion and non-lesion areas of the spinal cord following EAEinduction. EAE spinal cord tissues were immunostained using two specific markers to these reactive cells, which are the anti-CD11b and anti-Iba-1 antibodies, along with an anti-NgR antibody to localise any co-labelled cells. In addition, we mapped the populations of activated microglia that were expressing NgR in spinal cord grey and white matter to illustrate their relevance during the disease progression. Immunopanning was subsequently utilised to isolate spinal cord microglia from ngr-1-/- and ngr-1+/+ mice following EAE induction, performed by using Iba-1 (lectin) as the capture antibody and the population of these cells were examined by flow cytometry. Based upon both immunohistochemistry and flow cytometry analysis, we detected similar numbers of NgR positive microglial cells in our ngr-1-/- mice both with and without EAE induction; thus, we suggest that there may not be a direct signalling role for NgR1 in the activation of microglial cells during the neuroinflammatory and neurodegenerative processes. However, increases in the

number of NgR-positive microglial cells within the EAE progression suggest that other NgR homologues (NgR2 or/and NgR3) produce a response in the chronic stage of disease observed in ngr-1+/+ mice. We now aim to define the type and mechanism of NgR being expressed and activated in these microglial cells.

Oral presentations

1. The Amyloid-beta-dependent phosphorylation of CRMP-2 dissociates kinesin in Alzheimer's disease, Sara H Mokhtar, Maha M Bakhuraysah, Pei Mun Aui, Kylie A Magee, Amani A Alrehaili, David L Steer, Rachel Kenny, Catriona McLean, Michael F. Azari, Antonis Birpanagos, Speros Efthimiopoulos, Steven Petratos, 2015, *Alzheimer's Association Internation Conference,* Washington, D.C, USA

Poster presentations

1. The Beta-amyloid protein-induced phosphorylation of CRMP-2 at Thr555 site inhibits neurite outgrowth in Alzheimer's patients, Mokhtar S.H., Aui P.M., Magee K, Petratos S, 2013, *Australian Neuroscience Society*, Melbourne, Australia
2. The Beta-amyloid protein-induced phosphorylation of CRMP-2 at Thr555 site inhibits neurite outgrowth and axonal transport in Alzheimer's disease, Mokhtar S.H., Aui P.M., Magee K, Petratos S, 2014, *Australian Neuroscience Society*, Adelaide, Australia
3. The Amyloid-beta-dependent phosphorylation of CRMP-2 dissociates kinesin in Alzheimer's disease, Sara H Mokhtar, Maha M Bakhuraysah, Pei Mun Aui, Kylie A Magee, Amani A Alrehaili, David L Steer, Rachel Kenny, Catriona McLean, Michael F. Azari, Antonis Birpanagos, Speros Efthimiopoulos, Steven Petratos, 2015, *Australian Neuroscience Society*, Cairns, Australia

LIST OF AWARDS

1. Golden Key International Award, 2011, Golden Key International Honour Society
2. Most Outstanding Oral Presentation Award at the Department of Anatomy and Developmental Biology Postgraduate Student Symposium, 2013, Monash University, Australia
3. Winner of Three Minute Thesis Competition at the Department of Anatomy and Developmental Biology, 2013, Monash University, Australia
4. Winner of Three Minute Thesis Competition at the Central Clinical School, 2014, Monash University, Australia

CHAPTER 1: Introduction

DECLARATION FOR THESIS CHAPTER 1

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Key ideas and writing of article	70%

The following co-authors contributed to the work. The extent of co-authors' contribution is indicated in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Maha Bakhuraysah	Drawing figures	2%
David Cram	Critical examination of article	
Steven Petratos	Critical examination of article	

Candidate's signature

	Date
[redacted]	19/10/2015

Declaration by co-authors

The undersigned hereby certify that:

1) The above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2) They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise.
3) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication.
4) There are no other authors of the publication according to these criteria.
5) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit.
6) The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)

Central Clinical School, Monash University, Prahran

Co-authors signature

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Steven Petratos	[redacted]	

1.1 Introduction

Several neurodegenerative disorders share common characteristics including aggregation of misfolded mutant proteins in neurons leading to their deafferentation or loss with resultant structural or functional deficits in specific regions of the central nervous system (CNS) (Kozlowski et al., 2012). The most prevelant symptoms of age-related neurodegenerative disease are cognitive decline and movement disorders, along with brainstem and cerebellar signs. Such age-dependent disorders include Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD) and Spinal Cerebellar Ataxias (SCAs) (Kozlowski et al., 2012). There exists complexity in identifying fundamental molecular mechanisms precipitating neurodegeneration in these age-related brain diseases. However, common molecular signalling pathways have been defined in the specific neuronal populations associated with pathology (Kumar et al., 2012). Although the initiators of neuronal dysfunction may differ for each neurodegenerative disorder, there may be common molecular pathways which when dysregulated, drive and exacerbate neurodegeneration. For example, the degeneration seen in AD is a result of amyloid plaques and phosphorylated tau deposition in the cerebral cortex and specific subcortical regions, leading to degeneration in the temporal lobe and parietal lobe, along with parts of the frontal cortex and cingulate gyrus (Kumar et al., 2012). AD also displays dysregulation in kinase and phosphatase mechanisms along with microtubule motor proteins during the degeneration phase (Liu and Wang, 2009, Crews et al., 2010). Therefore, a major question that remains unresolved is whether the dysregulation in specific kinases/phosphatases and vesicular transport mechanisms are aetiological contributors to AD pathology. This literature review is discussing the normal brain development, anatomy and function versus the abnormal pathway. It will then discuss some of the neurodegenerative diseases focusing mainly on

Alzheimer's disease and its neuropathology. This will be followed by discussing the signalling molecules that are well known to play a major role in Alzheimer's disease and finishing off with the molecules that are well demonstrated to be affected during Alzhiemer's pathology.

1.2 Brain development

Different techniques have developed our understanding of brain development, anatomy and function. These techniques include magnetic resonance imaging (MRI), electroencephalography (EEG), magnetoencephalography (MEG) and physical sectioning (Coward, 2013). Human brain development is an extended process that begins in the third week of gestation and continues on until adulthood arguably throughout the lifespan (Stiles, 2008, Morange, 2001, Waddington, 1939). By the end of the embryonic development the basic structures of the central and peripheral nervous systems are determined. During foetal development, which extends until the end of gestation, there is rapid growth and expansion of the cortical and subcortical structures (for review see ((Stiles and Jernigan, 2010)). MRI studies have found that brain development continues throughout childhood and that different neural structures undergo different maturation pathways (Gogtay et al., 2004, Lebel et al., 2008, Westlye et al., 2010). Before the introduction of MRI, it was thought that biological development of the brain was complete by six years of age. However, MRI studies show major changes in the developing brain tissues during and after the postnatal brain growth spurt. Some of these changes include the rate of oligodendrocytal precursor cell proliferation and eventual deposition of myelin (Barkovich, 2000, Barkovich, 2005), grey matter volume in the cerebral cortex and in subcortical nuclei, which has been found to be larger in school-aged children than in young adults (Jernigan and Tallal, 1990, Jernigan et

al., 1991), the appearance of the brains at autopsy, the size of the cranial vault (Dekaban, 1978), the synaptic density in cortex, which has been found to be reduced throughout childhood (Huttenlocher and Dabholkar, 1997), neuronal connections, which in the early postnatal period far exceed that of adults (For review see (Innocenti and Price, 2005)) and the continuous age-related decrease in the volume of frontal cortex, nucleus accumbens, thalamus, and neurites across the lifespan. Therefore, tissue changes related to brain maturation are expanded during childhood, but some of these developmental alterations may undergo regression and involve tissue loss.

During the early gastrulation period, the cells of the epiblast layer start migrating to the primitive streak passing through the primitive node, which is located at the rostral end of the primitive streak, and differentiate into neural progenitor cells. While migrating, neural progenitors receive signals from the primitive node specifying their regional identity (For review see (Stiles and Jernigan, 2010)). The area containing the neural progenitor cells is known as the neural plate. Around the third week of gestation, two ridges appear to form along the two sides of the neural plate and then the ridges rise, fold inward and fuse to form the neural tube (For review see (Copp et al., 2003)). Fusion begins at the center of the neural tube and then continues in the rostral and caudal directions. Following the fusion of the neural tube, the neural progenitor cells form a single layer that lines the center, defined as the ventricular zone (VZ) (For review see (Stiles and Jernigan, 2010)). The progenitor cells in the rostral region of the neural tube forms the brain, while cells in the caudal region forms the hindbrain and the spinal cord. Before neural tube closure, the anterior end of the tube expands forming the three primary brain vesicles: the prosencephalon which becomes the forebrain,; the mesencephalon which becomes the midbrain;

and the rhombencephalon which forms the hindbrain. These three segments further subdivide forming the five secondary brain by the end of the embryonic period. Anatomically these are defined as the telencephalon, diencephalon, metencephalon, myelencephalon and mesencephalon, which together constitute the primary organization of the central nervous system. Foetal development extends from the ninth gestational week until the end of gestation. Throughout this time, the brain develops from a smooth "lissencephalic" structure to the typical mature pattern of gyral and sulcal folding. The first fissure to form is the one that separates the two hemispheres (For review see (Stiles and Jernigan, 2010)).

In humans, production of neurons starts at embryonic day 42 (For review see (Bystron et al., 2008)). The majority of cortical neurons are generated and migrate to their positions within the layers of the neocortex beginning to form networks by midgestation or early foetal development. Brain areas containing neuronal cell bodies look grey, hence the name. Different populations of neurons form grey matter structures in several area in the brain such as cerebellum, neocortex, midbrain structures, the hindbrain and spinal column, and deep subcortical nuclei (For review see (Stiles and Jernigan, 2010)). After their production, neurons that will form the neocortex migrate from the VZ forming the six-layered neocortical mantel anf differentiating to generate neurotransmitters and neurotrophic factors. This eventually extends the dendritic and axonal processes, making up the brain white matter (For review see (Stiles and Jernigan, 2010)).

Brain development also continues postnatally, and the brain increases fourfold in size during the preschool period (19-33 months of age), reaching approximately 90% of the adult volume by age six (Reiss et al., 1996, Iwasaki et al., 1997, Courchesne et al., 2000). However, neurogenesis

continues postnatally to only a limited degree apart from the subventricular zone (SVZ), from which new neurons develop and migrate to the olfactory bulb (For review see (Stiles and Jernigan, 2010)). Neurons are also produced in the dentate gyrus of the hippocampus then migrate from the subgranular layer to the nearby granular layer. These forms of neurogenesis are exceptional and appear to produce a small percentage of neurons throughout adult life (For review see (Stiles and Jernigan, 2010)). On the other hand, proliferation and migration of glial cells continue for an extended period in the adult brain and can differentiate in response to injury. Glial progenitors proliferate in the SVZ and migrate into the overlying white matter and cortex, striatum, and hippocampus then differentiate into oligodendrocytes and astrocytes (For review see (Cayre et al., 2009)).

There exist intrinsic and environmental factors that have impact upon the variability in neural architecture. These factors can include both genetic and epigenetic factors, such as puberty and the influence of experience. Studies have demonstrated that brain volume and cortical thickness are highly heritable (Baare et al., 2001, Peper et al., 2007, Thompson et al., 2001). However, evidence suggests that genetic influences on cortical thickness are regionally specific and that the expression of different genes appears to contribute to thickness in different brain regions (Rimol et al., 2010b). Recent studies have demonstrated that there exists a relationship between genetic variation and cortical surface area. They have also found that variants in the regions near genes related to microcephaly are associated with sex-linked cortical surface area expansion (Rimol et al., 2010a, Joyner et al., 2009).

Trials to determine whether plasticity-related changes in specific grey and white matter regions

can be found after intensive behavioural interventions or training have demonstrated that there are increases in the volume of the grey matter after training in the mid-temporal area (hMT/V5) bilaterally, along with the left posterior intraparietal sulcus, both of which are commonly known to be involved in visuomotor functions (Draganski et al., 2004). It has also been reported that juggling training increases fractional anisotropy (FA) in the white matter underlying the posterior intraparietal sulcus and that the increase in FA seems to be more related to the time spent on training, rather than the type of training (Scholz et al., 2009). Furthermore, to determine the effect of cognitive training on brain microstructure, Takeuchi et al. (2010) have applied a working memory training program in a 2-month period and maintained diffusion imaging before and after the program. They demonstrated an increased amount of time spent on training led to a higher FA observed in the white matter underlying the intraparietal sulcus and in the anterior body of the corpus callosum (Takeuchi et al., 2010). Moreover, a recent study involved the measurement of FA in poor readers before and after reading training revealing that before the training, the readers had significantly lower FA in the left anterior centrum semiovale. After training, readers revealed better reading performance, along with an increase in FA and decrease in perpendicular diffusivity in the left anterior centrum semiovale (Keller and Just, 2009). These data suggest that behavioural experience plays an integral role in the plasticity of both white matter and grey matter.

Puberty is a major developmental change in childhood and is related to increased levels of gonadal steroid hormones, such as testosterone and oestradiol, which have organizational and activating effects on the nervous system (Sisk and Zehr, 2005). It has been found that the testosterone and oestrogen sex-specific effect is mainly affects the amygdala and the

hippocampus (Neufang et al., 2009). It has also been demonstrated that the volume of the grey matter is negatively related to oestradiol levels in females, and positively related to testosterone levels in males (Peper et al., 2009). Furthermore, it has been shown that the testosterone-related increase in the volume of the white matter is more significant in males with a lower number of CAG repeats in the androgen receptor gene (Perrin et al., 2008). Moreover, it has been demonstrated that variation in androgen signalling has sex-linked effects on cortical thickness in adolescents (Raznahan et al., 2010). Finally, it has been shown more broadly that steroid hormone levels exhibit effects on the volume of grey matter in young adults (Witte et al., 2010).

Early postnatal experience of the human plays a significant role in brain development (Greenough et al., 1987). Postnatal experience is fundamental in neocortical organisation. At later ages, the developing nervous system continues to gain knowledge and to grow functional neural systems (For review see (Stiles and Jernigan, 2010)).

1.3 Brain anatomy

The adult human brain is divided into two hemispheres. The thick collection of nerve fibres connecting the two hemispheres is called the corpus callosum. Between the hemispheres and under the corpus callosum are different structures, including the thalamus, the hippocampus, the basal ganglia, the amygdala, and the cerebellum, with the spinal cord developing at the end of this sequence (Coward, 2013). In the human, the hippocampus borders the parahippocampal gyrus and belongs to the temporal lobe (Duvernoy, 2009). Opening the temporal horn and removing the choroid plexuses, reveals the hippocampus as an arc composing of three segments: a head, a body and a tail. The hippocampus is formed by two cortical laminae, the cornu

ammonis, and the gyrus dentatus. (Duvernoy, 2009). The basal forebrain is a group of nuclei located between the two cortical hemispheres, and these nuclei are unique because they have high numbers of three different types of projection neurons: cholinergic, GABAergic and glutamatergic (Coward, 2013). In AD, there is deterioration of the cholinergic neurons in the basal nuclei (Lyness et al., 2003). Lesions to the basal nuclei might result in learning deficits (Hepler et al., 1985), deficits in the performance of attention tasks (McGaughy et al., 2002), and loss of past memory, but in some cases these memories are gradually recovered over a period of a few weeks (Bartus et al., 1985).

1.4 Brain function

Improvement in neuroimaging techniques has enriched our understanding of brain anatomy and related brain function. The CNS is a complex integrated system, which is able to process an incredible number of different sensory inputs, store information and deliver behavioural, cognitive and motor outcomes. Because of basic neuronal structure, with dendrites receiving information and axons transporting it to another neuron, synaptic junctions between neurons are integral in maintaining the nervous system (Scarabino, 2003). In the cortex, billions of neurons are connected in a horizontal and vertical matrix in line with the six layers that form during foetal growth. The main functions of the brain as a whole include cognition, mobility, communication, biological maintenance, attention, emotions, intellectual discipline, consciousness and self-awareness, memory, learning and self-sustainability (Coward, 2013). The cortex and the hippocampus play a significant role in almost every cognitive and conscious process (Coward, 2013). Consciousness can be described as wakefulness, which is very distinct from being asleep or being in a coma. An important aspect of consciousness is self-awareness or

self-imaging. These self-images can be memories of past personal experiences, actions and emotions (Coward, 2013), implicating the hippocampus, which plays an integral role in certain types of memory. Most sensory organs, like the ear and nose, can work all day long. However, the memory zone, located in the hippocampus, cannot store every single piece of data without risking saturation. The hippocampus must therefore act as a filter, memorising events for a short period of time before they are rapidly forgotten. The hippocampus is part of a huge system, the limbic system, described as the generator of emotional memory. Hence, long-term memory is linked very strongly to emotions. This is why the oldest memories are always related to our emotions. Attention, which is another function governed by the cortex, is also able to select the quality and duration of memories (Scarabino, 2003).

In the 1950s, a clinical study reported that on an epilepsy patient who had undergone surgery that included the removal of large parts of the hippocampus and entorhinal cortex. This resulted in different types of memory deficits that were extensively investigated for many years (Coward, 2013). The ability to learn new words and facts was lost as well as memory for events that had occurred before the surgery were impaired (Sagar et al., 1985) but working memory, and memory for words learned before the surgery, was unaffected (Kensinger et al., 2001). It has been demonstrated that if the damage was limited to the mammillary bodies or anterior thalamic nucleus, it can result in loss of ability to create new fact or event memories in the context of the preservation of other cognitive capabilities (Tanaka et al., 1997, Van der Werf et al., 2003). It has also been found that damage to the amygdala or septal nuclei can lead to deficits in the memory of emotional events (Berti et al., 1990, Phelps, 2006).

1.5 Neurodegeneration

Over the past century, the aging of our population (the proportion of people aged ≥ 65 years) in industrialised countries has exceeded that of the population as a whole. It is predicted that in subsequent generations, the proportion of the elderly population will double, and so will the proportion of persons suffering from neurodegenerative disorders (Przedborski, 2003). Diagnosis of neurodegenerative disease is usually based on clinical symptoms, as there are no suitable non-invasive tests that can specifically predict the onset of these conditions. However, with the advent of specialised MRI techniques, it is now possible to detect early pathological changes in the brain (Chan et al., 2008), providing clinicians with a unique window for early therapeutic intervention. Nevertheless, it is imperative that biomarker(s) of neurodegeneration are identified in order to assist in the early detection of these idiopathic cognitive disorders. Such biomarkers may take the form of modified proteins or peptides that are released into the circulation, or alternatively sequestered intrathecally (Kumar et al., 2012, Kozlowski et al., 2012).

Biomarkers of neurodegeneration may well be derived from dysfunctional or modified proteins that form the basis of pathological signal transduction cascades (Kozlowski et al., 2012). The deregulation of signalling molecules central for maintaining neuronal function may stimulate the onset of neurodegeneration. For example, while Rho kinase (mainly ROCK2), glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase-5 (Cdk5) and phosphatases are all essential for normal neuronal function (For review see (Selkoe et al., 2012)), they may all also be involved in a plethora of neurodegenerative disorders through a central pathogenic mechanism.

1.6 Alzheimer's disease (AD)

Alzheimer's disease is the most common form of dementia in the elderly. The incidence of AD is raising significantly, with an estimated prevalence of 35.6 million individuals worldwide in 2010, which is expected to quadruple by 2050 (Prince et al., 2013). The global economic cost of the disease is massive, and was estimated to be 604 billion US dollars in 2010 (Wimo et al., 2013). AD has become the fourth most common cause of death in developed countries with no effective treatment (Aguzzi and O'Connor, 2010, Citron, 2010). The current AD therapy involves symptomatic treatment with cholinesterase inhibitors (ChEIs), N-methyl-D-aspartate receptor partial antagonists and other drugs, which are only used to treat the symptoms of the disease (Farlow et al., 2008, Porsteinsson et al., 2014).

Studies found that there are white matter deficits in patients with mild cognitive impairment (MCI) within the frontal, temporal, and parietal cortices, the corpus callosum, and the cholinergic system (Bozzali et al., 2011, Huang and Auchus, 2007, Medina et al., 2006). MCI is considered to be a symptomatic predementia stage of AD, as patients with MCI display cognitive deficits that are more severe than those seen in normal elderly brains but not severe enough to be considered dementia (Petersen et al., 2001). MRI and diffusion tensor imaging (DTI) studies in MCI patients has demonstrated axonal degeneration in the parahippocampal gyrus including the perforant pathway (Kalus et al., 2006, Rogalski et al., 2009), an axonal tract essential in connecting neurons in the entorhinal cortex to the dentate gyrus and other areas of the hippocampus (For review see (Witter, 2007)). It is now believed that these abnormalities can extend to medial temporal lobe regions and other cortical regions (For review see (Stebbins and Murphy, 2009)).

1.7 Alzheimer's disease pathology

1.7.1 Amyloid beta (Aβ) and amyloid plaque pathology

It is well documented that the aging process is the major determinant of developing amyloid plaques both in the presence or absence of the disease (Morimatsu M, 1975). These extracellular senile plaques are composed of accumulated Aβ protein aggregating as β-pleated sheets and are derived from the elevated cleavage of the transmembrane protein APP (Sisodia et al., 1990, Kirschner et al., 1986, Esch et al., 1990). Under normal physiological conditions, APP is a cell surface protein that is thought to be involved in signal transduction, axonal elongation and cell migration (Williamson et al., 1996, Small et al., 1996, Small et al., 1994, Qiu et al., 1995, Ohsawa et al., 1995, Milward et al., 1992, Jin et al., 1994, Breen et al., 1991, Allinquant et al., 1995). It was also demonstrated that the C-terminus of APP plays a central role in gene expression and neuronal cell survival (Nguyen et al., 2008). Such physiological mechanisms are only effective when APP is cleaved by various enzymes, including intramembranous degradation by beta-site AβPP-cleaving enzyme (BACE1) to form the β C-terminal fragment (βCTF) (Sinha and Lieberburg, 1999, Anderson et al., 1992), subsequently followed by gamma-secretase, which forms the small 4 kilodalton (kDa) amyloid-β (Aβ) peptides Aβ1-40 and Aβ1−42, which are released at the synapse (Figure. 1) (Snyder et al., 2005, Sinha and Lieberburg, 1999, Mucke et al., 2000). The normal function of Aβ is unclear; however, studies have suggested that Aβ1-40 can disrupt Aβ fibril formation and prevent neuronal death (Yan et al, 2007, Jan et al, 2008). It has also been suggested that in picomolar amounts, Aβ is neuroprotective and helps in the cellular mechanisms involved in learning and memory (Puzzo et al, 2008, Puzzo et al, 2011) but

in micromolar amounts, it becomes neurotoxic. It has been demonstrated that the extent of APP cleavage is amplified in AD brains and that Aβ treatment further enhances this cleavage (Nguyen et al., 2008). It has also been established that APP and its degradation products localise to neuritic vesicles (Muresan et al., 2009) in the axons of AD brains, along with other neurodegenerative diseases, suggesting that APP accumulation may represent a hallmark of axonal injury (Ahlgren et al., 1996, Cras et al., 1991). For instance, in APP transgenic mice, it has been demonstrated that elevated Aβ levels result in the loss of synapses and neuronal transmission along with behavioural abnormalities, before the formation of amyloid plaques (Nguyen et al., 2008). The rare early-onset familial AD (EOFAD) which is caused by mutations in the enzymes that cleave APP can lead to a rapid and increased cleavage with resultant overproduction of Aβ (Sisodia SS, 2007). On the other hand, the common late-onset AD (LOAD) is thought to result from one or more of the following processes: the failure of Aβ to be cleared from the brain (Chakravarthy et al., 2010, Hensley et al., 2011) by microglial cells; lower expression of Aβ degrading proteases such as insulysin (insulin degrading enzyme IDE); a decline in the availability of Aβ chaperone low density lipoprotein receptor-related protein (LRP1) to transport Aβ out of the brain; and reduced vascular and perivascular drainage (Donahue et al., 2006). Although Aβ monomers are relatively non-pathogenic, accumulating soluble Aβ oligomeric forms have been shown to be synaptotoxic and can prune dendritic spines, disconnecting the memory-encoding neuronal network in the entorhinal cortex, the parahippocampal gyrus and the hippocampus (Shankar et al., 2008). These oligomers eventually form large insoluble fibrillar aggregates or plaques. By themselves, these plaques do not directly induce neuronal death. Rather, they attract microglia and astrocytes that produce cytotoxic pro-inflammatory cytokines, and reactive oxygen species that may indirectly cause neuronal death

(Chiarini A, 2006). Additionally, other mechanisms proposed to contribute to neuronal damage include the vulnerability of cells to secondary insults, tau hyperphosphorylation, induction of the apoptosome and lysosomal protease activity, changes in calcium influx, and direct damage (peroxidation) of membranes (Behl et al., 1994).

In AD brains, insoluble Aβ exists either as diffuse amyloid or senile plaques. Diffuse amyloid can present in normal-aged and AD brains, but senile plaques are only found in AD brains, where they are associated with dystrophic neurons (Wang and Strittmatter, 1996), hence, a role for senile plaques in AD neurotoxicity has been proposed (Selkoe, 1991). Evidence from genetic (Games et al., 1995, Selkoe, 1995), pathological (Masliah et al., 1993, Wang and Strittmatter, 1996), and cell culture (Pike et al., 1993, Suzuki et al., 1994) studies have implicated Aβ1-40 and Aβ1-42 as toxic agents in AD. Several *in vitro* studies have demonstrated that Aβ can only be toxic when it forms fibrils (Lorenzo and Yankner, 1994, Pike et al., 1993, Simmons et al., 1994). These studies indicated that Aβ neurotoxicity is mediated by direct interactions between Aβ and membrane lipids. Arispe et al. (1993) demonstrated that Aβ1-40 can form cation-selective channels in membranes and that these channels can disrupt ion homeostasis and cause toxicity (Arispe et al., 1993). Terzi et al. (1994) investigated the interaction of phosphatidylcholine (PC)/phosphatidylglycerol (PG) vesicles with Aβ peptides using biophysical techniques and they have shown that Aβ1-40 forms β-sheet structures upon addition of PC/PG vesicles (Terzi et al., 1994, Terzi et al., 1995). Using AD brain homogenates, a ganglioside-bound Aβ species was isolated and idintified to be localized on the cell surface, where it can act as a seed for amyloid fibril formation (Yanagisawa et al., 1995). It was also found that Aβ disrupts membranes containing acidic phospholipids *in vivo*. This pH-dependence suggests that Aβ has the capacity to

disrupt endosomal and plasma membranes, and that this disruption might lead to the neurotoxic effect of the peptide. The authors also suggested that Aβ is initially soluble, and when binds to acidic lipids such as gangliosides it disrupts the bilayers in β-structured conformation (McLaurin and Chakrabartty, 1996).

Although the plaques are found extracellularly, it is thought that the production, oligomerisation and accumulation of Aβ occurs within neuronal processes, with the possibility that the incorporation of aggregates into plaques occurs after the neurites are dissolved (Muresan and Muresan, 2006). Certainly, studies performed in the well-established mouse models of AD have identified Aβ in several neuronal compartments such as the Golgi apparatus, the endoplasmic reticulum, the secretory vesicles, endosomes, and autophagic vacuoles, suggesting intraneuronal aggregation and pathology (Muresan and Muresan, 2006). However, recent evidence supports the extracellular deposition of Aβ as the initiating pathogenic mechanism in the AD brain (Jack Jr et al., 2010), with a direct correlation to the inhibition of anterograde axonal transport (Rodrigues et al., 2012a). Despite direct evidence of Aβ-dependant neurodegeneration, Aβ pathology occurs prior to the appearance of clinical symptoms (Jack Jr et al., 2010). Accordingly, determining the level of amyloid deposition in an AD patient's brain (the Aβ load) in a time-dependent manner would be informative in evaluating the progression of the disease and monitoring the patient's response to anti-amyloid therapies. Interestingly, through amyloid imaging, recent studies have demonstrated binding of the PET Pittsburgh compound B (PiB-PET) to Aβ peptides (Devanand et al., 2011). In this study, PET amyloid imaging with Pittsburgh compound B (PiB) showed increased cortical PiB binding in AD patients when compared to control subjects, and intermediate binding levels in patients with MCI (Devanand et

al., 2011). This means that the PiB compound could be beneficial in the early detection of AD and evaluation of disease progression.

Aβ1-40 and Aβ1-42 formation is well known to be a result of sequential cleavage of APP by β- and γ-secretases (Checler, 1995). APP cleavage occurs through two main pathways: The amyloidogenic pathway, in which APP is cleaved by β-secretase, followed by γ-secretase forming the toxic Aβ peptide; and the non-amyloidogenic pathway, in which APP is cleaved by α-secretase then by γ-secretase, producing the non-toxic P3 peptide. The highly amyloidogenic 42-residue Aβ peptide (Aβ1-42) aggregates rapidly, and is known to be more neurotoxic than the shorter Aβ peptide (Aβ1-40) (For review see (Querfurth and LaFerla, 2010)). It has been suggested that β-secretase activity is localized to endosomal vesicles, while γ-secretase activity is restricted near the external plasma membrane. Due to the spatial separation of β- and γ-secretase activities, Aβ generation may be concomitant with secretion and does not accumulate inside the cell (Checler, 1995). On the other hand, intracellular accumulation of Aβ has been shown in the human neuronal cell line, NT2N (Wertkin et al., 1993), and in COS cells transfected with APP cDNA containing the Swedish mutation (Martin et al., 1995). The pathway for Aβ intracellular accumulation appears to be different from that for Aβ secretion. Martin and colleagues suggest that while Aβ is usually produced through the secretory pathway, under specific conditions, such as the presence of the Swedish mutation, the intracellular pathway can be up-regulated (Martin et al., 1995).

Recently, it was demonstrated by a combination of *in vivo* and *in vitro* studies, that Aβ binding to the cellular prion protein (PrPc), an oligomer-specific high-affinity binding site for Aβ, can play

a central role in Aβ-induced memory deficits, axon degeneration, synapse loss and neuronal death in the AD brain through Fyn kinase activation (Um et al., 2012b). The activation of this kinase results in alterations in N-methyl-D-aspartate receptor (NMDAR) function by increasing surface NMDAR, NMDAR phosphorylation, and eventually leads to dendritic spine, in association with, surface receptor loss (Um et al., 2012b). The data suggest that by inhibiting PrPc in the APPswe/PSEN1-M146L double transgenic mouse, reversal of memory deficits and restoration of synaptic density could be achieved (Um et al., 2012b). It has been demonstrated that Fyn kinase associates with the tau protein, and that abnormal Fyn-tau interactions sensitise synapses to glutamate excitotoxicity (Um et al., 2012b). Together, these data suggest that PrPc-Fyn signalling may contribute to Aβ and tau pathologies and thus its downregulation may be a potential therapeutic approach.

Other studies suggested that Nogo-A and Nogo receptors (NgR) play a significant role in the formation of amyloid plaque and AD pathology (Park et al., 2006). Nogo-A is a protein expressed by oligodendrocytes and is a main constituent of myelin in the CNS. It disrupts neurite elongation *in vitro* and is believed to inhibit axonal outgrowth *in vivo* after injury in the adult CNS (Huebner and Strittmatter, 2009). NgR immunoreactivity was demonstrated in more than 50% of hippocampal neurons, and might be correlated with the development of tangles in AD (Zhu et al., 2007). Both Nogo and NgR are mislocalized in AD brains, and NgR interaction with APP limits Aβ plaque accumulation (Park et al., 2006). In AD, hippocampal neurons overexpress Nogo-A, and the protein is found to be associated with Aβ plaque (Gil et al., 2006). It has been demonstrated that Nogo-A overexpression limits neurite outgrowth and enhances Aβ secretion (Xiao et al., 2012). It has also been suggested that Nogo-66 inhibits neurite outgrowth in

different nerve cells, such as dorsal root ganglion cells (Chen et al., 2000b, GrandPre et al., 2000), PC12 (GrandPre et al., 2000), and cerebellar granule cells (Niederost et al., 2002). Also, it has been shown that Nogo-66 association to NgR on nerve cell membranes activates two downstream signalling molecules, ROCK and Protein Kinase C (PKC), and hence potentiates neurite outgrowth inhibition (Yiu and He, 2006). Down-regulation of MAP2 was found in cortical neurons treated with Nogo-P4 indicating that Nogo-P4 limits neurite outgrowth (Xiao et al., 2012). Treating the cells with the NgR antagonist NEP1-40 and/or inhibiting PKC and ROCK activity using GÖ6976 and Y-27632, respectively, attenuates the outgrowth inhibition mechanism and increases MAP2 gene expression (Xiao et al., 2012). Munc-18 interacting proteins (Mints)/X11s, members of the reticulon family (RTN-3 and RTN4-B/C), and NgR are some of the regulatory molecules that modulate APP processing and Aβ formation (For review see (Tang and Liou, 2007)). NgR interaction with APP and RTN-3, and RTN4-B/C interaction with β-secretase minimises Aβ production (Murayama et al., 2006, Park et al., 2006). Inhibiting NgR with NEP1-40 has no effect on Nogo-P4-induced Aβ1-42 secretion in cultured cortical neurons while ROCK inhibitor Y-27632 inhibits Nogo-P4-stimulated Aβ1-42 secretion (Xiao et al., 2012). This demonstrates that Y-27632 decreases Aβ1-42 by inhibiting the activation of ROCK, and Nogo-P4 raises Aβ1-42 secretion by activating the ROCK pathway. Other studies have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) decrease Aβ1-42 levels through inhibition of Rho activity in SH-SY5Y APP cells (Zhou et al., 2003). It has been demonstrated that the active ROCK1 molecule inhibits sAPPα shedding in cultured APP transfected cells (Pedrini et al., 2005). It has also been suggested that the activation of ROCK reduces sAPPα secretion, which may enhance APPβ processing resulting in an increase in Aβ1-42 production (Xiao et al., 2012). However, PKC inhibitor GÖ6976 increases Aβ1-42 secretion,

suggesting that a combined treatment will have no effect on Aβ1-42 (Xiao et al., 2012). This suggests that Nogo-P4 has no effect on Aβ1-42 levels and that it might be mediated by other signalling pathways apart from the ROCK pathway. From the viewpoint of a drug therapy target, NgR may be considered a target for promoting neurite outgrowth or axon regeneration, but not as a target for limiting Aβ1-42 production. PKC may be used to promote neurite outgrowth; however, it promotes Aβ1-42 production and therefore is not appropriate for use in AD. On the other hand, ROCK appears to be a key molecular target to induce neurite outgrowth and limit Aβ1-42 production, and thus may prove a potentially useful and effective treatment for AD.

Another potential therapeutic technique that can be effective in AD is targeting Aβ epitopes. AD is one of more than thirty human amyloid diseases (For review see (Chiti and Dobson, 2006)) in which amyloid proteins are produced having entirely different sequences that share conformational epitopes as a result from their β-pleated sheet structure (Kayed et al., 2003). Monoclonal antibodies (mAbs) that recognize these amyloid proteins have been isolated and demonstrated to have anti-amyloid properties (Hrncic et al., 2000, O'Nuallain et al., 2011, O'Nuallain and Wetzel, 2002, Zhao et al., 2014). Human intravenous immunoglobulins (IVIGs) isolated from collection of plasma from healthy donors contain IgGs that bind these epitopes (Du et al., 2001, Du et al., 2003, O'Nuallain et al., 2006). Aβ-reactive polyclonal IgGs in IVIGs can interact with different types of amyloid fibrils and oligomers, including Ig light chain amyloid (AL) (O'Nuallain et al., 2008, O'Nuallain et al., 2006), disrupting their fibrillogenesis *in vitro* and *in vivo*. Studies performed to test IVIG in transgenic AD mouse models demonstrated that short-term and long-term treatments promote cognitive function (Mengel et al., 2013, St-Amour et al., 2014). Moreover, clinical trials of IVIG in mild to moderate AD patients indicated cognitive

benefits (For review see (Relkin, 2014)). These data suggest that conformation-specific human IgGs exist and may have promising therapeutic anti-amyloid activities. However, it is not clear whether the conformational amyloid binding and anti-amyloid capabilities observed with polyclonal IVIGs can be replicated by individual mAbs, or if these may contribute to the potential utility of IVIG in AD (Gu et al., 2014, Magga et al., 2010, Relkin et al., 2009). Using a hybridoma method, Levites et al. (2015) tested the characterisation, cloning and functional activities of anti-amyloid IgG isolated from CD27- peripheral blood B cells from healthy individuals. They demonstrated that IgG mAb (3H3) recognises diverse amyloids, suppresses Aβ and LC amyloids elongation, and minimise the disturbance of synaptic plasticity in the rat hippocampus by AD brain extract (Levites et al., 2015). Furthermore, these authors demonstrated that the expression of a single-chain variable fragment (scFv), derived from 3H3, attenuates Aβ1-40 amyloid deposition in the brains of TgCRND8, a transgenic mouse model of CNS Aβ deposition, and cerebral amyloid angiopathy (CAA) from ADan deposition in a mouse model of Familial Danish dementia (FDD) (Levites et al., 2015). The ability of the 3H3 mAb to inhibit the amyloid toxicity in a living brain illustrates quite clearly that naturally occurring, conformation-specific antibodies in IVIG might exhibit a protective function in AD.

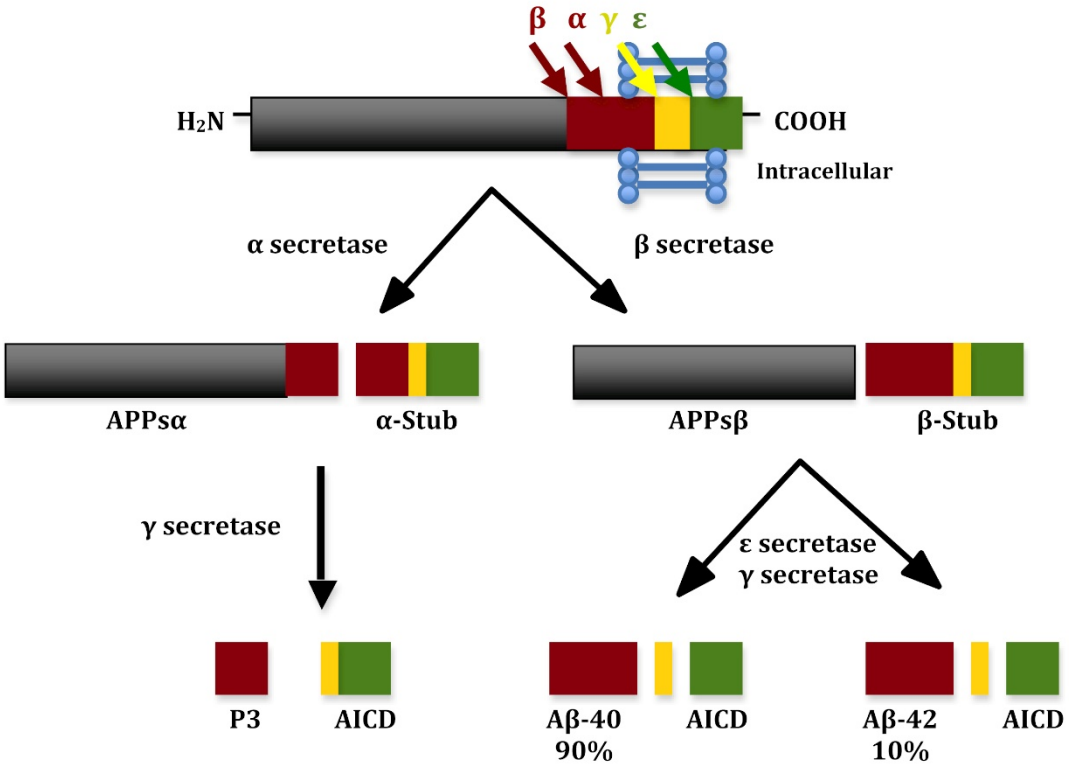
β α γ ε
H2N
COOH
Intracellular
α secretase
β secretase
APPsα
α-Stub
APPsβ
β-Stub
γ secretase
ε secretase
γ secretase
P3
AICD
Aβ-40
90%
AICD
Aβ-42
10%
AICD

Figure 1: The processing of APP through the beta-site AβPP-cleaving enzyme BACE1, followed by presenilin 1 (PS1).

Sequential beta and gamma-secretase cleavage of APP generates the synaptotoxic amyloid-β (Aβ) peptide species, Aβ1-40 and Aβ1−42 (Mokhtar et al., 2013).

1.7.2 Tau protein pathology

The tau protein is an integral component of the neuronal cytoskeleton with a molecular weight ranging from 45 kDa to 65 kDa (Hirokawa et al., 1988). It is responsible for the promotion of microtubule assembly in the normal brain (Scott et al., 1992). Microtubule assembly is tightly regulated by a combination of protein kinases and phosphatases that balance the amount of tau phosphorylation (Whiteman et al., 2009, Li et al., 2013). Tau pathology is commonly seen in AD as well as frontotemporal dementias (FTD) and PD (Arendt et al., 1998, Ittner et al., 2008). In the AD brain, tau exists in a hyperphosphorylated state, which leads to aberrant secondary structures and loss of function, in turn resulting in a reduced ability to bind to microtubules and to promote their assembly (Terry, 1998). The abnormal translocation of tau from axonal microtubules to neuropil thread inclusions, cell bodies and dendritic processes, where it aggregates and accumulates, are other prominent cytopathological hallmarks (Velasco et al., 1998). The tau protein is initially synthesised as a single chain polypeptide, then targeted by post-translational modifications that alter its conformation, promoting tau dimerisation in an anti-parallel manner (Martin et al., 2011). Stable tau dimers subsequently form tau oligomers, which aggregate at an increasing rate to form subunits of filaments called protomers. Two protomers twisted around each other, with a crossover repeat of 80 nm, constitute the width varying between w10 and w22 nm to form paired helical filaments (PHFs), a characteristic of AD neuronal pathology (Bulic et al., 2010, Martin et al., 2011). Assembly of PHFs finally establishes the neurofibrillary tangles (NFTs), which can be observed microscopically (Shelton and Johnson, 2004) (Figure 2). Hyperphosphorylated tau sequesters normal tau and other neuronal microtubule associated proteins (MAPs), such as MAP1A, MAP1B and MAP2, contributing further to disassembled microtubules, disruption of the axonal cytoskeleton and transport, culminating

neuronal damage (Iqbal et al., 2008). After neuronal death, tau oligomers are released into the extracellular environment, which leads to microglial cell activation, and as a consequence, further progressive bystander neuronal degeneration (Maccioni et al., 2010). It has been suggested that tau pathology results from elevated protein kinase activity, a reduction in the activity of protein phosphatase, or both (Arendt et al., 1998). Analysis of phosphorylated tau isolated from AD brains has identified numerous target serine or threonine residues (Arendt et al., 1998). It has been demonstrated that MAP-kinase, GSK-3β and/or Cdk5 are the main kinases involved in tau phosphorylation. However, in AD not all tau phosphorylation events can be attributed to these kinases (Arendt et al., 1998).

The mechanism by which tau exerts its neuronal toxicity is still controversial (Amadoro et al., 2011). Ittner et al, (2010) have suggested that Aβ toxicity is tau dependent and that tau has an important role in postsynaptic targeting of the Src kinase Fyn, a substrate of which is the NMDAR. Using transgenic mice expressing truncated tau (D tau) and tau knockout mice, they demonstrated disruption in postsynaptic targeting of Fyn, NMDAR-mediated excitotoxicity and consequently Aβ toxicity (Ittner et al., 2010). Furthermore, they have found that D tau expression and tau deficiency causes improvement in the memory and survival of Aβ-forming APP23 mice, a model of AD (Ittner et al., 2010). It has also been suggested that tau can activate a series of degenerative signals such as Aβ aggregation, iron overload (Lavados et al., 2008), oxygen free radicals (Zambrano et al., 2004), cholesterol levels in neuronal rafts, LDL species (Neumann et al., 2008), and homocysteine initiating an innate immune response (Maccioni et al., 2010). The activation of microglial cells, for instance, results in the subsequent release of pro-inflammatory cytokines that modify neuronal behaviour through anomalous signalling cascades, with the end

result being the promotion of tau hyperphosphorylation (Maccioni et al., 2010). However, numerous cellular and transgenic animal models indicate that tau is crucial for Aβ-induced neurotoxicity (Amadoro et al., 2011). For instance, cultured hippocampal neurons from tau-deficient mice are protected against Aβ pathology (Amadoro et al., 2011). Furthermore, in cultured hippocampal neurons from wild-type mice, the silencing of tau by siRNA has demonstrated that tau is required for pre-fibrillar Aβ-induced microtubule disassembly. Furthermore, it has been demonstrated that a reduction in soluble Aβ and tau, but not Aβ alone, causes cognitive decline in the triple transgenic AD mouse model with plaques and tangles (Roberson et al., 2007). These data suggest that although Aβ is the initial trigger, tau accumulation plays a central role in neurodegeneration. Finally, in the AD-like transgenic model that expresses human APP with familial mutations, suppression of endogenous tau prevents Aβ-dependent water maze learning and memory deficits without reversing the amyloid pathology (Roberson et al., 2007). Collectively, these data suggest a link between Aβ and tau that drive the neural pathologies and the manifestations of clinical symptoms. Preliminary data on the inhibition of tau aggregation by methylene blue chloride (MTC) has indicated a lower rate of cognitive decline in treated patients compared with sporadic AD patients on alternate therapies, implicating tau as the key initiator of cognitive deficits (Bulic et al., 2010). However, the exact role of Aβ in signal transduction cascades that are associated with pathogenic tau modifications, and their contribution to the progression of neuronal death, requires further investigation (Amadoro et al., 2011).

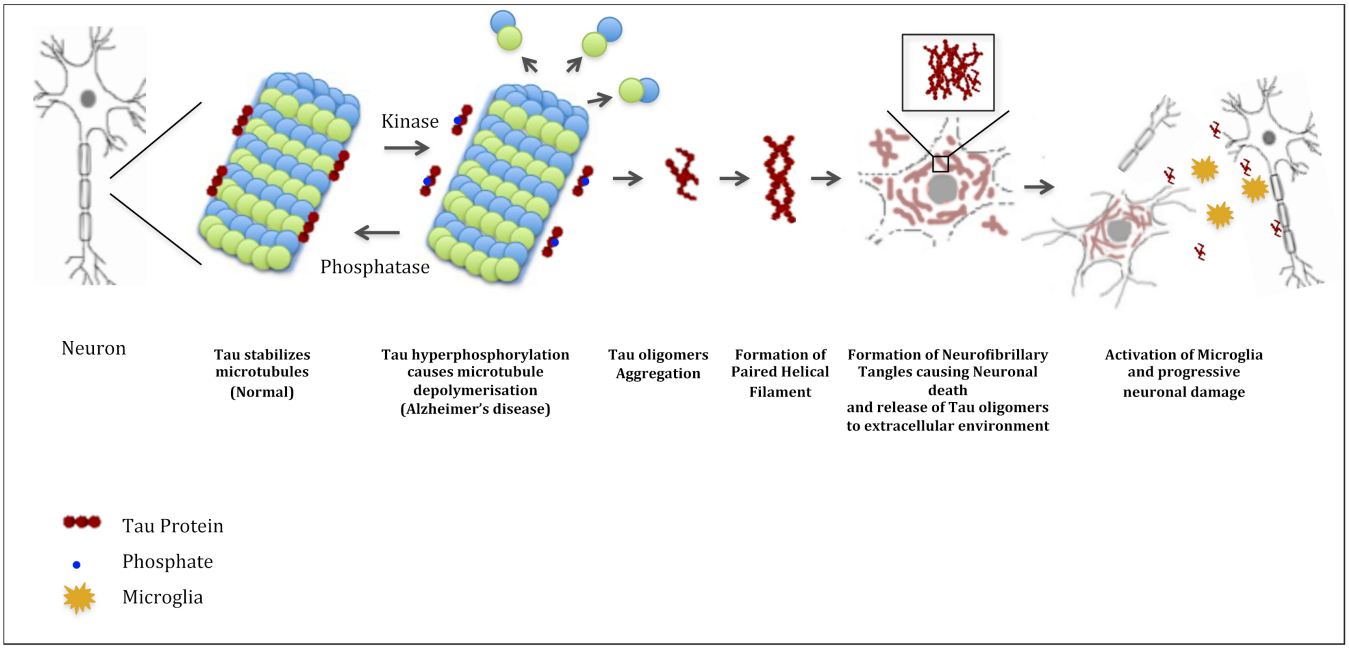
Kinase
Phosphatase
Neuron
Tau stabilizes microtubules (Normal)
Tau hyperphosphorylation causes microtubule depolymerisation (Alzheimer's disease)
Tau oligomers Aggregation
Formation of Paired Helical Filament
Formation of Neurofibrillary Tangles causing Neuronal death and release of Tau oligomers to extracellular environment
Activation of Microglia and progressive neuronal damage
Tau Protein
Phosphate
Microglia

Figure 2: Stabilisation of microtubules by the tau protein is regulated by kinases and phosphatases.

Abnormal hyperphosphorylation of tau proteins causes catastrophic microtubule depolymerisation and the formation of insoluble cytoplasmic tau oligomers, which aggregate to form protomers. Two protomers twisted around each other to form PHFs, which assemble to produce NFTs (Mokhtar et al., 2013).

1.7.3 Axonal transport and synaptic pathology

It has been suggested that synaptic dysfunction leads to memory and cognitive abnormalities in early AD stages (For review see (Bell and Claudio Cuello, 2006)). It has also been demonstrated that there are abnormalities in synaptic morphology, as well as reduction in the total number of synapses, in AD (DeKosky and Scheff, 1990, Masliah et al., 1991). Studies demonstrated that neurodegeneration in AD brains initially affects medial temporal lobe structures, such as the transentorhinal and entorhinal cortices, the subiculum and the hippocampus (Braak et al., 2006). Studies have found that dystrophic neurite pathology appears much earlier than NFT (Ghoshal et al., 2002, Su et al., 1997, Vana et al., 2011) and amyloid plaque depositions (Kowall and Kosik, 1987). Overexpression of APP mutants such as APPV717F, APPK670N and M671L (Mucke et al., 2000) in cell culture or mouse models such as 3XTg-AD (expressing mutant PS1M146V, APPSWE, and tauP301L) (Oddo et al., 2003, Cai et al., 2012) and 5xFAD (expressing APPK670N, M671L, I716V, V717I; PS1M146L, L286V) (Oakley et al., 2006) causes synaptic loss before the formation of amyloid plaques (Jawhar et al., 2011). On the other hand, DTI imaging of Tg2576 and PDAPP mouse models has demonstrated that white matter deficits coincide with amyloid plaque pathology (Song et al., 2004).

Neurons normally extend dendrites and axons, long projections that mediate the reception, processing and transmission of chemical information through synaptic contacts with other neurons. The length of the axons ranges from the short axons required to communicate with interneurons to the long axons required to communicate with neurons in distant areas of the nervous system (For review see (Mattson and Magnus, 2006)). Neurons synthesize molecules required for maintenance of synapses within the cell body and then transport them to the synapse

through the axon; hence, a proper axonal transport system is integral for the maintenance of synaptic function and for neuron survival (Morfini et al., 2001, Morfini et al., 2009a). Axonal transport includes slow transport, which is important for the transport of cytoskeletal structures and cytosolic proteins at rates of 0.1–6 mm/day and fast axonal transport (FAT), which is important in the transport of membrane-bound organelles (MBOs) at rates of 50–400 mm/day (Morfini et al., 2011). FAT includes anterograde transport towards the plus-end of microtubules (from cell body to synapse) and retrograde transport towards the minus-end of microtubules (from synapse to cell body). Kinesin-1 is a member of the kinesin protein superfamily, and is the most abundant anterograde-directed motor protein in neurons (Wagner et al., 1989). Kinesin-1 mediates the transport of several molecules such as mitochondria, synaptic vesicle precursors, axolemmal constituents, and secretory products (Morfini et al., 2011). Kinesin-1 is a heterotetramer molecule containing two heavy chains (KHC) and two light chains (KLC). KHCs mediate kinesin-1 binding to microtubules and ATP hydrolysis, while KLCs bind kinesin-1 to specific molecular cargoes (Stenoien and Brady, 1997). Studies have suggested that hypoactivity of kinesin and cDyn lead to neuronal degeneration (Morfini et al., 2009a, Pfister et al., 2006, Roy et al., 2005).

Drosophila models overexpressing Aβ (Zhao et al., 2010), APP (Gunawardena and Goldstein, 2001, Rusu et al., 2007), and tau (Mudher et al., 2004) demonstrate deficits in axonal transport. In addition to this, transgenic mouse models expressing mutant PS1 (Lazarov et al., 2007, Pigino et al., 2003), APP/PS1 (Chen et al., 2011, Wirths et al., 2007), APP/PS1/tau (Desai et al., 2009, Cai et al., 2012) and mutant tau (Gilley et al., 2012, Yoshiyama et al., 2007, Zhang et al., 2004) demonstrate defective axonal transport and synaptic degeneration. For example, a mouse model

expressing mutant PS1 demonstrates reduction in the transport of mitochondria, APP, synaptophysin, specific Trk receptors, and syntaxin (Lazarov et al., 2007, Pigino et al., 2003). Moreover, MRI studies of mutant APP transgenic mice (Smith et al., 2007) and APP/PSI/tau triple transgenic mice (Kim et al., 2011) found that the reduction in axonal transport precedes NFT and plaque deposition. Interestingly, environmental enrichment in APPswe/PS1Δ E9 mice promoted a reduction in tau hyperphosphorylation and concomitantly increased the expression levels of conventional kinesin subunits, suggesting that induction of brain plasticity modulates the toxic pathways elicited by pathogenic forms of APP and PS1 (Hu et al., 2010). Some studies have suggested that tau limits axonal transport by interfering with kinesin-1 binding to microtubules (Mandelkow et al., 2003, Ebneth et al., 1998) however this was not supported by other studies (Yuan et al., 2008, Morfini et al., 2007). It has been demonstrated that tau blocks microtubule binding of kinesin-1 and revert CDyn directionality (Dixit et al., 2008), a possibility that other studies disagree with (LaPointe et al., 2009, McVicker et al., 2011). It has been demonstrated that Aβ inhibits axonal transport through activation of N-methyl-D-aspartate receptors (NMDAR) and activation of specific kinases (Decker et al., 2010, Tang et al., 2012) including GSK3, Cdk5, and casein kinase 2 (CK2) (Morfini et al., 2009a).

It has been demonstrated that different protein kinases can regulate kinesin-1 activity. For instance, *in vivo* studies detected that cJun amino-terminal kinase 3 (JNK3) phosphorylates serine 176 within KHC and disrupts kinesin-1 association to microtubules (Morfini et al., 2009b). Other studies demonstrated that CK2 and GSK3 regulate kinesin binding to molecular cargoes via phosphorylation of KLCs (Morfini et al., 2002, Pigino et al., 2003).

APP cleavage within the plasma membrane by secretases produces Aβ which then undergoes aggregation forming oligomeric (oAβ) and fibrillar (fAβ) species (For review see (Thinakaran and Koo, 2008)). It has been demonstrated that Aβ, predominantly oAβ, disrupts axonal transport and synaptic connectivity and mediates neuronal degeneration (Ferreira and Klein, 2011, Moreno et al., 2009) through abnormal activation of kinases (Pigino et al., 2009). Cell culture (Hiruma et al., 2003, Wang et al., 2010) and animal model studies (Kasa et al., 2000, Smith et al., 2007) demonstrated that intracellular and extracellular Aβ deposition leads to axonal transport deficits. For example, studies have found that Aβ peptides, oligomers and fibrils limit mitochondrial transport in cultured neurons through activation of GSK3 (Rui et al., 2006), NMDAR (Decker et al., 2010, Tang et al., 2012) and CK2 (Pigino et al., 2009).

Presenilins (PSs) are the catalytic core of the γ-secretase that cleave APP (For review see (O'Brien and Wong, 2011)). Many AD cases result from PS1 and PS2 mutations. For instance, there are more than 170 PS1 and 14 PS2 mutations that lead to APP cleavage and formation of toxic Aβ (For review see (Thinakaran and Koo, 2008)). Evidence suggests that deletion or overexpression of mutant PS1 activates GSK3 and increases tau and KLC phosphorylation, decreases binding of kinesin-1 to molecular cargos, and limits anterograde axonal transport (Pigino et al., 2003). In addition, two transgenic mouse models overexpressing mutant forms of PS1 demonstrated increased phosphorylation of GSK3 substrates (tau and neurofilaments) and defective anterograde axonal transport of APP and neurotrophin receptors in the sciatic nerve (Lazarov et al., 2007). Studies using other transgenic mouse models expressing human wild-type tau (Probst et al., 2000), mutant P301L tau (Gilley et al., 2012, Lin et al., 2005), mutant P301S tau (Yoshiyama et al., 2007), mutant R406W tau (Zhang et al., 2004), and mutant G272V/P301S

tau (Leroy et al., 2007) demonstrated synaptic and axonal abnormalities and limited axonal transport.

Trials to link tau to axonal transport deficits suggested that tau can activate PP1, which in turn activates GSK3 via dephosphorylation at serine 9, ultimately increasing KLC phosphorylation and dissociation of kinesin-1 from molecular cargoes (LaPointe et al., 2009). Other studies demonstrated that there exists a phosphatase-activating domain (PAD) within the N-terminus of tau that can activate the PP1-GSK3 signalling cascade and limit kinesin-dependent axonal transport, and that this effect can be mediated without tau aggregation (Kanaan et al., 2011). Furthermore, studies demonstrated that increased PAD exposure occurs early in AD even before the accumulation of NFTs (Patterson et al., 2011a, Kanaan et al., 2011). Moreover, studies demonstrated that the oligomeric form of tau is a major toxic species that might lead to axonal transport inhibition (Patterson et al., 2011a, Patterson et al., 2011b). Although tau abnormality is a hallmark of AD, neurons appear to survive for decades with tau pathology, suggesting that other mechanisms are mediating neurodegeneration in AD. Aβ exposure in cultured neurons and in animal models leads to activation of several kinases, such as CK2 and GSK3, which can phosphorylate tau causing the formation of NFTs (Avila et al., 2006, Hernandez et al., 2010).

1.8 *Secretases and presenilins*

Evidence suggests that APP and its derivative, Aβ peptide, are involved in the initiation of AD (Oddo et al., 2003). APP gene mutations cause chromosome 21-linked familial AD (FAD) (Goate et al., 1991, Murrell et al., 1991) which has a similar neuropathological phenotype to sporadic AD. Aβ is composed of a set of 39 to 43 amino acid oligopeptides developed from the

cleavage of APP (Kang et al., 2007), by β-secretase at its NH2–terminus and the γ-secretase complex at its COOH-terminus (Ahn et al., 2010, Takasugi et al., 2003). BACE is an aspartyl transmembrane protease encoded by Asp 2 (For review see (Roberts, 2002)). Characterisation of the APP Swedish mutation at the BACE1 cleavage site emphasises the potency of modulating this pathway in regulating Aβ generation (Mullan et al., 1992, Citron et al., 1995). Furthermore, genetic studies in a population of Icelanders demonstrated that an APP amino acid substitution that inhibits BACE1 cleavage protects against AD (Jonsson et al., 2012). Development of BACE1 inhibitors has been challenging due to the size of the BACE1 enzymatic site and the lack of pharmacokinetic efficacy *in vivo* (For review see (Stachel, 2009)). Nevertheless, alternatives to reduce Aβ production have been developed, including β-secretase inhibitors/modulators and nonsteroidal anti-inflammatory drugs (NSAIDs) (For review see (De Strooper et al., 2010)).

The other major intramembranous enzyme is γ-secretase, which catalyses the processing of type I transmembrane proteins such as Notch, APP and E-cadherin. γ-secretase is a membrane-bound high molecular weight aspartyl protease that is highly expressed in the brain (Roberts, 2002, Kovacs et al., 2010). It consists of PS, nicastrin (NCT), anterior pharynx-defective-1 (Aph-1), and presenilin enhancer-2 (Pen-2). PS is the catalytic constituent while the other three are important in the maturation of active γ-secretase (Sanjo et al., 2010). Studies of knockout (KO) mice have demonstrated the important role of both enzymes in brain function. BACE1-deficient mice show cognitive and behavioural deficits such as spontaneous behavioural seizures, together with altered electrophysiological properties in their neurons (Kovacs et al., 2010).

A number of studies have suggested that altered BACE1 and/or PS/γ-secretase activities play an

important role in sporadic and familial AD (Kovacs et al., 2010, Pratt et al., 2011). APP is cleaved twice to generate Aβ proteins (Roberts, 2002, Kovacs et al., 2010, Bakshi et al., 2011, Crews et al., 2010). Initially, β-secretase cleavage generates the soluble extracellular domain (sAPP), along with a C-terminal fragment (C83) (Kovacs et al., 2010). Subsequent cleavage by γ-secretase produces the p3 fragment and an intracellular domain (AICD) (Figure 2) (Kovacs et al., 2010, Roberts, 2002). It appears that γ-secretase cleaves β-APP at multiple sites, to produce Aβ molecules that end at amino acids 38, 39, 40, 42 or 43. The cleavage peptides that are found mostly *in vivo* are Aβ1-40 and Aβ1-42 with higher ratio of Aβ1-40; however Aβ1-42 is thought to be the most toxic form (For review see (Roberts, 2002)). Studies have suggested that the most direct approach to reduce the amyloid load in the brain is by controlling its production, through the inhibition of β and/or γ-secretases (Roberts, 2002, Crews et al., 2010). Interestingly, Bakshi et al. (2011) showed that AD progression could be limited by blocking a chemokine receptor, CXCR2, this is believed to enhance γ-secretase activity and increase Aβ production (Bakshi et al., 2011). Moreover, studies have demonstrated that enhanced β-secretase cleavage of APP diminishes APP anterograde axonal transport while blocking β-secretase, which can enhance APP axonal transport (Rodrigues et al., 2012b). Furthermore, this study used a mouse model of familial AD (FAD) that carries the Swedish mutation in APP (Tg-swAPP^{Prp}), which enhances β-secretase cleavage of APP, and has been treated with γ-secretase inhibitor, to reduce Aβ formation, to show that this led to the development of plaque-independent axonal dystrophy in the absence of synaptic degradation (Rodrigues et al., 2012b). These results together suggest that inhibiting β-secretase activities may preserve APP structure and anterograde axonal transport and protect the axon.

PSs are basic membrane proteins that contain eight transmembrane domains and a hydrophilic loop (Li and Greenwald, 1998, Doan et al., 1996). They concentrate in the nuclear membranes, endoplasmic reticulum (ER), intermediate compartment, and growth cones of neurons (Pigino et al., 2001, Capell et al., 1997, Busciglio et al., 1997, Annaert et al., 1999). They undergo endoproteolytic cleavage, generating stable N- and C-terminal fragments (NTF and CTF) that interact with other proteins to form a macromolecular complex containing the γ-secretase activity (Marambaud et al., 2002, Ebinu and Yankner, 2002). PS play a role in protein trafficking, calcium homeostasis, and regulation of β-catenin signalling. They can be found within the γ-secretase complex or outside (Wolfe et al., 1999). Studies have demonstrated that patients with familial AD have mutations in PS1 and PS2 on chromosome 14q24.3 and chromosome 1q42.2, respectively (Mirnics et al., 2008). Furthermore, mutations in PS1 and PS2 have been reported in numerous families with both late and early onset disease (Devi et al., 2000, Jayadev et al., 2010, Kauwe et al., 2007). Strong and progressive neurodegeneration in the hippocampus and frontal cortex, which is characterized by both anatomical and behavioral changes, was demonstrated in PS1/PS2 double KO mice which is characterized by both anatomical and behavioural changes (Mirnics et al., 2008). Other studies suggested that the specific binding between APP and PS is fundamental in Aβ formation, and that in cell cultures in media containing an excess of the water-soluble NH2–terminal domain of PS1 (residues 1– 80) conjugated to FLAG protein, Aβ production was significantly decreased (Dewji and Singer, 1996, Dewji et al., 2006). It has been demonstrated that a family of peptides containing the DEEEDEEL sequence (P6, P7 and P8) and another independent peptide (P4) are capable of significantly decreasing Aβ production *in vitro* and *in vivo* in the brains of mThy1-hAPP Tg mice (Dewji et al., 2015). It has also been demonstrated using biolayer interferometry and fluorescence confocal microscopy, that they are

able to perform specific and biologically relevant binding with the purified ectodomain of human APP 695. Moreover, these authors demonstrated that this reduction in Aβ secretion does not affect β- or γ-secretase catalytic activities or APP level (Dewji et al., 2015). These findings have bearing on the therapeutic potential of the inhibitory peptides.

1.9 Signalling molecules linked with neuronal cytoskeleton disassembly

1.9.1 *Rho kinase (ROCK)*

The Rho-associated coiled-coil forming protein kinases (ROCKs) include the ROCK1 and ROCK2 isoforms. These two kinases contain highly conserved amino-terminal but different carboxy-terminal domains (Nakagawa et al., 1996). Both ROCK1 and ROCK2 were originally shown to be involved in cell differentiation, essential for the regulation of myogenesis from embryonic fibroblasts along with skeletal muscle maturation and differentiation (Sordella et al., 2003). Both Rho kinase (ROCKs) and p21-activated kinase (PAKs) are members of the serine/threonine class of protein kinases. However, they are known to have antagonistic effects on the actin cytoskeleton and therefore on the plasticity of synapses. PAK also has two major isoforms, PAK1 and PAK3, and they have downstream signalling effects on Rho/Rac (For review see (Zhao et al., 2006)). PAK can stimulate actin polymerisation (Gorovoy et al., 2005), axon outgrowth and the formation of dendritic spines (Daniels et al., 1998) through LIM kinase stimulation (Gorovoy et al., 2005). PAK can also inhibit the myosin light chain kinase (MLCK) which diminishes actomyosin contractility (Goeckeler et al., 2000, Chew et al., 1998).

It has been reported that 13-month-old AD-like mice (PDAPP) displayed a substantial decrease in PAK 1-3 activity compared to normal controls (Nguyen et al., 2008). Furthermore, the hippocampus of patients exhibiting the early clinical signs of AD have displayed high PAK 1-3 activity, which was then shown to decline in the late stages of AD pathology (Nguyen et al., 2008). It was further suggested that C-terminal cleavage of APP at the Asp664 site mediates PAK abnormalities, and that an Asp664 mutation may potentially prevent these abnormalities

(Nguyen et al., 2008). On the other hand, ROCKs stimulate the retraction of axonal and dendritic growth cones by activating MLCK through the phosphorylation of myosin light chain proteins to promote an interaction with actin (Gallo, 2004). In addition, ROCK2 can phosphorylate collapsin response mediator protein-2 (CRMP-2), another microtubule-associated protein, to induce growth cone collapse (Arimura et al., 2000).

Moreover, many developmentally or pathologically regulated molecules can also activate the RhoA/ROCK pathway to inhibit axonal growth, including semaphorins, ephrins and myelin inhibitory factors such as Nogo and myelin-associated glycoprotein (MAG). On the other hand, there are some signalling molecules such as Sema4D/plexin-B1 that activate the RhoA/ROCK pathway, especially in hippocampal neurons, and may induce dendritic spine formation. It has been speculated that this may be due to the activation of LIM kinase and the PAK-type response via actin-depolymerising factor ADF/cofilin (Niederöst et al., 2002, Heredia et al., 2006). In AD, dendritic spine defects play a major role in cognitive impairments (Zhao et al., 2006). It has been reported that dendritic post-synaptic proteins are excessively distorted with disease progression (Gylys et al., 2004). For instance, neuronal loss in the hippocampus of AD patients is approximately 5–40% while the loss of postsynaptic proteins such as the developmentally regulated actin-regulating brain protein (drebrin), which is targeted by Aβ oligomers, reaches 70–95% (Ma et al., 2008). This study in particular suggested that Aβ-induced alteration in postsynaptic PAK may have a central role in the massive drebrin loss and cognitive deficits found in AD. This means that it could be prevented by an antibody to Aβ and/or by *in vivo* or *in vitro* overexpression of wild-type PAK (Ma et al., 2008).

Cognitive defects and eventually dementia are important clinical features of AD (For review see (Kumar et al., 2012)). It has been reported that there exist a relationship between the cognitive decline occurring in AD, along with genetic mental retardation syndromes, and synaptic dysfunction, primarily since the post-synaptic maintenance of dendritic spines is lost. To maintain synaptic balance, both ROCK1 and 2 transduce signals to retract the growth cones and dendritic spines (For review see (Salminen et al., 2008)). It has been shown that ROCK may provoke APP breakdown to the toxic Aβ1–42 peptide. For example, ROCK inhibitors, such as Y27632, inhibit the toxic processing of APP (Leuchtenberger et al., 2006). An intriguing conundrum is that the binding of Aβ on neurons may activate RhoA and ROCK2 to potentiate the phosphorylation of its substrates (Del Pozo et al., 2004, Guirland et al., 2004). One of the specific substrates that our group has recently defined is CRMP-2 (Figure 3). It has been shown that CRMP-2 exhibits hyperphosphorylation in the cortex of AD post-mortem brains (Cole et al., 2007). Experimentally, it has been illustrated that other kinases such as GSK-3 and Cdk5 can also phosphorylate CRMP-2 and produce growth cone collapse in neurons (Uchida et al., 2005b) (Figure 3). Our data suggest that Aβ can increase the RhoA-GTP level in differentiated SH-SY5Y cells increasing CRMP-2 phosphorylation and reducing the neurite lengths in cultured neuroblastoma cells. Additionally, RhoA and CRMP-2 levels are elevated in neurons surrounding amyloid plaques in the cerebral cortex of the APP (Swe) Tg2576 AD mouse model. Our work indicates that Aβ induces Rho GTPase activity and ROCK2 to promote CRMP-2 phosphorylation which can lead to the inhibition of neurite outgrowth (Petratos et al., 2008b) (Figure 3). However, a direct link with the reduction of ROCK2-dependent CRMP-2 phosphorylation and the limitation of cognitive decline is yet to be established in the context of Aβ-dependent neurodegeneration.

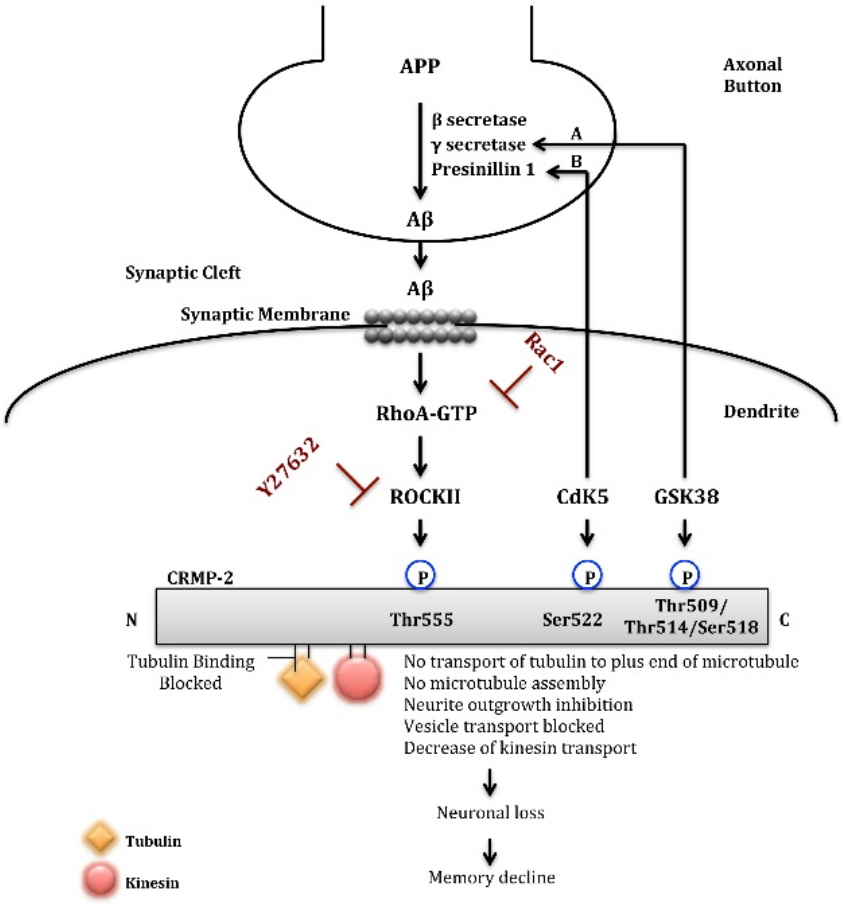
APP
Axonal Button
β secretase
γ secretase
Presinillin 1
A
B
Aβ
Synaptic Cleft
Aβ
Synaptic Membrane
Rac1
RhoA-GTP
Dendrite
Y27632
ROCKII
CdK5
GSK38
P
P
P
CRMP-2
N
Thr555
Ser522
Thr509/ Thr514/Ser518
C
Tubulin Binding Blocked
No transport of tubulin to plus end of microtubule
No microtubule assembly
Neurite outgrowth inhibition
Vesicle transport blocked
Decrease of kinesin transport
Neuronal loss
Memory decline
Tubulin
Kinesin

Figure 3: Model of Aβ-mediated neurite outgrowth inhibition. Aβ (oligomeric) activates the small GTPase, RhoA, which inhibits the pro-neurite outgrowth GTPase Rac1.

RhoA-GTP activates ROCK2 to effect microfilament rearrangement and also potentiate microtubule disassembly. Microtubule disassembly occurs when ROCK2 directly phosphorylates CRMP-2 at the Thr555 position preventing the association of CRMP-2 with tubulin heterodimers, thereby leading to neurite outgrowth inhibition. Neurite outgrowth is further impeded by CRMP-2 phosphorylation since this prevents the microtubule motor protein, kinesin, from associating with CRMP-2 and transport growth-related vesicular cargo, such as BDNF, anterogradely to the distal end of the neurite. It is demonstrated that CRMP-2 is also phosphorylated by GSK-3β at the Thr509, Thr514 and Ser518 sites after priming phosphorylation of CRMP-2 with Cdk-5 at the Ser522 site. Studies have demonstrated that GSK-3β activity can also regulate the processing of APP, resulting in the production of Aβ, which in turn can further increase GSK-3β activity through PI3K inhibition, illustrating as a potential feedback loop (A). Additionally, it has been suggested that Cdk5 may phosphorylate presenilin-1 at Thr354, destabilising its carboxy-terminal fragment and leading to increased APP processing (B) (Mokhtar et al., 2013).

1.9.2 *Glycogen synthase kinase-3β (GSK-3β)*

The proline-directed serine/threonine kinase, GSK-3, is important for several cellular processes such as metabolism, cell structure, apoptosis and in the regulation of gene expression (For review see (Proctor and Gray, 2010)). The GSK-3 family contains two members, GSK- 3α and GSK-3β, which are highly expressed in the brain and spinal cord. GSK-3β in particular plays a central role in neuronal differentiation and the maintenance of neurons (For review see (Kim and Snider, 2011)). As shown in Figure 3, activation of GSK-3β requires pre-phosphorylation of CRMP-2 by other priming kinases such as Cdk5 at serine or threonine sites located 4 residues, C-terminal to the site phosphorylated by GSK-3β (For review see (Cole et al., 2004)). Abnormal GSK-3 function has been implicated in different brain pathologies indicating its fundamental role in controlling basic mechanisms of neuronal function, modulation of neuronal polarity, migration, proliferation and survival, not to mention the establishment of neuronal circuits (For review see (Frame and Cohen, 2001)). It has been demonstrated that phosphorylation of GSK-3 may influence cytoskeletal proteins altering neuronal plasticity (For review see (Salcedo-Tello et al., 2011)). Neuronal cytoskeletal changes occur due to an altered rate in the stabilisation and destabilisation of microtubules (MT), thereby altering the dynamics of dendrites, spines, axons and synapses. Intensified efforts in the identification of enzymes involved in regulating tau phosphorylation *in vivo*, have revealed GSK-3β as a candidate kinase for therapeutic targeting (Proctor and Gray, 2010) during AD pathology.

GSK3β is involved in many aspects of AD pathogenesis (Medina and Avila, 2014). Firstly, it has been suggested that GSK3β-dependent activation of BACE1 enhances Aβ deposition and plaque formation (Ly et al, 2013). In addition, there is evidence that GSK3β can phosphorylate PS1 and

activate γ-secretase (Twomey and McCarthy, 2006). Additionally, it has been suggested that the APP intracellular domain (AICD) is a substrate for GSK3β *in vitro* and *in vivo* (Aplin et al, 1997, Aplin et al, 1996). In Drosophila, GSK3β phosphorylates and minimises the number of motors that are bound to microtubules (Weaver et al, 2013). In a squid axoplasm model of AD, PP1-GSK3β signalling phosphorylates KLC-1 and dissociates KIF5 from vesicular cargos (Morfini et al, 2002).

It has been hypothesised that GSK-3 overactivity may potentiate sporadic and familial forms of AD by enhancing tau hyperphosphorylation (Qian et al., 2010), APP processing and possibly through the phosphorylation of CRMP-2 leading to profound memory impairment (Cole et al., 2004) (Figures. 2 and 3). It has been established that the expression of full-length unmodified or unphosphorylated CRMP-2, in primary hippocampal neurons or SH-SY5Y neuroblastoma cells, promotes axon elongation. Moreover, cultured neurons expressing CRMP-2 with mutant GSK-3 phosphorylation sites (T509A, S518A) display significantly reduced axon elongation (Cole et al., 2004). On the other hand, studies have demonstrated that GSK-3β phosphorylation of the CRMP-2 T509 site can play a crucial role in mediating the repulsive action of Sema3A (Ryan and Pimplikar, 2005) and promoting growth cone collapse (Uchida et al., 2005b). More recently, Cole et al (2008) have demonstrated that dephosphorylation of CRMP-2 at the GSK-3β-dependent sites (Ser-518/Thr-514/Thr-509) can be carried out by a protein phosphatase 1 (PP1) in vitro, observed in neuroblastoma cells and primary cortical neurons, showing that the inhibition of GSK-3β by insulin-like growth factor-1 or the highly selective inhibitor CT99021, results in dephosphorylation of CRMP-2 at these sites (Cole et al., 2008). How can this be translated to real therapeutic outcomes during AD pathology is yet to be demonstrated.

1.9.3 *Cyclin-dependent kinase-5 (Cdk5)*

The other proline-directed serine/threonine kinase, identified as a major priming enzyme for tau phosphorylation, is Cdk5 (Piedrahita et al., 2010). Although Cdk5 is ubiquitously expressed in most tissues, it is not directly involved in mediating progression through the cell cycle as it requires prior activation by p35 and p39, which are expressed almost exclusively in the CNS (Peterson et al., 2010). Cdk5 plays an important role in CNS development, possibly by mediating interactions between neurons and glia during radial migration, which is essential for developing appropriate cortical laminar architecture (Chae et al., 1997, Ohshima et al., 1996). Furthermore, Cdk5 has also been reported to play a role in neuronal differentiation, axonal guidance, synaptic plasticity, cellular motility, cellular adhesion and neurodegeneration (For review see (Cheung and Ip, 2012)).

Studies have shown that inhibition of Cdk5 reduces Aβ-induced neurodegeneration in cortical neurons (Wen et al., 2008), highlighting the possibility that targeting Cdk5 could be a future therapeutic strategy for neurodegenerative disorders. The critical microtubule-associated protein, CRMP-2, has been also demonstrated to be a substrate for Cdk5 (Uchida et al., 2005b). This study showed an orderly phosphorylation process of CRMP-2 by Cdk5 (defining it as the priming kinase) followed by GSK-3β as a consequence of Sema3A stimulation that inhibits axonal growth (Uchida et al., 2005b). A non-phosphorylated form of CRMP-2 cannot respond to Sema3A signalling. This study also demonstrated that Sema3A promotes phosphorylation of CRMP-2 at Ser522, which is the established Cdk5 phosphorylation site (Uchida et al., 2005b). It has been demonstrated that Cdk5 phosphorylates APP at T668, leading to Aβ overproduction

(Lee et al., 2003, Iijima et al., 2000a). It has also been found that Cdk5 hyperphosphorylates neurofilaments and limits their association with kinesin, interrupting their transport through the axons (Lee et al., 2011). On the other hand, studies have suggested that Cdk5 inhibition activates GSK3β, leading to dissociation of kinesin from cargo (Morfini et al, 2004). Thus, targeted kinase inhibitors may possibly be therapeutically beneficial in AD to limit both tau and CRMP-2 phosphorylation. The process of deciphering which of the kinases are most critical in neurodegeneration is still on going, but when elucidated, finding novel inhibitors against those kinases may possibly prevent the cognitive decline associated with AD.

1.9.4 *Phosphatases*

Protein phosphatases provide unique endogenous signalling mechanisms for the dephosphorylation of proteins, reversing such post-translational modifications, which may limit protein dysfunction. Protein phosphatase 2A (PP2A) is one of the most important serine/threonine phosphatases in the mammalian brain. It is present in most tissues, comprising up to 1% of total cellular protein. It has major roles in development, cell growth, transformation (For review see (Liu and Wang, 2009)), regulation of protein phosphorylation and cell signalling pathways (Hill et al., 2006). PP2A is composed of three subunits: subunit A (scaffolding/structural); subunit B (regulatory/targeting); and subunit C (catalytic) (Braithwaite et al., 2012). PP2A with PP1 collectively account for more than 80% of the total serine/threonine phosphatase activity in all mammalian cells (Liu and Wang, 2009, Martin et al., 2013) making these enzymes integral to cellular physiology.

In situ, PP2A, PP1, PP5 and PP2B accounts for 71%, 11%, 10% and 7%, respectively, of the total tau phosphatase activity in the human brain (Liu et al., 2005). PP2A is the most prevalent phosphatase involved in tau dephosphorylation (Xu et al., 2008). Knockdown of PP2A phosphatase activity was shown to lead to tau hyperphosphorylation (Kins et al., 2001). Furthermore, when PP2A was inhibited in cultured cells and in transgenic mice with mutant PP2A, hyperphosphorylation of tau was observed (Kins et al., 2001). Moreover, the naturally abundant SET protein, a potent PP2A inhibitor, is found to be elevated in AD brains (Tanimukai et al., 2005), possibly illustrating reduced PP2A activity that in turn allows for the hyperphosphorylation of cellular substrates to occur unabated and the potentiation of neurodegeneration. Interestingly, autopsy studies of brains from AD patients, non-AD dementia patients and normal human brains demonstrate that there is a loss of PP2A protein, mRNA and enzymatic activity in areas of the brain affected by AD (the hippocampus and cortex) but not in the cerebellum (Sontag et al., 2004). In addition, the inhibition of PP2A activity mimics most of the phosphorylation events seen in AD, such as tau hyperphosphorylation (Gong et al., 2000).

Phosphorylation of APP by an array of kinases has been shown to influence its cleavage by β-secretase resulting in Aβ production (Ando et al., 2001). It has also been demonstrated that PP2A has the ability to dephosphorylate APP at the Thr668 site and thus inhibit Aβ generation (Iijima et al., 2000b). Studies of cells expressing the (APPswe) mutation, transgenic mice expressing both APPswe and PS mutations, and sections of hippocampus and entorhinal cortex from human AD patients show that PP2A levels are decreased and Y307 levels (an inhibitor of PP2A) are increased (Liu et al., 2008). This implies that the phosphatase affects the processing of APP and highlighting its importance in limiting AD pathology. In N2a cells, where PP2A was inhibited

with okadaic acid (OA), the phosphorylation of APP and the secretion of both sAPPα and sAPPβ were all elevated(Sontag et al., 2007). In addition, inhibition of the protein phosphatases PP1 and PP2A in the rat brain by OA results in the accumulation of hyperphosphorylated tau and Aβ species (Braithwaite et al., 2012, Arendt et al., 1998). Even though incubation of different types of cells with OA resulted in the stimulation of APP secretion, it was not proven that the effect was mediated by PP1 (Da Cruz Silva et al., 1995) and/or PP2A (Holzer et al., 2000). Moreover, it was demonstrated that demethylation of PP2A by nuclear phosphatase methylesterase-1 (PME-1) reduces its activity and thus leads to tau hyperphosphorylation along with APP phosphorylation, promoting APP cleavage and Aβ production (De Baere et al., 1999, Sontag et al., 2008, Vogelsberg-Ragaglia et al., 2001). Collectively, these results suggest that downregulation of PP2A may induce Aβ production and tau phosphorylation, precipitating AD pathology.

A direct link of PP2A activity with the progression of AD pathology has been associated with the fact that CRMP-2 phosphorylation may actually be a result of lowered PP2A activity (Hill et al., 2006). Since CRMP-2 hyperphosphorylation is commonly observed to correspond with progressive neurodegeneration, decreased PP2A may well regulate such a disease-specific event.

1.9.5 *Collapsin Response Mediator Protein (CRMP)*

CRMPs are members of the dihydropyrimidinase-related neuronal phosphoprotein family (Wang and Strittmatter, 1997). The CRMP family has five isoforms, CRMP1-5 (Hamajima et al., 1996). The most well-described of these, CRMP-2, is highly expressed in the adult mammalian CNS

localising in the cytoplasm and neurites of post-mitotic neurons (Wang and Strittmatter, 1997). CRMP-2 is also highly expressed in the areas of the adult brain that show the greatest plasticity such as the hippocampus, olfactory bulb and cerebellum (Wang and Strittmatter, 1996). In neurons, CRMP-2 is concentrated within the distal portions of neurites, in synapses and in growth cones (Inagaki et al., 2001). It regulates the polarity and differentiation of neurons through the assembly and trafficking of microtubules (Morita and Sobuě, 2009). CRMP-2 has no known enzymatic activity by itself but through an interaction with other binding partners it can regulate neural differentiation, dendrite/axon fate specification, Ca^{2+} homeostasis, neurotransmitter release, regulation of cell surface receptor endocytosis, kinesin-dependent axonal transport, growth cone collapse, neurite outgrowth, and microtubule dynamics (For review see (Arimura et al., 2004, Petratos et al., 2008b)). The last three functions have shown to be regulated by phosphorylation near the C-terminus of CRMP-2 by kinases (Arimura et al., 2005b, Yoshimura et al., 2005b) including Cdk5, GSK-3β (Hensley et al., 2011, Soutar et al., 2009, Cole et al., 2008, Cole et al., 2007), Tau-tubulin kinase-1 (TTBK1) (Asai et al., 2012), and ROCK2 (Arimura et al., 2005b, Petratos et al., 2008b, Yoneda et al., 2012), all of which culminate in neurite retraction (For review see (Petratos et al., 2008b)). CRMP-2 hyperphosphorylation in AD was suggested to be a result of increased kinase activity, decreased phosphatase activity, or both (Cole et al., 2008). All phosphorylation events can disrupt the association of mature full-length CRMP-2 with tubulin heterodimers, possibly resulting in the destabilisation of the neuronal microtubule system rendering axonal retraction (Arimura et al., 2000). Moreover, disruption of the binding between CRMP-2 and tubulin due to the phosphorylation of CRMP-2 can block tubulin transport to the plus ends of microtubules for assembly (Figure. 3) (Petratos et al., 2008b), blocking neurite outgrowth and elongation. In

primary neurons and neuroblastoma cells, it has been demonstrated that overexpression of CRMP-2 results in axon elongation (Inagaki et al., 2001), while overexpression of truncated CRMP-2, lacking the C-terminus tubulin binding domain, inhibits axon growth. These data implicate this region of CRMP-2 in playing a central role in axonal growth (Inagaki et al., 2001). Both Cdk5 and GSK-3β phosphorylation of CRMP-2 have been shown to be increased in the cortex and hippocampus of the triple transgenic mouse (PS1/APP/Tau mutant), along with the double transgenic mouse (PS1/APP mutant), which develop AD-like plaques along with NFTs. However, in transgenic mice, which display only mutant tau (P301L) and develop tangles but do not develop amyloid plaques, Cdk5 phosphorylation of CRMP-2 does not occur. These results indicate that hyperphosphorylation of CRMP-2 might be induced by APP overexpression and/or its enhanced processing, thereby generating a high amyloid load within the brain of these transgenic mice (Cole et al., 2007).

Our laboratory has recently demonstrated that in human neuroblastoma SH-SY5Y cells and in the Tg2576 mouse model of AD, Aβ can reduce the length of neurites by deactivating the neurite outgrowth-signalling molecule Rac1 (Petratos et al., 2008b). Furthermore, the data suggested that Aβ-mediated reduction in neurite length could be reversed by the Rho Kinase inhibitor, Y27632. Additionally, the Aβ-mediated decrease in neurite length was linked to the promotion of a threonine phosphorylation of CRMP-2 (unrelated to GSK-3β-dependant phosphorylation), conferring a reduced binding capacity to tubulin. Both of these processes can be reversed by inhibiting RhoA activity (Petratos et al., 2008b). These data suggest that Aβ-mediated neurite outgrowth inhibition results from the activity of RhoA-GTP and the dysregulation of CRMP-2 to bind tubulin for neurite outgrowth (Petratos et al., 2008b) (Figure. 3).

Studies using transgenic mouse models expressing the Swedish familial AD mutant (APP/ TTBK1) demonstrated that the induced up-regulation of TTBK1 can promote axonal degeneration via phosphorylation of CRMP-2 and tau within the entorhinal cortex and hippocampus, implicating TTBK1 as a potential therapeutic target for AD (Asai et al., 2012).

Despite the profound link to CRMP-2-dependent degeneration through kinase-mediated phosphorylation, another function of CRMP2 is mediated through its known association with kinesin, facilitating the anterograde molecular transport of growth-promoting vesicles along axonal microtubules (Kimura et al., 2005a). The exact mechanism of binding and transport and its contribution to AD will be discussed in detail below.

1.10 CRMP2-Tubulin binding

The microtubule and actin cytoskeleton orchestrates axonal growth cone dynamics through a process of signal transduction, leading to either depolymerisation or polymerisation events for directional growth (Soutar et al., 2009). As already discussed above, the binding of CRMP-2 to tubulin heterodimers can enhance microtubule assembly, leading to axon outgrowth (Kawano et al., 2005, Nishimura et al., 2003). Semaphorin-3A (Sema3A) is an extracellular protein that can block axonal outgrowth (Uchida et al., 2005b) through the activation of Cdk5, with downstream phosphorylation of both tau and CRMP-2 (Uchida et al., 2005b, Hensley et al., 2011). Such phosphorylation can disrupt their tubulin association limiting axonal growth. Following the Cdk5 phosphorylation of CRMP-2, the latter may potentiate a conformational change leading to subsequent phosphorylation by GSK-3β (Uchida et al., 2005b, Hensley et al., 2011). However, it has been demonstrated that in GSK-3β overexpressing mice, no hyperphosphorylation of CRMP-2 can be identified at the GSK-3β phosphorylation sites, and furthermore, phosphorylation of tau does not increase (Tan et al., 2013). This may explain the finding that activation of GSK-3β alone cannot induce growth cone collapse (For review see (Soutar et al., 2009)). Interestingly, protein lysates from human AD cortex and animal models of AD show hyperphosphorylation of CRMP-2 at residues Thr509, Thr514 and Ser518, which are known to be GSK-3β phosphorylation sites, as well as Ser522, a well-known Cdk5 phosphorylation site (For review see (Petratos et al., 2008b)). These findings indicate that Sema3A signalling may regulate microtubule polymerisation through the physiological actions of tau and CRMP-2, which regulate the dynamics of microtubules and tubulin dimers respectively (Fukata et al., 2002b). Phosphorylation of CRMP-2 by Rho kinase at the Thr555 site however, can also reduce the CRMP-2 association with tubulin heterodimers and induce growth cone collapse. This is

unrelated to Sema3A signalling and could quite possibly be the result of Aβ-dependent signalling (Uchida et al., 2005b, Hensley et al., 2011). The phosphorylation of CRMP-2 by Cdk5, GSK-3β and Rho kinase may therefore play a central role in coordinating cytoskeletal activities in response to multiple axon guidance cues (Uchida et al., 2005b, Hensley et al., 2011).

There is a plausible hypothesis that the activation of the three kinases Cdk5, GSK-3β and ROCK2 contributes to the destabilisation of the neuronal microtubule system in AD. Consequently, tau and CRMP-2 have some similarities in that both control microtubule polymerisation and stability, and they both respond to the growth cone guidance molecule Sema3A (Uchida et al., 2005b). Therefore, it might be theorised that a balanced treatment which may successfully decrease CRMP-2 phosphorylation could also be effective in regards to tau aggregation and vice versa in AD (For review see (Hensley et al., 2011)).

1.11 Microtubules (MT)

One of the most important physiological features of the multipolar neuron is that it has a polarised axon, one that can extend to more than 1 meter in the human CNS (Reis et al., 2012). For a neuron to function normally, it should be able to transport vital molecular cargo from its body to synaptic terminals, and vice versa, in a timely manner through the axon via anterograde and retrograde transport mechanisms respectively (Reis et al., 2012, Falzone and Stokin, 2012). Therefore, it stands to reason that the integrity of the microtubule transport system is crucial for axonal transport (Iqbal and Grundke-Iqbal, 1995). The microtubule system facilitates ATP driven transport through molecular motors of the cell's vital components, which include vesicles,

proteins, mitochondria, chromosomes, and large macromolecules such as microtubule heterodimers themselves (Potter et al., 2011, Falzone and Stokin, 2012). The transport machinery directly interacts with microtubules and includes two families of proteins categorised according to their directional movement. These proteins include either microtubule plus end–directed kinesins or the microtubule minus end–directed cytoplasmic dynein (Reis et al., 2012).

Many neurodegenerative diseases, such as AD, display a blockade in microtubule transport, emphasising the significance of this transport in normal physiology and highlighting abnormal neuronal vesicle trafficking as a potential pathogenic mechanism (Potter et al., 2011, Brunden et al., 2011, Ye et al., 2012). It is believed that Aβ may cause mitochondrial dysfunction and therefore, axonal transport defects (Ye et al., 2012). It has been demonstrated that APP processing and Aβ overproduction in the mitochondria leads to mitochondrial dysfunction and therefore reduction of mitochondrial energy supply and inhibition of axonal transport (Mao and Reddy, 2011). Enhancing the energy supply of neurons could be critical to compensate for the Aβ-dependent loss of energy and thus facilitate axonal transport.

Microtubule depolymerisation has been proposed as a contributing factor in the gross loss of memory, as it is necessary to stabilise newly formed microtubules in spines for long-lasting memory (Goldstein, 2009, Mitsuyama et al., 2012). There is evidence implicating tubulin sequestration (Paula-Barbosa et al., 1987) and blockade in microtubule assembly as a pathogenic mechanism in AD (Iqbal and Grundke-Iqbal, 1995). It has been demonstrated that *in vitro*, microtubules can be assembled from the cytosol of normal autopsy brain tissue obtained within five hours post-mortem, while this is not possible from identically treated AD post-mortem brain

tissue (Iqbal and Grundke-Iqbal, 1995). Furthermore, it has been documented that axonal transport is defective in neurons from AD post-mortem brains, indicating the destruction of the microtubule cytoskeleton in the axons of diseased neurons (Goldstein, 2009). There is also data suggesting that the abnormality in axonal transport might stimulate the formation of, or enhance the accumulation of Aβ (Goldstein, 2009, Flala, 2007), through autophagocytosis of mitochondria without normal lysosomal degradation (Flala, 2007).

One of the main physiological functions of tau is to stimulate microtubule assembly by polymerising with tubulin, maintaining the microtubule structure and stability through its capacity to anchor polymerised microtubules to the internal axolemma (Iqbal and Grundke-Iqbal, 1995). Evidence for the role of tau and microtubule destabilisation arises from tau transgenic mice that show spinal cord tau inclusions (Brunden et al., 2011). In this animal model, the inability of tau to stabilise microtubules can be compensated for by the MT-stabilising agent paclitaxel, resulting in increased MT density and marked improvement in motor function (Brunden et al., 2011). However, paclitaxel is thought to have poor blood–brain barrier permeability, and thus is an unlikely candidate for human therapy during neurodegeneration (Brunden et al., 2011).

In the early-stages of AD pathogenesis, observations within the neuropil demonstrate that there exists an abnormal aggregation of the activated actin-associated protein cofilin, a protein that modulates actin-rich dendritic spine architecture and is therefore important for learning and memory (Whiteman et al., 2009). Those neuropil threads can disrupt the cytoskeletal network by blocking cargo trafficking to synapses, resulting in memory and cognition impairment

(Whiteman et al., 2009). It is also suggested that abnormal activation of cofilin may trigger the accumulation of phosphorylated tau in neuropil threads (Whiteman et al., 2009). The activities of cofilin and the protein actin-depolymerising factor (ADF) are regulated by phosphorylation and dephosphorylation through LIM and other kinases, along with chronophin phosphatases, respectively (Whiteman et al., 2009). Heredia et al. (2006) found that Aβ may activate LIMK1 and thus stimulate ADF/cofilin phosphorylation in cultured neurons (Heredia et al., 2006). Moreover, these authors demonstrated, in the AD brain, that the number of P-LIMK1-positive neurons was extensively increased in the affected regions (Heredia et al., 2006). A recent study of AD transgenic mice, demonstrated that neuronal cell bodies are viable although the neurites are damaged (Adalbert et al., 2009). Taken together, these studies highlighted the possibility that the development of *in vivo* methods targeting the disruption of LIMK1 activation, the formation of the cofilin–actin rods and/or the interaction between cofilin and pMAP may be a plausible way to stop the disease early in its presentation.

Evidence suggests that AD is caused by increased processing of APP, leading to elevated Aβ levels and formation of amyloid plaques. Besides amyloid plaque pathology, NFT pathology occurs along with loss of synaptic connectivity, neuritic dystrophy and neuronal death. As tau in NFTs is hyperphosphorylated and dissociated from MTs, its plausible to suggest that Aβ overproduction causes tau hyperphosphorylation and its relocation into a dendritic compartment, in turn causing MT depolymerisation and neuronal death (Busciglio et al., 1995, Ferreira et al., 1997, Ittner and Gotz, 2011, LaFerla, 2010, Takashima et al., 1996, Takashima et al., 1993, Takashima et al., 1995, Zheng et al., 2002). Although several studies hypothesised the 'Aβ to tau' pathogensis in AD neurotoxicity, the exact pathway connecting Aβ to tau is poorly

understood (Ittner and Gotz, 2011). One hypothesis that is yet to be tested is that Aβ may disrupt MT stability, leading to tau hyperphosphorylation as a reaction to compensate for the changes in MT. It has been demonstrated in non-neuronal cells that do not express tau, that Aβ disrupts MT assembly and maintenance of the mitotic spindle through the inhibition of motor proteins called kinesins (Borysov et al., 2011). It has also been demonstrated that Aβ can interrupt non-mitotic MT integrity of non-neuronal cells in the presence of tau (King et al., 2006). Deregulation of Rho GTPase signalling has been implicated in neurotoxicity by Aβ (Chacon et al., 2011, Petratos et al., 2008b, Pozueta et al., 2013). Actin dynamic modifications are considered to be the mechanism by which Rho contributes to the inhibition of dendritic spine outgrowth and thus inhibits synaptic transport, all early events occurring in neurons treated with Aβ (Lambert et al., 1998, Lefort et al., 2012, Lesne et al., 2006, Shankar et al., 2007). In neurons, APP and caspase-2 regulate RhoA activation, and cells deficient in either APP or caspase-2 are immunised against Aβ dependent synaptic damage (Pozueta et al., 2013, Troy et al., 2000). Additionally, cell surface APP dimerization by cross-linking with a divalent antibody is enough to mimic Aβ toxic effects in neurons (Lefort et al., 2012, Rohn et al., 2000). Activation of RhoA in neuronal cells by lysophosphatidic acid (LPA) is regulated by caspase-2 activity, suggesting that Aβ and LPA share pathways (Pozueta et al., 2013). Studies have shown that oligomeric Aβ1-42 enhances the formation of stable Glu MTs in non-neuronal cells and in primary neurons. In fibroblasts, they have showed that Glu MTs were generated through RhoA and its effector mDia1, demonstrating that Aβ has a conserves its function in the MT cytoskeleton independently of tau, and that Aβ regulates the stabilization of MTs and modifies the levels of tubulin detyrosination associated with MT longevity (Pianu et al., 2014). These authors have also demonstrated that Aβ induces RhoA-GTP, FAK activation and focal adhesion assembly and increases the levels of F-actin and

phospho-myosin light chain. It has been shown that integrins or FAK signalling are involved in Aβ-dependent neurotoxicity. Aβ binds to integrins (Sabo et al., 1995) and these regulate Aβ uptake and toxicity (Bi et al., 2002). Primary cortical neurons treated with Aβ show FAK activation (Williamson et al., 2002), and activation of focal adhesion proteins mediating Aβ–dependent neuronal dystrophy (Grace et al., 2002, Williamson et al., 2002). Moreover, APP, a potential receptor for Aβ (Lorenzo et al., 2000), is found at focal contacts (Sabo et al., 2001), it colocalises with b1 integrins in neuronal cells (Yamazaki et al., 1997), and antibody-mediated clustering of APP enhances significant loss of dendritic spines (Lefort et al., 2012) and neuronal injury through FAK activation (Xu et al., 2009). Studies have demonstrated that the clustering of APP is enough to induce Glu MTs, and both APP and caspase-2 expression were required for the formation of stable Glu MTs by Aβ (Pianu et al., 2014), suggesting that induction of MT stability by Aβ is initiated by the same APP/caspase-2/RhoA pathway that leads to Aβ neurotoxicity in neurons (Lefort et al., 2012, Pozueta et al., 2013, Troy et al., 2000). More importantly, should the importance of these findings to AD be confirmed, this work will provide novel targets for the development of new therapeutic strategies to rescue the impairment of cell function and cognition in AD.

1.12 Kinesin

All eukaryotic cells require a microtubule-based transport system to mediate the intracellular transport of important proteins and organelles. This transport system is fundamental for neuronal cells because of their morphology and polarity, and it is required for proper communication between the following structures: the cell body; dendrites, which receive information; and axons, which transmit information to other neurons (Arimura and Kaibuchi, 2007, Craig and Banker, 1994). Throughout FAT, axons can supply nerve endings with neurotrophic factors, lipids and mitochondria, and inhibit the accumulation of toxic molecules (Hinckelmann et al., 2013, Millecamps and Julien, 2013). Kinesin and dynein are key motor proteins for the transport of molecular cargo anterogradely towards the synapse and retrogradely towards the cell body, respectively.

Studies have demonstrated that there are more than 40 kinesin genes in the human and mouse genomes. These have been categorized into 14 subfamilies based on phylogenetically conserved similarities in their motor domains and in other parts of the proteins (Hirokawa et al., 2010, Lawrence et al., 2004). Kinesin-1 is highly expressed in neurons and is vital in the transport of various membranous and nonmembranous molecules. It consists of two heavy chains (KHCs) and two light chains (KLCs) (Bloom et al., 1988) that have both an ATP, and the microtubule-binding motif which are essential for vesicle transport (Dhaenens et al., 2004). In mammals, KHC is expressed in threes isoforms, KIF5A, B, and C, with specificity of KIF5A and 5C to the nervous system (Niclas et al., 1994). Each KHC contains the following elements: a motor head domain that binds to microtubules and hydrolyzes ATP; a neck linker; a stalk that is involved in dimerisation; and a tail that limits the ATPase activity of the head and also binds to microtubules

(Kaan et al., 2011, Dietrich et al., 2008, Wong and Rice, 2010). Adaptor proteins mediate molecular cargo binding and keep kinesin-1 in an active state, thus enhancing the anterograde axonal transport system. In mammals, four KLC isoforms (KLC 1–4) stimulate kinesin-1 activation by suppressing tail-head and tail-microtubule interactions (Dietrich et al., 2008, Gyoeva et al., 2004, Wong and Rice, 2010). For instance, in the absence of molecular cargo, KLC1 keeps kinesin-1 in an autoinhibited state, which can be relieved upon cargo binding to KLC1. KLCs are also important in the selection of the molecular cargo. They express a coiled-coil domain, which interacts with the stalk domain of KHC to form a tetramer (Diefenbach et al, 1998), and a series of six tandem repeats, termed tetratricopeptide repeats (TPRs), which mediate protein-protein interactions (Stenoien et al, 1997, Gindhart et al, 1998, Verhey et al, 2001, Pernigo et al, 2013). Downstream of the TPR domain is the C-terminal domain, which varies in size and sequence. It has been suggested that variation in the C-terminal sequences is integral for kinesin-1 association to different structures (McCart et al, 2003). It has been demonstrated that variant B binds to mitochondria (Khodjakov et al, 1998), while D and E bind to rough ER (Woznaik et al, 2006) and Golgi membranes (Gyoeva et al, 2000). In humans, 19 variants of KLC1 have been found, representing a potential to produce 285,919 spliceforms (McCart et al, 2003). The expression levels, functions, and regulatory mechanisms of many neuronal KLC variants have not yet been identified in either healthy or disease individuals.

Proper FAT requires the kinesin-dependent transport of neurotransmitter-containing vesicles (Goldstein, 2012), as well as proteins required for neurotransmitter exocytosis, a process regulated by protein zeta-1 (FEZ1) and other protein kinases (Fang et al., 2014). There is evidence for defective FAT in some neurodegenerative diseases, such as HD and AD

(Hinckelmann et al., 2013, Millecamps and Julien, 2013, Goldstein, 2012). Neuropathology studies have also demonstrated microtubule depolymerisation, impaired transport systems, synaptic loss, and neuritic dystrophy (Terry, 1998). Early studies correlated these changes to sporadic mutations that enhance the aggregation of toxic molecules and disrupt neuronal metabolism and homeostasis (Hinckelmann et al., 2013, Goldstein, 2012). However, more recent studies have suggested that genetic mutations in kinesins, dynein, adaptors, and microtubule-associated proteins lead to neurodegeneration, suggesting that FAT has a fundamental role in disease progression (Farrer et al., 2009, Hinckelmann et al., 2013, Lazarov et al., 2007). In AD, arguments as to whether deficits in FAT lead to or arise from Aβ toxicity have not been resolved. Early symptoms associated with AD include defects in spatial relationships, perception, and orientation. It has been suggested that KLC1vE enrichment can disrupt the transport of APP and other KLC1 cargoes that regulate amyloidogenesis, leading to intracellular Aβ accumulation and release of Aβ into the extracellular space. In several AD transgenic mouse models (3xTg-AD, Tg2576, and 5xFAD), synaptic failure and cell death precede amyloid plaque formation. Intracellular Aβ forms wherever APP and secretases present, such as the plasma membrane, trans-Golgi network, ER, and endosomal, lysosomal, and mitochondrial membranes (For review see (LaFerla et al, 2007)). In AD, APP is insufficiently cleared leading to BACE cleavage and subsequent Aβ generation (Li et al, 2015, Kudu et al, 2006).

Proper axonal transport systems require other protein complexes, called adaptors, which are associated with the molecular motor proteins to regulate cargo interactions through extracellular and intracellular signals (For review see (Fu and Holzbaur, 2014)). It is now well established that CRMP-2 plays a central role in negotiating fast axonal transport by acting as an adaptor protein

to the microtubule motor kinesin-1. In this way it propagates the anterograde vesicle transport of key traffic molecules, such as the high-affinity neurotrophin receptor tyrosine kinase (TrkB). Following distal localisation of this receptor, TrkB is inserted into the cell membrane and activated by its cognate ligand brain-derived neurotrophic factor (BDNF), resulting in axonal growth through signalling within the growth cone, thereby establishing the accumulation and polymerisation of F-actin and tubulin. In AD, phosphorylated CRMP-2 releases kinesin-1, inhibiting TrkB function and limiting the structural integrity of the actin-based cytoskeleton in distal axons, growth cones and synapses (Quach et al., 2004). Inhibiting CRMP-2 phosphorylation could be beneficial to restore tubulin and kinesin-1 binding to CRMP-2 thus promoting axonal outgrowth and transport of important molecular cargo.

APP axonal transport is mediated by direct binding to KLC1 (Kamal et al., 2000). APP is one of the molecular candidates for receptors that attach kinesin-1 to vesicular cargo (For review see (Goldstein, 2001)). The carboxy-terminus of APP binds directly to the light-chain subunits of kinesin-1 (Dhaenens et al., 2004) and thus plays a major role in the recruitment of kinesin-1 to axonal vesicles (Szpankowski et al., 2012). Moreover, the level of axonal APP has been suggested to play a central role in determining expression levels of kinesin-1 decorating vesicles, providing the ability to determine the anterograde movement behaviour of APP-containing vesicles (Szpankowski et al., 2012). It has been reported that kinesin blockade and axonal swellings are involved in the pathogenesis of the early stages of AD even before the formation of amyloid plaques and neurofibrillary tangles, although the initiating events are not clear (Stokin et al., 2005b). Moreover, in animal models, Aβ formation and its subsequent transport is enhanced when kinesin transport is abrogated or impaired (Szpankowski et al., 2012, Rodrigues et al.,

2012a). Axonal transport damage results in the development of axonal swellings where APP is processed into smaller Aβ species. Genetic manipulation designed to damage APP axonal transport in AD mouse models, such as Tg-swAPP^{Prp} demonstrated an enhancement in the incidence of axonal swellings, elevated Aβ levels, and potentiated the production of amyloid deposition (Stokin et al., 2005b). Also, it has been shown that decreased KLC1 transport may stimulate tau hyperphosphorylation and formation of NFTs as well as axonal swellings producing catastrophic damage to axons. Such damage may arise from increased Aβ levels and tau hyperphosphorylation, further disrupting axonal transport (Falzone et al., 2010).

1.13 Conclusion

AD is an age-related progressive neurodegenerative disorder and is the most common form of dementia in the elderly. The hallmarks of AD pathology are the extracellular deposition of a 4 kDa amyloid beta (Aβ) polypeptide and the formation of intracellular NFTs, along with dystrophic neurites, degenerating neurons, and activated astrocytes and microglia, a part of the reactive pathology observed around senile plaques. Neuritic plaques result from the aggregation of Aβ, which is a consequence of the aberrant processing of APP. The corresponding accumulation of filamentous inclusions within the CNS as NFTs, resulting from the hyperphosphorylation of the microtubule-associated protein, tau, and amyloid deposition, are both pathognomonic to sporadic AD. There is an impressive list of genes and proteins involved in AD pathologies, including APP, presenilins, secretases, kinases and phosphatases, all of which have been touted as being responsible for either increasing the production of the neurotoxic Aβ protein or promoting the hyperphosphorylation of CRMP-2 or tau, processes that lead in turn to the devastating neurodegenerative sequelae. Gaining an understanding of the major gene players and how they cooperate with key environmental factors that contribute to the manifestation of AD pathology is key to a comprehensive understanding of AD pathogenesis. This comprehensive understanding is essential in the development of specific and more effective treatments for this devastating age-dependent disease.

1.14 Research rationale, hypothesis and aims

Research Rationale

The primary aim of this project was to investigate how Aβ aberrantly regulates CRMP-2 phosphorylation to induce neuritic abnormalities through the dysregulation of axonal transport. These experiments may aid in the identification of novel therapeutic targets that specifically inhibit aberrant phosphorylation of CRMP-2, thereby facilitating abnormal axonal transport and thereby reduced integrity.

This study sought to identify which Aβ-mediated kinase activity is initially responsible for inducing neurite retraction events by transducing neuron-like cells with phospho-specific mutant CRMP-2 constructs to correlate the perturbations in kinesin-dependent axonal transport and outgrowth. In addition, the specific CRMP-2 phosphorylation events that regulate neurodegeneration in the transgenic animal models of AD, as well as in the sporadic human form of AD, were identified and described.

Hypothesis

Phosphorylation of CRMP-2 can be potentiated by Aβ to alter microtubule dynamics in neurons of the cerebral cortex and hippocampus initiating neurite dysfunction and cognitive decline.

Aims

Aim 1: To determine the CRMP-2 phosphorylation sites regulated by Aβ, and to ascertain the Aβ-dependent phospho-CRMP-2 modifications that regulate axonal elongation and transport in SH-SY5Y cells.

Aim 2: To demonstrate the prominent kinases involved in Aβ-induced inhibition of neurite outgrowth and axonal transport in cultured neuron-like cells.

Aim 3: To investigate the: (i) Phosphorylation of CRMP-2 and its relationship to elevated Aβ levels in the Tg2576 mouse model of AD and (ii) Phosphorylation of CRMP-2 and its relationship to the dissociation from kinesin-1 and tubulin in human brain samples.

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Review Article

The Beta-Amyloid Protein of Alzheimer's Disease: Communication Breakdown by Modifying the Neuronal Cytoskeleton

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Alzheimer's disease (AD) is one of the most prevalent severe neurological disorders afflicting our aged population. Cognitive decline, a major symptom exhibited by AD patients, is associated with neuritic dystrophy, a degenerative growth state of neurites. The molecular mechanisms governing neuritic dystrophy remain unclear. Mounting evidence indicates that the AD-causative agent, β-amyloid protein (Aβ), induces neuritic dystrophy. Indeed, neuritic dystrophy is commonly found decorating Aβ-rich amyloid plaques (APs) in the AD brain. Furthermore, disruption and degeneration of the neuronal microtubule system in neurons forming dystrophic neurites may occur as a consequence of Aβ-mediated downstream signaling. This review defines potential molecular pathways, which may be modulated subsequent to Aβ-dependent interactions with the neuronal membrane as a consequence of increasing amyloid burden in the brain.

1. Introduction

Several neurodegenerative disorders share common characteristics including aggregation of misfolded mutant proteins in neurons leading to their deafferentation or loss with resultant structural or functional deficits in specific regions of the central nervous system (CNS) [1]. The most prevalent symptoms of age-related neurodegenerative disease are cognitive decline and movement disorders, along with brainstem and cerebellar signs. Such age-dependent disorders include Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and Spinal Cerebellar Ataxias (SCAs) [1]. There exists complexity in identifying fundamental molecular mechanisms precipitating neurodegeneration in these age-related brain diseases. However, common molecular signalling pathways have been defined in the specific neuronal populations associated with pathology [2]. Although the initiators of neuronal dysfunction may differ for each neurodegenerative disorder, there may be common molecular pathways which when being dysregulated, drive and exacerbate neurodegeneration. For example, the degeneration seen in AD is a result of amyloid plaques and phosphorylated tau deposition in the cerebral cortex and specific subcortical regions, leading to degeneration in the temporal lobe and parietal lobe, along with parts of the frontal cortex and cingulate gyrus [2]. AD also displays dysregulation in kinase and phosphatase mechanisms along with microtubule motor proteins during the degeneration phase [3, 4]. Therefore, a major question that remains unresolved is whether the dysregulation in specific kinases/phosphatases and vesicular transport mechanisms are aetiological contributors to AD pathology.

2. Neurodegeneration and Alzheimer's Disease (AD)

Over the past century, the ageing of our population (≥65 years) in industrialised countries has exceeded that of the population as a whole. It is predicted that in subsequent

generations, the proportion of the elderly population will double and so will the proportion of persons suffering from neurodegenerative disorders [5]. Diagnosis of neurodegenerative disease is usually based on clinical symptoms as there are no suitable noninvasive tests that can specifically predict onset of these conditions. However, with the advent of specialised magnetic resonance imaging (MRI) techniques, it is now possible to detect early pathological changes in the brain [6], providing clinicians with a unique window for early therapeutic intervention. Nevertheless, it is imperative that biomarker(s) of neurodegeneration are identified to assist in the early detection of these idiopathic cognitive disorders. Such biomarkers may take the form of modified proteins or peptides that are released into the circulation or alternatively sequestered intrathecally [1, 2].

Biomarkers of neurodegeneration may well be derived from dysfunctional/modified proteins that form the basis of pathological signal transduction cascades [1]. The dysregulation of signalling molecules central for maintaining neuronal function may stimulate the onset of neurodegeneration. For example, while Rho kinase (mainly ROCKII), glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase-5 (Cdk5), and phosphatases are all essential for normal neuronal development [7], they may all be involved in a plethora of neurodegenerative disorders through a central pathogenic mechanism.

3. Amyloid Beta (Aβ) and Amyloid Plaque Pathology

It is well documented that the aging process is the major determinant of developing amyloid plaques with or without disease [8]. These extracellular senile plaques are composed of accumulated Aβ protein aggregating as β-pleated sheets and are derived from the aberrant cleavage of the transmembrane protein, APP [9–11]. Under normal physiological conditions, APP is a cell surface protein that is thought to be involved in signal transduction, axonal elongation, and cell migration [12–20]. It was also demonstrated that the C-terminus of APP plays a central role in gene expression and neuronal cell survival [21]. Such physiological mechanisms are only effective when APP is cleaved by various enzymes which can include intramembranous degradation by beta-site AβPP-cleaving enzyme (BACE1) to form the β C-terminal fragment (βCTF) [22, 23], subsequently followed by gamma-secretase which forms the small 4 kilodalton (kDa) amyloid-β (Aβ) peptides Aβ1-40 and Aβ1-42, which are released at the synapse (Figure 1) [22, 24, 25]. It has been demonstrated that the extent of APP cleavage is amplified in AD brains and that Aβ treatment further enhances this cleavage [21]. It has also been established that APP and its degradation products localise to neuritic vesicles [26] in the axons of AD brains, along with other neurodegenerative diseases, suggesting that APP accumulation may represent a hallmark of axonal injury [27, 28]. For instance, in APP transgenic mice, it has been demonstrated that elevated Aβ levels result in the loss of synapses and neuronal transmission along with behavioural abnormalities, before the formation of amyloid

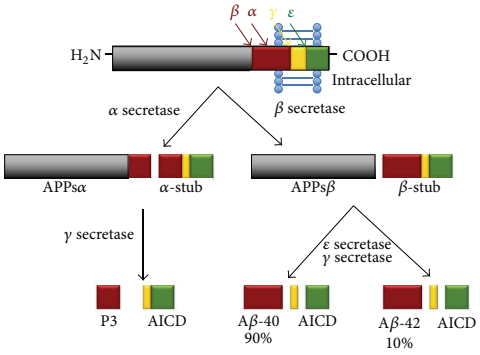

Figure 1: The processing of APP through the beta-site AβPP-cleaving enzyme BACE1, followed by presenilin-1 (PS1). Sequential beta and gamma-secretase cleavage of APP generates the synaptotoxic amyloid-β (Aβ) peptide species, Aβ1-40 and Aβ1-42.

plaques [21]. Accumulation of Aβ, mainly Aβ1-42, results in the rare early-onset familial AD (EOFAD) which is caused by mutations in the enzymes that cleave APP, leading to rapid and aberrant cleavage with resultant overproduction of Aβ [29]. On the other hand, the common late-onset AD (LOAD) is thought to result from either the failure of Aβ to be cleared from the brain [30, 31] by microglial cells, lower expression of Aβ degrading proteases such as insulysin (insulin degrading enzyme IDE), a decline in the availability of Aβ chaperone low density lipoprotein receptor-related protein (LRP1) to transport Aβ out of the brain, reduced vascular and perivascular drainage, or a combination of the above [32]. Although Aβ monomers are relatively nonpathogenic, accumulating soluble Aβ oligomeric forms have been shown to be synaptotoxic and can prune dendritic spines, disconnecting the memory-encoding neuronal network in the entorhinal cortex, the parahippocampal gyrus, and the hippocampus [33]. These oligomers eventually form large insoluble fibrillar aggregates or plaques that by themselves do not directly induce neuronal death but rather attract microglia and astrocytes that produce cytotoxic proinflammatory cytokines and reactive oxygen species that may indirectly cause neuronal death [34].

Additionally, other proposed mechanisms that contribute to neuronal damage include the vulnerability of cells to secondary insults, tau hyperphosphorylation, induction of the apoptosome and lysosomal protease activity, changes in calcium influx, and direct damage (peroxidation) of membranes [35].

Although the plaques are found extracellularly, it is thought that production, oligomerisation, and accumulation of Aβ occur within neuronal processes with the possibility that the incorporation of aggregates into plaques occurs after the neurites are dissolved [36]. Certainly, studies performed in the well-established mouse models of AD have identified Aβ in several neuronal compartments such as the Golgi

apparatus, the endoplasmic reticulum, the secretory vesicles, endosomes, and autophagic vacuoles, suggesting intraneuronal aggregation and pathology [36]. However, recent evidence supports the extracellular deposition of Aβ as the initiating pathogenic mechanism in the AD brain [37], with a direct correlation with the inhibition of anterograde axonal transport [38]. Despite direct evidence of Aβ-dependent neurodegeneration, Aβ pathology occurs prior to the appearance of clinical symptoms [37]. Accordingly, determining the level of amyloid deposition in an AD patient's brain (Aβ load) in a time-dependent manner would be informative in evaluating the progression of the disease and monitoring patient's response to antiamyloid therapies. Interestingly, through amyloid imaging, recent studies have demonstrated binding of the PET Pittsburgh compound B (PiB-PET) to Aβ peptides [39]. In this study, PET amyloid imaging with Pittsburgh compound B (PiB) showed increased cortical PiB binding in AD patients when compared to control subjects and intermediate binding levels in patients with mild cognitive impairment (MCI) [39]. This compound could be beneficial in the early detection of AD and evaluation of disease progression.

Recently, it was demonstrated by a combination of *in vivo* and *in vitro* studies that Aβ binding to the cellular prion protein (PrPc), an oligomer-specific high-affinity binding site for Aβ, can play a central role in Aβ-induced memory deficits, axon degeneration, synapse loss, and neuronal death in the AD brain through Fyn kinase activation [40]. The activation of this kinase results in alterations in N-methyl-D-aspartate receptor (NMDAR) function by increasing surface NMDAR expression along with its phosphorylation, and eventually leading to dendritic spine, in association with surface receptor loss [40]. The data suggests that by inhibiting PrPc in the APPswe/PSEN1-M146L double transgenic mouse, reversal of memory deficits and restoration of synaptic density could be achieved [40]. It has been demonstrated that Fyn kinase associates with the tau protein and that abnormal Fyn-tau interactions sensitise synapses to glutamate excitoxicity [40]. Together, these data suggest that PrPc-Fyn signalling may contribute to Aβ and tau pathologies and thus its downregulation may be a potential therapeutic approach.

4. Tau Protein Pathology

The tau protein is an integral component of the neuronal cytoskeleton [41] with a molecular weight ranging from 45 kDa to 65 kDa [41] and is responsible for the promotion of microtubule assembly in the normal brain [42]. Microtubule assembly is tightly regulated by a combination of protein kinases and phosphatases that balance the amount of tau phosphorylation [43, 44]. The most common tau pathology is seen in AD, but it is manifest in other diseases such as frontotemporal dementias and Parkinson's disease [45, 46]. In the AD brain, tau exists in a hyperphosphorylated state, which leads to aberrant secondary structures and loss of function, resulting in a reduced ability to bind to microtubules and to promote their assembly [47]. The abnormal translocation of tau from axonal microtubules to neuropil thread inclusions, cell bodies and dendritic processes, where tau aggregates and accumulates, are pther prominent cytopathological hallmarks observed within AD brain sections [48]. The tau protein is initially synthesised as a single chain polypeptide and then targeted by posttranslational modifications that alter its conformation, promoting tau dimerisation in an antiparallel manner [49]. Stable tau dimers subsequently form tau oligomers, which aggregate at an increasing rate to form subunits of filaments, called protomers. Two protomers twisted around each other with a crossover repeat of 80 nm, constitute the width varying between w10 and w22 nm to form paired helical filaments (PHFs), a characteristic of AD neuronal pathology [49, 50]. Assembly of PHFs finally establishes the neurofibrillary tangles (NFTs), which can be observed microscopically (Figure 2) [51]. Hyperphosphorylated tau sequesters normal tau and other neuronal microtubule associated proteins (MAPs), such as MAP1A, MAP1B, and MAP2, contributing further to disassembled microtubules, disruption of the axonal cytoskeleton, and transport, culminating as damaged neurons [52]. After neuronal death, tau oligomers are released into the extracellular environment which leads to microglial cell activation and, as a consequence, further progressive bystander neuronal degeneration [53]. It has been suggested that tau pathology results from elevated protein kinase activity, a reduction in the activity of protein phosphatase, or both [45]. Analysis of phosphorylated tau isolated from AD brains has identified numerous target serine or threonine residues [45]. It has been demonstrated that MAP-kinase, GSK-3β, and/or Cdk5 are the main kinases involved in tau phosphorylation. However, in AD not all tau phosphorylation events can be attributed to these kinases [45].

The mechanism by which tau exerts its neuronal toxicity is still controversial [54]. It has been suggested that a series of degenerative signals such as Aβ aggregation, iron overload [55], oxygen free radicals [56], cholesterol levels in neuronal rafts, LDL species [57], and homocysteine can activate the innate immune response [53]. The activation of microglial cells, for instance, results in the subsequent release of pro-inflammatory cytokines that modify neuronal behaviour through anomalous signalling cascades, with the end result being the promotion of tau hyperphosphorylation [53]. However, numerous cellular and transgenic animal models indicate that tau is crucial for Aβ-induced neurotoxicity [54]. For instance, cultured hippocampal neurons from tau deficient mice are protected against Aβ pathology [54]. Furthermore, in cultured hippocampal neurons from wild-type mice, the silencing of tau by siRNA has demonstrated that tau is required for prefibrillar Aβ-induced microtubule disassembly. Furthermore, it was demonstrated that a reduction in soluble Aβ and tau but not Aβ alone causes cognitive decline in the triple transgenic AD mouse model with plaques and tangles [58]. These data suggest that although Aβ is the initial trigger, tau accumulation plays a central role in neurodegeneration. Finally, in the AD-like transgenic model that expresses human APP with familial mutations, suppression of endogenous tau prevented Aβ-dependent water maze learning and memory deficits without reversing the amyloid pathology [58]. Collectively, these data

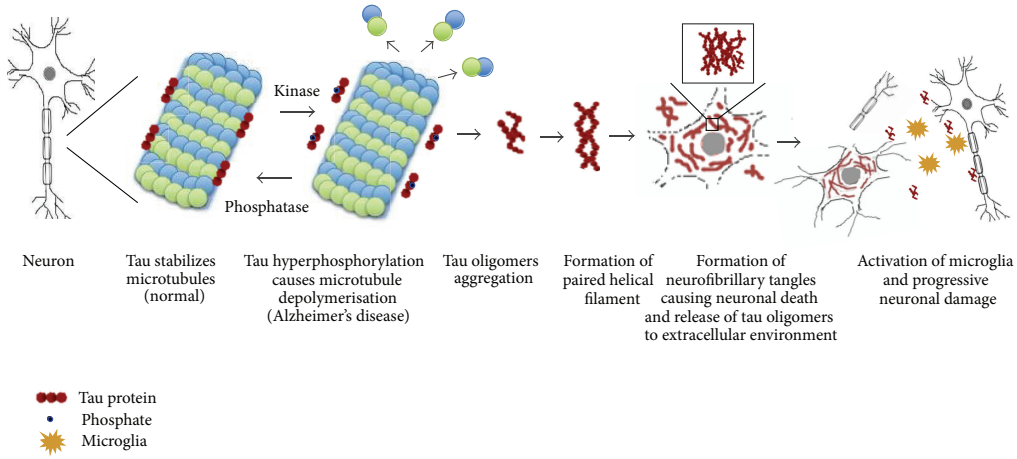

Figure 2: Stabilisation of microtubules by the tau protein is regulated by kinases and phosphatases. Abnormal hyperphosphorylation of tau proteins causes catastrophic microtubule depolymerisation and the formation of insoluble cytoplasmic tau oligomers, which aggregate to form protomers. Two protomers twisted around each other to form paired helical filaments (PHFs), which assemble to produce neurofibrillary tangles (NFTs).

suggest a link between Aβ and tau that drive the neural pathologies and the manifestations of clinical symptoms. Preliminary data on the inhibition of tau aggregation by methylene blue chloride (MTC) has indicated a lower rate of cognitive decline in treated patients compared with those sporadic AD patients on alternate therapies, implicating tau as the key initiator of cognitive deficits [50]. However, the exact role of Aβ dependent in signal transduction cascades that are associated with pathogenic tau modifications and the contribution to the progression of neuronal death require further investigation [54].

5. Signalling Molecules Linked with Neuronal Cytoskeleton Disassembly

5.1. Rho Kinase (ROCK). The Rho-associated coiled-coil forming protein kinases (ROCKs) include the ROCK-1 and ROCK-2 isoforms. These two kinases contain highly conserved aminoterminal but different carboxy-terminal domains [59]. Both ROCK-1 and ROCK-2 were originally shown to be involved in cell differentiation, essential for the regulation of myogenesis from embryonic fibroblasts along with skeletal muscle maturation and differentiation [60]. Both Rho kinase (ROCKs) and p21-activated kinase (PAKs) are members of the serine/threonine class of protein kinases. However, they are known to have antagonistic effects on the actin cytoskeleton and therefore on the plasticity of synapses. PAK also has two major isoforms, PAK1 and PAK3, and they have downstream signalling effects on Rho/Rac (for review see [61]). PAK can stimulate actin polymerisation [62], axon outgrowth, and the formation of dendritic spines [63] through LIM kinase stimulation [62]. PAK can also inhibit the myosin light chain kinase (MLCK) which diminishes actomyosin contractility [64, 65].

It has been reported that 13-month-old AD-like mice (PDAPP) displayed a substantial decrease in PAK 1-3 activity compared to normal controls [21]. Furthermore, the hippocampi of patients exhibiting the early clinical signs of AD have displayed high PAK 1-3 activity which was then shown to decline in the late stages of AD pathology [21]. It was further suggested that C-terminal cleavage of APP at the Asp664 site mediates PAK abnormalities and that an Asp664 mutation may potentially prevent these abnormalities [21]. On the other hand, ROCKs stimulate the retraction of axonal and dendritic growth cones by activating MLCK through the phosphorylation of myosin light chain proteins to promote an interaction with actin [66]. In addition, ROCK2 can phosphorylate collapsin response mediator protein 2 (CRMP2), another microtubule associated protein, to induce growth cone collapse [67].

Moreover, many developmentally or pathologically regulated molecules can also activate the RhoA/ROCK pathway to inhibit axonal growth including semaphorins, ephrins, and myelin inhibitory factors, such as Nogo and myelin-associated glycoprotein (MAG). On the other hand, there are some signalling molecules such as Sema4D/plexin-B1 that activate the RhoA/ROCK pathway especially in hippocampal neurons that may induce dendritic spine formation. It has been speculated that this may be due to the activation of LIM kinase and the PAK-type response via actin-depolymerising factor ADF/cofilin [68, 69].

In AD, dendritic spine defects play a major role in cognitive impairments [61]. It has been reported that dendritic postsynaptic proteins are excessively distorted with disease progression [70]. For instance, neuronal loss in the

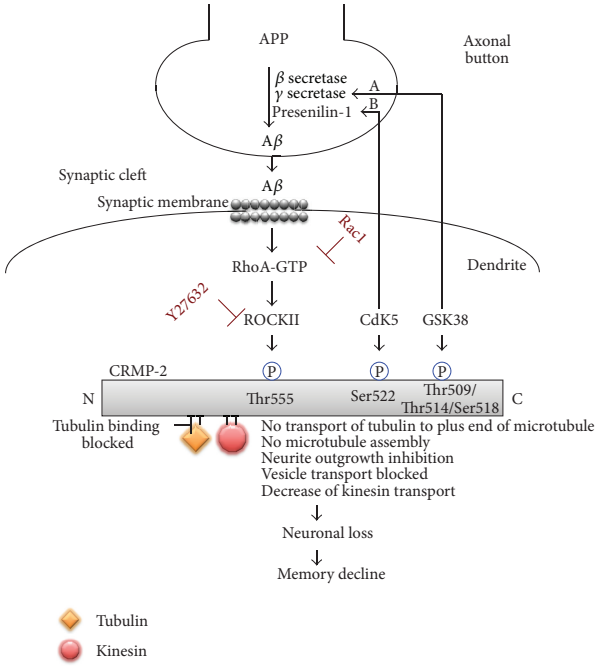

Figure 3: Model of Aβ-mediated neurite outgrowth inhibition. Aβ (oligomeric) activates the small GTPase, RhoA, which inhibits the proneurite outgrowth GTPase Rac1. RhoA-GTP activates Rho Kinase (ROCK II) to effect microfilament rearrangement and also potentiate microtubule disassembly. Microtubule disassembly occurs when ROCK II directly phosphorylates CRMP-2 at the Thr555 position preventing the association of CRMP-2 with tubulin heterodimers, thereby affecting neurite outgrowth inhibition. Neurite outgrowth is further impeded by CRMP-2 phosphorylation since this prevents the microtubule motor protein, kinesin, to associate with CRMP-2 and transport growth-related vesicular cargo, such as BDNF, antergradely to the distal end of the neurite. It is demonstrated that CRMP-2 is also phosphorylated by GSK-3β and Cdk-5. (A) Studies have demonstrated that GSK-3β activity can also regulate the processing of APP resulting in the production of Aβ, which in turn can further increase GSK-3β activity through PI3K inhibition, illustrating as a potential feedback loop. (B) Additionally, it has been suggested that Cdk5 may phosphorylate presenilin-1 at Thr354 destabilising its carboxy-terminal fragment, leading to increased APP processing.

hippocampi of AD patients is approximately 5–40% while the loss of postsynaptic proteins such as the developmentally regulated actin-regulating brain protein (drebrin), which is targeted by Aβ oligomers, reaches 70–95% [71]. This study in particular suggested that Aβ-induced alteration in postsynaptic PAK may have a central role in the massive drebrin loss and cognitive deficits found in AD, which could be prevented by an antibody to Aβ and/or by *in vivo* or *in vitro* overexpression of wild-type PAK [71].

Cognitive defects and eventually dementia are important clinical features of AD (for review see [2]). It has been reported that there exists a relationship between the cognitive-decline occurring in AD along with genetic mental retardation syndromes and synaptic dysfunction, primarily since the postsynaptic maintenance of dendritic spines is lost. To maintain synaptic balance, both ROCK1 and 2 transduce signals to retract the growth cones and dendritic spines (for review see [72]). It has been shown that ROCK may provoke APP breakdown to the toxic β-amyloid 1-42 peptide. For example, ROCK inhibitors, such as Y27632, inhibit the toxic processing of APP [73]. An intriguing conundrum is that the binding of Aβ on neurons may activate RhoA and ROCK2 to potentiate the phosphorylation of its substrates [74, 75]. One of the specific substrates that our group has recently defined is CRMP-2 (Figure 3). It has been shown that CRMP-2 exhibits hyperphosphorylation in the cortex of AD postmortem brains [76]. Experimentally, it has been illustrated that other kinases such as GSK-3 and Cdk5 can also phosphorylate CRMP-2 and produce growth cone collapse in neurons [77] (Figure 3). Our data suggest that β-amyloid can increase the RhoA-GTP level in differentiated SH-SY5Y cells increasing CRMP-2 phosphorylation and reducing the neurite lengths in cultured neuroblastoma cells. Additionally, RhoA and CRMP-2 levels are elevated in neurons surrounding amyloid plaques in the cerebral cortex of the APP (Swe) Tg2576 AD mouse model. Our work indicates that Aβ induces

Rho GTPase activity and ROCK2 to promote CRMP-2 phosphorylation which can lead to the inhibition of neurite outgrowth [78] (Figure 3). However, a direct link with the reduction of ROCK2-dependent CRMP-2 phosphorylation and the limitation of cognitive decline is yet to be established in the context of Aβ-dependent neurodegeneration.

5.2. Glycogen Synthase Kinase-3β (GSK-3β). The proline-directed serine/threonine kinase, glycogen synthase kinase-3 (GSK-3), is important for several cellular processes such as metabolism, cell structure, and apoptosis and in the regulation of gene expression (for review see [79]). The GSK-3 family contains two members, GSK-3α and GSK-3β, that are highly expressed in the brain and spinal cord with GSK-3β playing a central role in neuronal differentiation and the maintenance of neurons (for review see [80]). Activation of GSK-3 requires prephosphorylation by other priming kinases such as Cdk5 at serine or threonine sites located 4 residues, C-terminal to the site phosphorylated by GSK-3 (for review see [81]) (Figure 3). Abnormal GSK-3 function has been implicated in different brain pathologies indicating its fundamental role in controlling basic mechanisms of neuronal function, modulation of neuronal polarity, migration, proliferation, and survival, not to mention the establishment of neuronal circuits (for review see [82]). It has been demonstrated that phosphorylation of GSK-3 may influence cytoskeletal proteins altering neuronal plasticity (for review see [83]). Neuronal cytoskeletal changes occur due to an altered rate in the stabilisation/destabilisation of microtubules (MT), thereby altering the dynamics of dendrites, spines, axons, and synapses. Intensified efforts in the identification of enzymes involved in regulating tau phosphorylation *in vivo* have revealed GSK-3β as a candidate kinase for therapeutic targeting [79] during AD pathology.

It has been hypothesised that GSK-3 overactivity may potentiate sporadic and familial forms of AD by enhancing tau hyperphosphorylation [84] and APP processing and possibly through the phosphorylation of CRMP-2 leading to profound memory impairment [81] (Figures 2 and 3). It has been established that the expression of full-length unmodified or unphosphorylated CRMP-2, in primary hippocampal neurons or SH-SY5Y neuroblastoma cells, promotes axon elongation. Moreover, cultured neurons expressing CRMP-2 with mutant GSK-3 phosphorylation sites (T509A, S518A) display significantly reduced axon elongation [81]. On the other hand, studies have demonstrated that GSK-3β phosphorylation of the CRMP-2 T509 site can play a crucial role in mediating the repulsive action of Sema3A [85] and promoting growth cone collapse [77]. Recently, Cole et al. have demonstrated that dephosphorylation of CRMP-2 at the GSK-3β-dependent sites (Ser-518/Thr-514/Thr-509) can be carried out by a protein phosphatase 1 (PP1) *in vitro*, observed in neuroblastoma cells and primary cortical neurons, and that the inhibition of GSK-3β by insulin-like growth factor-1 or the highly selective inhibitor CT99021 results in dephosphorylation of CRMP-2 at these sites [86]. How this may be translated to real therapeutic outcomes during AD pathology is yet to be demonstrated, even within animal models of disease.

5.3. Cyclin-Dependent Kinase-5 (Cdk5). The other proline-directed serine/threonine kinase, identified as a major priming enzyme for tau phosphorylation, is cyclin-dependent kinase-5 (Cdk5) [87]. Although Cdk5 is ubiquitously expressed in most tissues, it is not directly involved in mediating progression through the cell cycle as it requires prior activation by p35 and p39, which are expressed almost exclusively in the CNS [88]. Cdk5 plays an important role in CNS development possibly by mediating interactions between neurons and glia during radial migration, which is essential for developing appropriate cortical laminar architecture [89, 90]. Furthermore, Cdk5 has been reported to also play a role in neuronal differentiation, axonal guidance, synaptic plasticity, cellular motility, cellular adhesion, and neurodegeneration (for review see [91]).

Studies have shown that inhibition of Cdk5 reduces Aβ-induced neurodegeneration in cortical neurons [92] which highlights that targeting Cdk5 could be a future therapeutic strategy for neurodegenerative disorders. The critical microtubule associated protein, CRMP-2, has been also demonstrated to be a substrate for Cdk5 [77]. This study showed an orderly phosphorylation process of CRMP-2 by Cdk5 (defining it as the priming kinase) followed by GSK-3β as a consequence of Sema3A stimulation that inhibits axonal growth [77]. Alternatively, a non-phosphorylated form of CRMP-2 cannot respond to Sema3A signalling. This study also demonstrated that Sema3A promotes phosphorylation of CRMP-2 at Ser522, which is the established Cdk5 phosphorylation site [77]. Thus, targeted kinase inhibitors may possibly be therapeutically beneficial in AD to limit both tau and CRMP-2 phosphorylation. Deciphering which of the kinases precipitate neurodegeneration is still under investigation but when elucidated, the possibility exists that formulation of specific inhibitors to prevent cognitive decline associated with AD is achievable.

5.4. Phosphatases. Protein phosphatases provide unique endogenous signalling mechanisms for the dephosphorylation of proteins, reversing such posttranslational modifications, which may limit protein dysfunction. Protein phosphatase 2A (PP2A) is one of the most important serine/threonine phosphatases in the mammalian brain. It also exists in most tissues comprising up to 1% of total cellular protein. It has major roles in development, cell growth, transformation (for review see [3]), regulation of protein phosphorylation, and cell signalling pathways [93]. PP2A is composed of 3 subunits: subunit A (scaffolding/structural), subunit B (regulatory/targeting), and subunit C (catalytic) [94]. PP2A with PP1 collectively account for more than 80% of the total serine/threonine phosphatase activity in all mammalian cells [3, 95] making these enzymes integral to cellular physiology.

In situ, PP2A, PP1, PP5, and PP2B account for 71%, 11%, 10%, and 7%, respectively, of the total tau phosphatase activity in the human brain [96]. PP2A is the most prevalent phosphatase involved in tau dephosphorylation [97]. Knockdown of PP2A phosphatase activity was shown to lead to tau hyperphosphorylation [98]. Furthermore, when PP2A was inhibited in cultured cells and in transgenic mice with mutant

PP2A, hyperphosphorylation of tau was observed [98]. Moreover, the naturally abundant SET protein, a potent PP2A inhibitor, is found to be elevated in AD brains [99], possibly illustrating reduced PP2A activity allowing for the hyperphosphorylation of cellular substrates to occur unabated and the potentiation of neurodegeneration. Interestingly, autopsy studies of brains from AD patients, non-AD dementia, and normal human brains demonstrate that there is loss in PP2A protein, mRNA, and enzymatic activity in areas of the brain affected by AD, the hippocampus and cortex, but not in the cerebellum [100]. In addition, the inhibition of PP2A activity mimics most of the phosphorylation events seen in AD, such as tau hyperphosphorylation [101].

Phosphorylation of APP by an array of kinases has been shown to influence its cleavage by β-secretase resulting in Aβ production [102]. It was demonstrated that PP2A has the ability to dephosphorylate APP at the Thr668 site and thus inhibit Aβ generation [103]. Studies of cells expressing the (APPswe) mutation, transgenic mice expressing both APPswe and presenilin mutations, and sections of hippocampus and entorhinal cortex from human AD patients, show that PP2A levels are decreased and Y307 levels (an inhibitor of PP2A) were increased [104] implying that the phosphatase affects the processing of APP and highlighting its importance in limiting AD pathology. In N2a cells, where PP2A was inhibited with okadaic acid (OA), the phosphorylation of APP and the secretion of both sAPPα and sAPPβ were all elevated [105]. In addition, inhibition of the protein phosphatases PP1 and PP2A in rat brain by OA results in the accumulation of hyperphosphorylated tau and Aβ species [45, 94]. Even though incubation of different types of cells with OA resulted in the stimulation of APP secretion, it was not proven that the effect was mediated by PP1 [106] and/or PP2A [107]. Moreover, it was demonstrated that demethylation of PP2A by nuclear phosphatase methylesterase-1 (PME-1) reduces its activity and thus leads to tau hyperphosphorylation along with APP phosphorylation, promoting APP cleavage and Aβ production [108–110]. Collectively, these results suggest that downregulation of PP2A may induce Aβ production and tau phosphorylation, precipitating AD pathology.

A direct link of PP2A activity with the progression of AD pathology has been affiliated to the fact that CRMP-2 phosphorylation may actually be a result of lowered PP2A activity [93]. Since CRMP-2 hyperphosphorylation was commonly observed to correspond with progressive neurodegeneration, decreased PP2A may well regulate such a disease-specific event. However, such a hypothesis would need to be substantiated beyond a causal link.

5.5. Collapsin Response Mediator Protein (CRMP). The collapsin response mediator proteins (CRMPs) are members of the dihydropyrimidinase-related neuronal phosphoprotein family [111]. The CRMP family has five isoforms, CRMP1-5 [112]. The most well characterised of these, CRMP-2, is highly expressed in the adult mammalian CNS localising in the cytoplasm and neurites of postmitotic neurons [111]. CRMP-2 is also highly expressed in the areas of the adult brain of greatest plasticity such as the hippocampus, olfactory bulb, and cerebellum [113]. In neurons, CRMP-2 is concentrated within the distal portions of neurites, in synapses and in growth cones [114]. It regulates the polarity and differentiation of neurons through the assembly and trafficking of microtubules [115]. CRMP-2 has no known enzymatic activity by itself but through an interaction with other binding partners it can regulate neural differentiation, dendrite/axon fate specification, Ca^{2+} homeostasis, neurotransmitter release, regulation of cell surface receptor endocytosis, kinesin-dependent axonal transport, growth cone collapse, neurite outgrowth, and microtubule dynamics (for review see [78, 116]). The last three functions have been demonstrated to be regulated by phosphorylation near the C-terminus of CRMP-2 by kinases [117, 118] including cyclin-dependent kinase 5 (Cdk5), glycogen synthase kinase-3β (GSK-3β) [31, 76, 86, 119], Tau-tubulin kinase-1 (TTBK1) [120], and Rho kinase II (ROCKII) [78, 117, 121], all of which culminate in neurite retraction (for review see [78]). CRMP-2 hyperphosphorylation in AD was suggested to be a result of increased kinase activity, decreased phosphatase activity, or both [86]. All phosphorylation events can disrupt the association of mature full-length CRMP-2 with tubulin heterodimers possibly resulting in the destabilisation of the neuronal microtubule system rendering axonal retraction [67]. Moreover, disruption of the binding between CRMP-2 and tubulin due to the phosphorylation of CRMP-2 can block tubulin transport to the plus ends of microtubules for assembly (Figure 3) [78], blocking neurite outgrowth/elongation. In primary neurons and neuroblastoma cells, it has been demonstrated that overexpression of CRMP-2 results in axon elongation [114] while overexpression of truncated CRMP-2, lacking the C-terminus tubulin binding domain, inhibits axon growth. These data implicate this region of CRMP-2 to play a central role in axonal growth [114]. Both the Cdk5 and GSK-3β phosphorylation of CRMP-2 have been shown to be increased in the cortex and hippocampus of the triple transgenic mouse (PS1/APP/Tau mutant), along with the double transgenic mouse (PS1/APP mutant), that develop AD-like plaques along with NFTs. However, in transgenic mice, which display only mutant tau (P301L) that develop tangles but do not develop amyloid plaques, Cdk5 phosphorylation of CRMP-2 does not occur. These results indicate that hyperphosphorylation of CRMP-2 might be induced by APP overexpression and/or its enhanced processing, thereby generating a high amyloid load within the brain of these transgenic mice [76].

Our laboratory has recently demonstrated that, in human neuroblastoma SH-SY5Y cells and in the Tg2576 mouse model of AD, Aβ can reduce the length of neurites by inactivating the neurite outgrowth-signalling molecule Rac1 [78]. Furthermore, the data suggested that Aβ-mediated reduction in neurite length could be reversed by the Rho Kinase inhibitor (Y27632). Additionally, the Aβ-mediated decrease in neurite length was linked to the promotion of a threonine phosphorylation of CRMP-2 (unrelated to GSK-3β-dependant phosphorylation), conferring a reduced binding capacity to tubulin, both of which can be reversed by inhibiting RhoA activity [78]. These data suggested that Aβ-mediated neurite outgrowth inhibition results from the

activity of RhoA-GTP and the dysregulation of CRMP-2 to bind tubulin for neurite outgrowth [78] (Figure 3).

Studies using transgenic mouse models expressing the Swedish familial AD mutant (APP/TTBK1) demonstrated that the induced upregulation of tau tubulin kinase-1 (TTBK1) can promote axonal degeneration via phosphorylation of CRMP-2 and tau within the entorhinal cortex and hippocampus, implicating TTBK1 as a potential therapeutic target for AD [120].

Despite the profound link to CRMP-2-dependent degeneration through kinase-mediated phosphorylation, another function of CRMP2 is mediated through its known association with kinesin, facilitating the anterograde molecular transport of growth promoting vesicles along axonal microtubules [122]. The exact mechanism of binding and transport and its contribution to AD will be discussed in detail below.

6. CRMP2-Tubulin Binding

The microtubule and actin cytoskeleton orchestrates axonal growth cone dynamics by a process of signal transduction leading to either depolymerisation or polymerisation events, for directional growth [119]. As already discussed above, the binding of CRMP2 to tubulin heterodimers can enhance microtubule assembly leading to axon outgrowth [123, 124]. Semaphorin-3A (Sema3A) is an extracellular protein that can block axonal outgrowth [77] through the activation of Cdk5, with downstream phosphorylation of both tau and CRMP-2 [31, 77]. Such phosphorylation can disrupt their tubulin association limiting axonal growth. Following the Cdk5 phosphorylation of CRMP-2, the latter may potentiate a conformational change leading to subsequent phosphorylation by GSK-3β [31, 77]. However, it has been demonstrated that in GSK-3β overexpressing mice, no hyperphosphorylation of CRMP-2 can be identified at the GSK-3β phosphorylation sites and furthermore phosphorylation of tau does not increase [125]. This may explain the finding that activation of GSK-3β alone can not induce growth cone collapse (for review see [119]). Interestingly, protein lysates from human AD cortex and animal models of AD show hyperphosphorylation of CRMP-2 at residues Thr509, Thr514, and Ser518 which are known to be the GSK-3β phosphorylation sites as well as Ser522, the well-known Cdk5 phosphorylation site (for review see [78]). These findings indicate that Sema3A signalling may regulate microtubule polymerisation through the physiological actions of tau and CRMP-2, which regulate the dynamics of microtubules and tubulin dimers, respectively [126]. Phosphorylation of CRMP-2 by Rho kinase at the Thr555 site, however, can also reduce the CRMP-2 association with tubulin heterodimers and induce growth cone collapse unrelated to Sema3A signalling and quite possibly be the result of Aβ-dependent signalling [31, 77]. The phosphorylation of CRMP-2 by Cdk5, GSK-3β, and Rho kinase may therefore play a central role in coordinating cytoskeletal activities in response to multiple axon guidance cues [31, 77].

The plausible hypothesis exists that activation of all thre kinases Cdk5/GSK-3b/ROCK2, contribute to the destabilisation of the neuronal microtubule system in AD. Consequently, tau and CRMP-2 have some similarities in that both control microtubule polymerisation and stability and they both respond to the growth cone guidance molecule Sema3A [77]. Therefore, it can be theorised that a balanced treatment which may successfully decrease CRMP-2 phosphorylation could also be effective in regard to tau aggregation and vice versa in AD (for review see [31]).

7. Microtubules (MT)

One of the most important physiological features of the multipolar neuron is to have a polarised axon, that can extend to more than 1 meter in the human CNS [127]. For the neuron to function normally, it should be able to transport vital molecular cargo from its body to synaptic terminals and vice versa in a timely manner through the axon via anterograde and retrograde transport mechanisms, respectively [127, 128]. Therefore, it stands to reason that the integrity of the microtubule transport system is crucial for axonal transport [129]. The microtubule system facilitates ATP driven transport through molecular motors of the cell's vital components which include vesicles, proteins, mitochondria, chromosomes, and large macromolecules such as microtubule heterodimers themselves [128, 130]. The transport machinery directly interacts with microtubules and includes two families of proteins categorised according to their directional movement. These proteins include either microtubule plus end-directed kinesins or the microtubule minus end-directed cytoplasmic dynein [127].

Many neurodegenerative diseases, such as AD, display a blockade in microtubule transport, emphasising its significance in normal physiology and highlighting abnormal neuronal vesicle trafficking as a potential pathogenic mechanism [130–132]. It is believed that Aβ may cause mitochondrial dysfunction and, therefore, axonal transport defects [132]. It has been demonstrated that APP processing and Aβ overproduction in the mitochondria lead to mitochondrial dysfunction and therefore reduction of mitochondrial energy supply and inhibition of axonal transport [133]. Enhancing energy supply of neurons could be critical to compensate for the Aβ-dependent loss of energy and thus facilitate axonal transport.

Microtubule depolymerisation has been touted as a contributing factor in the gross loss of memory, as it is necessary to stabilise newly formed microtubules in spines for long-lasting memory [134, 135]. There exists evidence implicating tubulin sequestration [136] and blockade in microtubule assembly as a pathogenic mechanism of AD [129]. It has been recently demonstrated that *in vitro*, microtubules can be assembled from the cytosol of normal autopsy brain obtained within five hours postmortem, while this is not possible from identically treated AD postmortem brain tissue [129]. Furthermore, it has been documented that axonal transport is defective in neurons from AD postmortem brains indicating the destruction of the microtubule cytoskeleton in axons of diseased neurons [134]. There also exist data suggesting that the abnormality in axonal transport might stimulate the formation of, or enhance the accumulation of, Aβ [134, 137],

through autophagocytosis of mitochondria without normal lysosomal degradation [137].

One of the main physiological functions of tau is to stimulate microtubule assembly by polymerising with tubulin, maintaining the microtubule structure and stability through its capacity to anchor polymerised microtubules to the internal axolemma [129]. Evidence for the role of tau and microtubule destabilisation arises from tau transgenic mice which show spinal cord tau inclusions [131]. In this animal model, an inability of tau to stabilise microtubules can be compensated with the MT-stabilising agent paclitaxel resulting in increased MT density and marked improvement in motor function [131]. However, paclitaxel is thought to have poor blood-brain barrier permeability and thus is an unlikely candidate for human therapy during neurodegeneration [131].

In the early stages of AD pathogenesis, observations within the neuropil demonstrate that there exists an abnormal aggregation of the activated actin-associated protein cofilin, a protein that modulates actin-rich dendritic spine architecture, which is important for learning and memory [43]. Those neuropil threads can disrupt the cytoskeletal network by blocking cargo trafficking to synapses, resulting in memory and cognition impairment [43]. It is also suggested that abnormal activation of cofilin may trigger the accumulation of phosphorylated tau in neuropil threads [43]. The activities of cofilin and the protein actin-depolymerising factor (ADF) are regulated by phosphorylation and dephosphorylation through LIM and other kinases, along with chronophin phosphatases, respectively [43]. Heredia et al. found that β-amyloid may activate LIMK1 and thus stimulate ADF/cofilin phosphorylation in cultured neurons [69]. Moreover, they demonstrated, in the AD brain, that the number of P-LIMK1-positive neurons was extensively increased in the affected regions [69]. A recent study of AD transgenic mice demonstrated that neuronal cell bodies are viable although the neurites are damaged [138]. Taken together, these studies highlighted that the development of *in vivo* methods to disrupt LIMK1 activation, the formation of the cofilin-actin rods, and/or the interaction between cofilin and pMAP, may be a plausible way to stop the disease early in its presentation.

8. Kinesin

The microtubule motor protein complex, kinesin-1, has a fundamental role in the vesicular transport from the neuronal cell body, along the axon and anterograde, to the synapse (for review see [139]). The motor protein complex consists of two kinesin heavy chains (KHC) that have both an ATP and the microtubule binding motif which are essential for vesicle transport [140]. Two kinesin light chains (KLC) that associate with the heavy chain and vesicular cargo membranes [140] complete the structure of the transport protein. APP is one of the molecular candidates for receptors that attach kinesin-1 to vesicular cargo [139]. The carboxy terminus of APP binds directly to the light-chain subunits of kinesin-1 [140] and thus plays a major role in the recruitment of kinesin-1 to axonal vesicles [141]. Moreover, the level of axonal APP is suggested to play a central role in determining expression levels of kinesin-1 decorating vesicles, providing the ability to determine the anterograde movement behaviour of APP-containing vesicles [141]. It has been reported that kinesin blockade and axonal swellings are involved in the pathogenesis of the early stages of AD even before the formation of amyloid plaques and neurofibrillary tangles, although the initiating events are not clear [142]. Moreover, in animal models, β-amyloid formation and its subsequent transport are enhanced when kinesin transport is abrogated or impaired [38, 141]. Axonal transport damage results in the development of axonal swellings where APP is processed into smaller Aβ species. APP axonal transport is mediated by direct binding to KLC1 [143]. Genetic manipulation designed to damage APP axonal transport in AD mouse models, such as Tg-swAPP^{Prp}, demonstrated the enhancement in the incidence of axonal swellings, elevated Aβ levels, and potentiated the production of amyloid deposition [142]. In particular, APP directly interacts with KLC1 (the microtubule transport machinery) through its carboxy terminus, suggesting that impaired interaction of APP and KLC1 might play a central role in the AD pathogenesis [144]. Decreased KLC1 transport may also stimulate tau hyperphosphorylation and formation of NFTs as well as axonal swellings producing catastrophic damage to axons. Such damage may arise from increased Aβ levels and tau hyperphosphorylation, further disrupting axonal transport [145].

It is now well established that CRMP-2 plays a central role in negotiating fast axonal transport by acting as an adaptor protein to the microtubule motor kinesin-1, for propagation of anterograde vesicle transport of key traffic molecules such as the high affinity neurotrophin receptor, tyrosine kinase (TrkB). Following distal localisation of this receptor, TrkB is inserted into the cell membrane and activated by its cognate ligand brain-derived neurotrophic factor (BDNF), resulting in axonal growth through signalling within the growth cone, thereby establishing the accumulation and polymerisation of F-actin and tubulin. In AD, phosphorylated CRMP-2 releases kinesin-1, inhibiting TrkB function and limiting the structural integrity of the actin-based cytoskeleton in distal axons, growth cones, and synapses [146]. Inhibiting CRMP-2 phosphorylation could be beneficial to restore tubulin and kinesin-1 binding to CRMP-2 and thus promoting axonal outgrowth and transport of important molecular cargo.

9. Conclusion

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder and is the most common form of dementia in the elderly. The hallmarks of AD pathology are the extracellular deposition of a 4 kDa amyloid beta (Aβ) polypeptide and the formation of intracellular neurofibrillary tangles (NFTs) along with dystrophic neurites, degenerating neurons, and activated astrocytes and microglia, a part of the reactive pathology observed around senile plaques. Neuritic plaques result from the aggregation of the amyloid β protein (Aβ) which is a consequence of amyloid precursor protein (APP) aberrant processing. The corresponding accumulation of filamentous inclusions within the CNS as neurofibrillary

tangles (NFTs), resulting from the hyperphosphorylation of the microtubule-associated protein, tau and amyloid deposition, are both pathognomonic to sporadic AD. There is an impressive list of genes and proteins involved in AD pathologies including APP, presenilins, secretases, kinases, and phosphatases all touted as being responsible for either increasing the production of the neurotoxic Aβ protein or promoting the hyperphosphorylation of CRMP-2 or tau, leading to the devastating neurodegenerative sequelae. The understanding of the major gene players cooperating with key environmental factors that contribute to the manifestation of AD pathology is fundamental in the derivation of a more comprehensive understanding of AD pathogenesis and for the development of specific and more effective treatments of this devastating age-dependent disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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CHAPTER 2: Methods

Materials

The following primary antibodies were used: rabbit polyclonal anti-phospho-Thr555 CRMP-2 (developed in-house); rabbit polyclonal anti-phospho-Thr514 CRMP-2 (Cell Signaling Technology Inc., Boston, MA); rabbit polyclonal anti-phospho-Ser522 CRMP-2 (ECM Biosciences, USA); mouse monoclonal anti-CRMP-2 (Immuno-Biological Laboratories Co. Ltd, Gunma, Japan); mouse monoclonal anti-alpha tubulin (Merck-Millipore, CA); mouse monoclonal anti-FLAG (Sigma-Aldrich, St Louis, MO); mouse monoclonal anti-Myc tag, clone 4A6 (Merck-Millipore, CA); mouse monoclonal anti-neurofilament 200 (NF200) (Invitrogen, USA); mouse monoclonal anti-human phospho-PHF-tau pSer202/Thr205 (AT8) (Thermo Scientific, Belgium); mouse monoclonal anti-tau, A.A 210-241, clone Tau-5 (Calbiochem, USA); mouse monoclonal anti-kinesin light chain antibody (Merck-Millipore, CA). The following secondary antibodies were used: rabbit anti-mouse HRP conjugated IgG and goat anti-rabbit HRP conjugated IgG (Calbiochem, USA); Alexa-Fluor 488 goat anti-rabbit IgG; Alexa-Fluor 546 goat anti-rabbit IgG; and Alexa-Fluor 555 goat anti-mouse IgG (Invitrogen-Molecular Probes, Eugene, OR). The following reagents were used: Ab40, Ab42, scrambled peptide Ab40 and scrambled peptide Ab42, as well as fluorescein-conjugated Ab40 (all purchased from rPeptides Inc., Athens, GA); retinoic acid (Sigma-Aldrich, St Louis, MO); Thioflavin T (Sigma-Aldrich, St Louis, MO); Pureproteome protein G magnetic beads (Merck-Millipore, CA), recombinant AlexaFluor-555-conjugated Cholera toxin B subunit (FluoAb1-40; Life Technologies-Molecular Probes, Mulgrave, Australia).

2.1 Human post-mortem brain tissue

Human CNS tissue was obtained from the Victorian Brain Bank Network (VBBN) under the National Health and Medical Research Council guidelines and Monash University Human Ethics committee approval number CF13/1646-2013000831. The following tissues were used in the current study: MS = Multiple Sclerosis; AD = Alzheimer's disease; HD = Huntington's disease; FTD = Fronto-Temporal Dementia; Control = Non-neurological disease control brain tissue (Table 1). Postmortem interval did not exceed 57 hrs. All specimens were obtained from the frontal lobe inclusive of grey and white matter, frozen under liquid nitrogen and then stored in the Brain Biobank (-80^{o}C until required). All frozen brain tissues were ground with a mortar and pestle on dry ice, then lysed using RIPA buffer for western blotting analysis.

Table 1: Human brain tissue

Age	Gender	PMI[#]	Diagnosis
65.1	Female	12	MS/AD
38.5	Male	25	MS
66.2	Male	28.5	MS
71.4	Male	34	MS
49.9	Male	43	MS
62.7	Female	56	MS
49.9	Male	56.5	MS

51.1	Male	62	MS
71.4	Male	9.5	AD
73	Male	9.5	AD
83.8	Male	10	AD
88.4	Female	11	AD
60.6	Male	12	FTDu
61.4	Female	20.5	FTDu
77	Male	6	FTDu
66.8	Male	37	FTDu
61.1	Male	17	HD
66.7	Female	18.5	HD
57.2	Female	22	HD
72.2	Female	22	HD
52.1	Male	33	Control
82.7	Male	27	Control
63.4	Female	30.5	Control
63.9	Male	31.5	Control

73.6	Male	49	Control

Post-mortem interval

2.2 Immunohistochemistry

2.2.1 Paraffin sections

Fixed, paraffin-embedded wild-type (n=6) and transgenic Tg2576 (n=16) mouse brains were cut into 10 μm serial sections on a conventional microtome and processed for immunohistochemistry. The sections were de-waxed and antigen retrieval was performed. For antigen retrieval, the sections were washed in phosphate-buffered saline (PBS) and microwaved twice with 0.1 M citrate buffer (pH 6.0) for 5 min each. Sections were washed with PBS after each citrate buffer treatment. They were then incubated with proteinase K (20 μg/ml) (Qiagen) for 1 hour at 37°C, followed by three washes with PBS. The sections were post-fixed with 4% paraformaldehyde for 30 min at room temperature followed by three PBS washes. They were then blocked with 10% (v/v) FBS/0.3% (v/v) Triton X-100 in PBS overnight at 4°C. Sections were incubated with monoclonal anti-phospho-PHF-Tau (AT8) antibody (1:50) and polyclonal anti-phospho-Thr555 CRMP-2 antibody (1:200) diluted in blocking buffer overnight at 4°C. They were washed 3 times for 10 min with PBS, followed by 1 hour incubation with Alexa-Fluor 488 goat anti-rabbit IgG (1:200) and Alexa-Fluor 555 goat anti-mouse IgG (1:200) in blocking buffer at room temperature. The sections were washed three times in PBS and incubated with DAPI for 15 min at room temperature. After three washes in PBS, sections were incubated with 0.2% (w/v) Thioflavin T in PBS for 30 min at room temperature. Finally, the sections were washed three times with PBS and cover-slipped using Dako fluorescent mounting medium. Images were captured under an oil 20x, 40x and 60x objective lens on the NikonC1 upright

confocal microscope. The 16-bit images were converted to TIFF files using ImageJ software and they were formatted using Adobe Photoshop CS3 software. Quantification of double-labelled neurons was performed by counting them at three different fields per slide (3 different slides per animal n=6 animals per age group and genotype). The percentage of pT555CRMP-2 positive neurons was calculated by counting the number of positive neurons and the overall number of neurons surrounding each amyloid plaque in three different slides per animal.

2.2.2 Frozen sections

Freshly dissected brain tissue blocks from Multiple Sclerosis (n=8), Alzheimer's disease (n=8), Huntington's disease (n=4), Fronto-Temporal Dementia (n=7) and Non-neurological disease control patients (n=7) were placed onto a pre-labelled tissue base mold. The tissue blocks were covered completely with Optimal Cutting Temperature compound (OCT) and the mold was placed into liquid nitrogen until the entire tissue block was submerged into liquid nitrogen, to ensure that the tissue was frozen completely. The blocks were then cut into 10 μm serial sections on a cryostat and processed for immunohistochemistry. The sections were washed with PBS after each citrate buffer treatment. They were then incubated with proteinase K (20 μg/ml) (Qiagen) for 1 hour at 37°C, followed by three washes with PBS. The sections were post-fixed with 4% paraformaldehyde for 30 min at room temperature, followed by three PBS washes. They were then blocked with 10% (v/v) FBS/0.3% (v/v) Triton X-100 in PBS overnight at 4°C. Sections were incubated with monoclonal anti-phospho-PHF-Tau (AT8) antibody (1:50) and polyclonal anti-phospho-Thr555 CRMP-2 antibody (1:200) diluted in blocking buffer overnight at 4°C. They were washed 3 times for 10 min with PBS, followed by 1 hour incubation with Alexa-Fluor 488 goat anti-rabbit IgG (1:200) and Alexa-Fluor 555 goat anti-mouse IgG (1:200) in blocking

buffer at room temperature. The sections were washed three times in PBS and incubated with DAPI for 15 min at room temperature. After three washes in PBS, sections were incubated with 0.2% (w/v) Thioflavin T in PBS for 30 min at room temperature. Finally, the sections were washed three times with PBS and cover-slipped using Dako fluorescent mounting medium. Images were captured under an oil 20x, 40x and 60x objective lens on the NikonC1 upright confocal microscope. The 16-bit images were converted to TIFF files using ImageJ software and they were formatted using Adobe Photoshop CS3 software. The percentage of pT555CRMP-2 - AT8 positive neurons was calculated by counting the number of double-labelled neurons and the overall number of neurons from 3 fields per slide.

2.3 Nucleofector transfection of SH-SY5Y and immunocytochemistry

Human CRMP-2 with N-terminal FLAG tag cloned into pRK5 mammalian expression vector was obtained from Dr. Lisa Ooms (Department of Biochemistry and Molecular Biology, Monash University). Site-directed mutagenesis was performed (Agilent Technologies) to produce the following CRMP-2 mutants: T509A, T514A, S518A, S522A, and T555A using the following primers: T509A forward 5'-gtgtgaagtgtctgtggcgcccaagacagtcac-3', T509A reverse 5'-gtgactgtcttgggcgccacagacacttcacac-3', T514A forward, 5'-gcccaagacagtcgctccagcctcctc-3', T514A reverse 5'-gaggaggctggagcgactgtcttgggc-3', S518A forward 5'-cactccagcctccgcggccaagacgtc-3', S518A reverse 5'-gacgtcttggccgcggaggctggagtg-3', S522A forward 5'-ctcggccaagacggctcctgccaagca-3', S522A reverse 5'-tgcttggcaggagccgtcttggccgag-3', T555A forward 5'-cccgccgcaccgcccagcgtatc-3', T555A reverse 5'-gatacgctgggcggtgcggcggg-3'. The CRMP-2 mutant constructs with FLAG tag were then subcloned into a pCMV-Tag5 mammalian

expression vector (Agilent Technologies) containing a C-terminal Myc tag and Kanamycin resistance. Human neuroblastoma SH-SY5Y cells were cultured in an 8-well slide in DMEM/F12 medium until they were 80% confluent. On the day prior to transfection, medium was replaced with OPTI-MEM antibiotic and serum free media (Invitrogen) for 24 hrs. The cells in each well were then transfected using Nucleofector kit (Lonza) with 2 μg/well of pCMV-Tag5, pCMV-Tag5-CRMP2-T555A, pCMV-Tag5-CRMP2-T514A, pCMV-Tag5-CRMP2-T509A, pCMV-Tag5-CRMP2-S518A or pCMV-Tag5-CRMP2-S522A. Cells were then treated with Aβ1-40 peptide at concentration of 10.0 μM for 24 hrs before they were washed and fixed in 4% paraformaldehyde for 30 min at room temperature followed by three PBS washes. The cells were then blocked with 10% (v/v) FBS/0.3% (v/v) Triton X-100 in PBS overnight at 4°C. Cells were incubated with monoclonal anti-FLAG (1:200), monoclonal anti-neurofilament 200 (NF200) (1:200) and polyclonal anti-phospho-Thr555 CRMP-2 antibody (1:200) diluted in blocking buffer overnight at 4°C. They were washed 3 times for 10 min with PBS, followed by 1 hour incubation with Alexa-Fluor 488 goat anti-rabbit IgG (1:200) and Alexa-Fluor 555 goat anti-mouse IgG (1:200) in blocking buffer at room temperature. The cells were washed three times in PBS and incubated with DAPI for 15 min at room temperature. After three washes in PBS, sections were incubated with 0.2% (w/v) Thioflavin T in PBS for 30 min at room temperature. Finally, the cells were washed three times with PBS and cover-slipped using Dako fluorescent mounting medium. Images were captured under an oil 20x, 40x and 60x objective lens on the NikonC1 upright confocal microscope. The 16-bit images were converted to TIFF files using ImageJ software and they were formatted using Adobe Photoshop CS3 software.

2.4 Transfection of primary cortical neurons with APP-FLAG-pDendra and BDNF-FLAG-pDendra

2.4.1 Isolation and culture of primary cortical neurons from P7 rat

Brains were obtained from P7 rat (7 days postnatal) and the meninges were gently removed from the cortex using a forceps. The two hemispheres were separated then cut into small pieces (<1 mm) with a razor blade. Tissues were then transferred into a Falcon tube containing 15 ml of 1x Krebs (refer Appendix) and 1x Trypsin. The tube was incubated with gentle shaking in a water bath at 37°C for 15-20 mins. 10ml of 1X Krebs stock containing DNase/ trypsin inhibitor (tube #2) were then added into the tube and was gently mixed until DNA was digested and tissue pieces are well seperated. The tube was incubated for 10 mins in room tempretaure, and the supernatent was removed gently without disturbing the cortical pellet and placed in new tube (tube #3). The pellet in tube #1 was resuspended with 1.5 ml of tube #3 using a pasteur pipette until tissue pieces were hard to see. The contents from tube #1 were removed and added to tube #3 and was incubated for 10 mins in room tempretaure. The undigested clumps will settled at the bottom of the tube, and the single-cell suspension was removed into a new tube (tube #4). Tube #4 containing the single-cell suspension was centrifuged at 1200 rpm for 5 mins and the supernatant was discarded. The pallet was then resuspended in 10 ml of plating medium (refer Appendix). Cell count was performed and 120,000 cells were seeded per well in a 24 well plate. The 24 well plate had been previously coated with poly-D-lysine for 24 hours, and, were washed three times with Dulbecco's phosphate buffered saline (DPBS), containing potassium chloride and magnesium chloride, before it was air dried in sterile conditions. Plates were incubated at 37°C in 10% O_2 and 5% CO_2 incubator. After two hours, the medium was changed onto neurobasal medium containing L-glutamine, penicillin/streptomycin antibiotics and B27

supplement. The cells have been checked and the medium has been changed every second day till the cells were 80% confluent.

2.4.2 Cloning of APP-FLAG-pDendra and BDNF-FLAG-pDendra

2.4.2.1 Transformation into stellar competent cells

50 ml of competent cells were incubated with 5 ng of APP-FLAG-PCMV, BDNF-FLAG-PCMV or pDendra for 30 min on ice. The cells were then heat shocked for 45 sec in 42°C then placed back on ice for 1-2 min. 500 ml of SOC medium was added and the tube was incubated by shaking (160-225 rpm) for 1 hr at 37°C. 200 ml of bacteria were plated on Kanamycin containing medium and incubated overnight at 37°C. PUC19 was used as a positive control and was plated on Ampecillin-containing medium.

2.4.2.2 Culturing bacteria for plasmid preparation

100 ml of LB broth and an appropriate antibiotic (either Ampecillin or Kanamycin) were added into an autoclaved glass flask. Using a sterile pipette tip, bacteria were swept and the tip was thrown into the flask. The flask was covered loosely with foil and incubated by shaking (225 rpm) overnight at 37°C. Plasmid preparation was performed using Qiagen Mediprep kit.

2.4.2.3 Culturing bacteria for glycerol stock

5 ml of LB broth and an appropriate antibiotic (either Ampecillin or Kanamycin) were added into 50 ml falcon tube. Using a sterile pipette tip, bacteria were swept and the tip was thrown into the tube. The tube was covered loosely with foil and incubated by shaking (225 rpm) overnight at 37°C.

2.4.2.4 Glycerol stock

A 1:1 LB broth to glycerol solution was prepared and added to the bacteria containing LB broth after overnight incubation. The glycerol stock solution was aliquoted in 1.6 ml Eppendorf tubes and stored at -80°C.

2.4.2.5 Plasmid linearisation

2 μg of pDendra2-N vector (Clontech #632545) was linearised using 1 μl xhol restriction enzyme and 5 μl 1x cut smart buffer. pDendra2-N was then digested for 2 hrs at 37°C. It was then heat-inactivated for 20 min at 65°C. Finally, PCR clean-up was performed using the Nucleospin kit. No DNA and no xhol negative controls were used.

2.4.2.6 PCR amplification of APP and BDNF inserts

Mouse APP/PN2 gene cDNA (full length ORF clone), expression ready and N-FLAG tagged was purchased from Sino Biologicals (# MG50402-NF). Mouse BDNF gene cDNA (full length ORF clone), expression ready and N-FLAG tagged was purchased from Sino Biologicals (#

MG50240-NF). Primers used to amplify both APP-FLAG or BDNF-FLAG insert + 15 bp complementary to pDendra were designed using in-fusion clontech primer design tool (Table 3) (review appendix). Both In-FusionR primers are in 5' -> 3' orientation. APP-FLAG and BDNF-FLAG were finally amplified using 12.5 µl of 2x Hifi PCR premix, 2.5 µl of 10x forward primer, 2.5 µl of 10x reverse primer and 100 µg template DNA. The PCR machine was set for 98°C for 10 sec, 55°C for 15 sec and 72°C for 1 min (35 cycles) PCR product was purified using Nucleospin PCR clean up kit followed by elution with 20 µl NE buffer.

Table 2: The result of the In-Fusion Primer design

Fragment 1 FW Primer (F1_FW)	**GGACTCAGATCTCGAGGGTACCATGCCACTGCTG**	Tm : 108	GC : 58.8 %
Fragment 1 RV Primer (F1_RV)	**GAAGCTTGAGCTCGAGTAGTCTTCCCCTTTTAATGG**	Tm : 106	GC : 47.2 %

Cloning Diagram

1. PCR Product.
Fragment1(+) :
GGACTCAGATCTCGAGGGTACCATGCCACTGCTG…....CCATTAAAAGGGGAAGACTACTCGAGCTCAAGCTTC
Fragment1(-) :
CCTGAGTCTAGAGCTCCCATGGTACGGTGACGAC…....GGTAATTTTCCCCTTCTGATGAGCTCGAGTTCGAAG

2. Linearized vector *Xho* I *Xho* I
Vector(+) : ….CAGATCCGCTAGCGCTACCGGACTCAGATC TCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTA….
Vector(-) : ….GTCTAGGCGATCGCGATGGCCTGAGTCTAGAGCT CGAGTTCGAAGCTTAAGACGTCAGCTGCCAT….

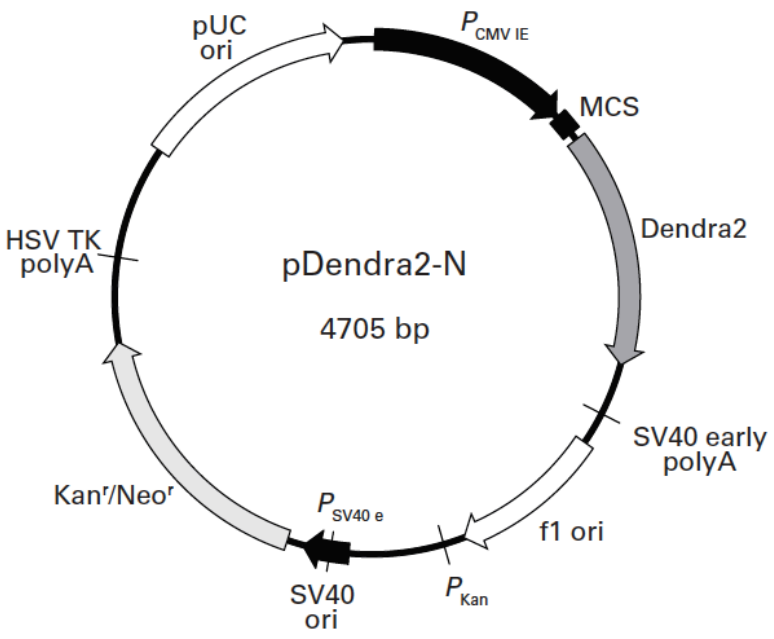
4. Annealing
Fragment1(+) : 5’-
GGACTCAGATCTCGAGGGTACCATGCCACTGCTG….CCATTAAAAGGGGAAGACTACTCGAGCTCAAGCTTC -3’
Fragment1(-) : 3’-
CCTGAGTCTAGAGCTCCCATGGTACGGTGACGAC….GGTAATTTTCCCCTTCTGATGAGCTCGAGTTCGAAG -5’
Vector(+) : ….CAGATCCGCTAGCGCTACCGGACTCAGATC -3’ 5’-
TCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTA….
Vector(-) : ….GTCTAGGCGATCGCGATGGCCTGAGTCTAGAGCT -5’ 3’-
CGAGTTCGAAGCTTAAGACGTCAGCTGCCAT….

* Highlighted text: 15 bp of fragment complementary to pDendra (blue text) to insure successful anneaaling

2.4.2.7 Infusion cloning of FLAG-APP and FLAG-BDNF into linearised pDendra

Infusion cloning was performed using In-Fusion® HD Cloning Kit (Clontech laboratory). 2 μl 5x infusion HD enzyme premix, 50g linearised vector and 50 ng purified PCR insert were prepared. The mixture was incubated for 15 min on ice and then transformed into bacteria using the previously described transformation protocol. No insert negative control and PUC19 positive control were used (Figure. 4).

A


pUC
ori
$P_{CMV\ IE}$
MCS
Dendra2
SV40 early
polyA
f1 ori
P_{Kan}
SV40
ori
$P_{SV40\ e}$
Kanʳ/Neoʳ
HSV TK
polyA
pDendra2-N
4705 bp

B

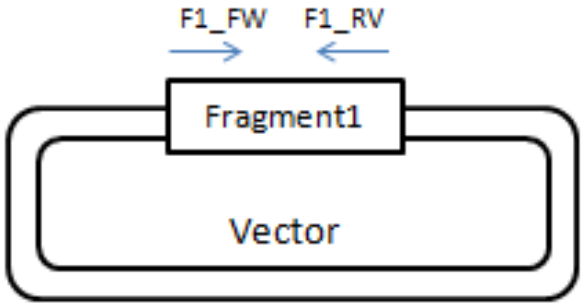

F1_FW
F1_RV
Fragment1
Vector

Figure 4: Infusion cloning of FLAG-APP and FLAG-BDNF into pDendra.

A) Shows pDendra-N vector map and multiple cloning sites (MCS). MCS is located between the cytomegalovirus immediate early promoter ($P_{CMV\ IE}$) and the Dendra2 coding sequence. SV40 polyadenylation signals downstream of the Dendra2 coding sequence direct proper processing of the 3' end of the Dendra2 mRNA. A neomycin-resistance cassette (Neo^r) allows stably transfected eukaryotic cells to be selected using G418. This cassette consists of the SV40 early promoter ($P_{SV40\ e}$), the Tn5 neomycin/kanamycin resistance gene, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSVTK) gene. A bacterial promoter (P_{Kanr}) upstream of the cassette confers kanamycin resistance in *E. coli* (Clontech laboratory, vector guide). **B)** shows the result of the in-Fusion primer design. Both In-FusionR primers are in 5' -> 3' orientation.

2.4.2.8 Screening with Xhol

To insure that APP-FLAG and BDNF-FLAG were successfully insterted into pDendra vector, screening using Xhol enzyme was performed. As Xhol was previously used to linearise the vector, screening using Xhol will produce 2 or 3 bands on the gel (depending on the number of cutting sites) if the insert was successfully bound to the vector or one band if not. Xhol digestion was performed using 1 μg of DNA, 5 μl 1x cut smart buffer and 1 ul Xhol. Reaction tubes were then topped up to 50 μl with dH_2O. Digestion reaction went for 2 hrs at 37°C followed by 20 min heat inactivation at 65°C. Digested samples were run onto 1% agarose get @ 100V for 1.5 hrs.

2.4.2.9 DNA and primer preparation for sequencing

To insure that APP-FLAG and BDNF-FLAG were successfully insterted into pDendra, APP-FLAG pDendra and BDNF-FLAG pDendra plasmids were prepared along with the primers and were sent to Micromon DNA Sequencing Facility at Monash Univeristy. 50:50 and 50:100 dilutions in dH_2O were used for APP-FLAG pDendra and BDNF-FLAG pDendra. APP/BDNF-pDendra sequencing forward and reverse primers were obtained from Micromon DNA Sequencing Facility (#38073- #38074). Plasmids and primers were sent for sequencing. All plasmids had a perfectly matched sequence except for one plasmid which had a mismatch sequence.

2.4.3 Transfection of primary cortical neurons with APP-FLAG-pDendra and BDNF-FLAG-pDendra

Primary cortical neurons were cultured in 24 well plates in neurobasal media until they were 80% confluent. Two hours prior to transfection, the medium was replaced with OPTI-MEM antibiotic and serum free media (Invitrogen). The cells in each well were then transfected using Lipofectamine 3000 (Invitrogen-Molecular Probes) with 1.5, 2 or 3 mg/well of either APP-FLAG-pDendra or BDNF-FLAG-pDendra. Images were captured under 20x objective lens on the NikonC1 upright confocal microscope at 24hrs, 48hrs and 72hrs post transfection. Transfection efficiency was calculated by counting the number of pDendra positive neurons and the overall number of neurons from 3 different fields per well.

2.5 Human neuroblastoma SH-SY5Y cell culture

Human neuroblastoma SH-SY5Y cells were seeded at a density of 100,000 cells per well in 24-well plates in Dulbecco's modified Eagle's medium DMEM/F12 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) and 1% v/v penicillin/streptomycin (Invitrogen). After 24 hours, the cells were differentiated into a neuronal phenotype using 10 μM retinoic acid (RA) (Sigma-Aldrich) over a 7-day period. On day 8, RA treatment was removed and the medium was replaced with a medium containing Aβ1-40 peptide, Aβ1-40 scrambled peptide, Aβ1-42 peptide or Aβ1-42 scrambled peptide at concentrations of 0.5 μM, 1.0 μM or 10.0 μM. The cells were treated with these peptides for 24 hrs before they were washed and lysed for protein collection. Alternatively, SH-SY5Y cells were differentiated on coverslips and incubated with FluoAβ1-40 (1 μM) for 24 hrs and then incubated on ice with AlexaFluor-555-conjugated Cholera toxin B subunit [250 ng/mL] for 15 mins, rinsed with ice-cold PBS

(containing Mg^{2+} and Ca^{2+}) and fixed in 4% paraformaldehyde for 30 min at room temperature. The cells were washed three times with ice-cold PBS and mounted on Superfrost Plus slides using Dako fluorescent mounting medium. Images were captured under an oil 20x, 40x and 60x objective lens on the NikonC1 upright confocal microscope.

2.6 CRMP-2 mutant constructs and Lipofectamine transfection of human neuroblastoma SH-SY5Ycells

Human CRMP-2 with N-terminal FLAG tag cloned into pRK5 mammalian expression vector was obtained from Dr. Lisa Ooms (Department of Biochemistry and Molecular Biology, Monash University). Site directed mutagenesis was performed (Agilent Technologies) to produce the following CRMP-2 mutants: T509A, T514A, S518A, S522A, and T555A as described earlier. The CRMP-2 mutant constructs with FLAG tag were then subcloned into pCMV-Tag5 mammalian expression vector (Agilent Technologies) containing a C-terminal Myc tag and Kanamycin resistance. Human neuroblastoma SH-SY5Y cells were cultured in 6-well plates in DMEM/F12 medium until they were 80% confluent. On the day prior to transfection, the medium was replaced with OPTI-MEM antibiotic and serum-free media (Invitrogen) for 24 hrs. The cells in each well were then transfected using Lipofectamine 2000 (Invitrogen-Molecular Probes) with 2 μg/well of pCMV-Tag5, pCMV-Tag5-CRMP2-T555A, pCMV-Tag5-CRMP2-T514A, pCMV-Tag5-CRMP2-T509A, pCMV-Tag5-CRMP2-S518A or pCMV-Tag5-CRMP2-S522A. Cells were then treated with amyloid-beta peptides as described earlier. Cells were lysed and proteins were collected for immunoprecipitation and western blotting analyses.

2.7 Preparation of cell and tissue lysates for western blotting

Human neuroblastoma SH-SY5Y cells and brain tissues (Alzheimer's disease, Fronto-Temporal Dementia, Huntington's disease, Multiple Sclerosis and non-neurological control) were lysed using cell lysis buffer (Cell Signaling Technology) containing 1% (v/v) protease and phosphatase inhibitors (Calbiochem, USA) (Table 1). Cell lysates were centrifuged at 10,000 x*g* for 15 min at 4°C and the supernatants were collected. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL).

2.8 Immunoprecipitation

Immunoprecipitation was performed by adding 1 μg of mouse monoclonal anti-CRMP-2 antibody to 100 μg of total protein from each sample. Samples were incubated for 24 hrs at 4°C followed by overnight incubation with 100 μl of Protein G magnetic beads on a rotating wheel. Beads were separated using a magnetic stand (Merck Millipore) and the supernatants were removed. Three washes were then done using radio-immunoprecipitation (RIPA) buffer or cell lysis buffer (Cell Signaling Technology) containing 1% (v/v) protease and phosphatase inhibitors (Calbiochem, USA). Proteins were dissociated from the beads using heat at 95°C for 5 min in 1x sample loading buffer (Invitrogen) containing beta-mercaptoethanol. Electrophoresis of the samples was done using 4-12% Bis-Tris gels (Invitrogen).

2.9 Western blotting

Five μg of total protein was loaded and run on a 4-12% Bis-Tris gel (Invitrogen). Proteins were then transferred onto polyvinylidene fluoride membranes (PVDF) (Millipore) and blocked with 5% skim milk powder in TBST for one hour at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer that included: mouse monoclonal anti-CRMP-2, 1:1000; anti-a-tubulin, 1:5000; anti-phosphothreonine555 CRMP-2, 1:5000; anti-phosphothreonine514 CRMP-2, 1:500; anti-phosphoserine522 CRMP-2, 1:2000; anti-phosphoserine199/202 tau, 1:2000; anti-tau5, 1:2000; anti-FLAG, 1:2000; or anti-Myc, 1:2000. After three 10 min washes with TBST, membranes were incubated with secondary anti-rabbit (1:10,000) or anti-mouse (1:10,000) HRP-conjugated antibodies diluted in TBST for 2 hours at room temperature. Proteins were detected using Luminata ECL chemiluminescence (Merck-Millipore, CA). The films were scanned using the Alpha Imager (Alpha Innotech, San Leandro, CA) and the intensities of the bands were measured using ImageQuant TL v2003 software (Nonlinear Dynamics Ltd, All Saints, Newcastle, UK).

2.10 Plaque assay with human neuroblastoma SH-SY5Y cells

Autoclaved coverslips were placed in a 24-well plate. 10 μL of Aβ1-40 peptide or Aβ1-40 scrambled peptide at a concentration of 10 μM was dropped in the middle of the coverslips or plastic wells, which were then air dried overnight to form plaques. Cells were seeded at a density of 100,000 cells per well for wells or coverslips that had been treated with Aβ1-40 peptide, or, at a density of 50,000 cells per well for wells or coverslips that had been treated with Aβ1-40 scrambled peptide. Cells were treated with 10 μM Retinoic Acid in DMEM/F12 medium

(Invitrogen) supplemented with 10% Fetal Bovine Serum (Invitrogen) and 1% v/v penicillin/streptomycin (Invitrogen) for 24 hours. After three washes with PBS (Ca^{2+}, Mg^{2+}) (Invitrogen), the cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Cells were then stained with 0.002% (w/v) Thioflavin T for 30 minutes at room temperature, washed three times, followed by overnight incubation with monoclonal anti-NF200 antibody (1:200) and polyclonal anti-pCRMP2 antibody (1:200) prepared in PBS (Ca^{2+}, Mg^{2+}) with 5% (v/v) Normal Goat Serum (Invitrogen) and 0.2% (v/v) Triton-X100 (Sigma) at 4°C. After three washes with PBS (Ca^{2+}, Mg^{2+}), cells were stained with Alexa-Fluor 555 goat anti-rabbit IgG (1:200) and Alexa-Fluor 488 goat anti-mouse IgG (1:200) for two hours at room temperature, followed by a final three washes with PBS (Ca^{2+}, Mg^{2+}). Coverslips were removed from the wells and mounted upside down onto slides with Dako Mounting Medium (Dako) while glycerol was added to plastic wells. Images were captured under an oil objective lens (60x) on a NikonC1 Upright confocal microscope. The 16-bit images were converted to TIFF files using ImageJ software and were formatted using Adobe Photoshop CS3 software. The percentage of pT555CRMP-2 positive SH-SY5Y cells was calculated by counting the number of pT555CRMP-2 positive SH-SY5Y cells and the overall number of cells from 3 fields inside and 3 different fields outside the plaque.

2.11 Trypsin digestion with reduction and alkylation

The protein was reduced in 2.5 mM DTT at 50°C for 30 minutes followed by alkylation with 10 mM Iodoacetamide for 30 minutes in the dark at room temperature. Following alkylation, a solution containing 1 µg Trypsin (Promega corp., Madison, WI, USA) in 20 mM Ammonium bicarbonate was added and the samples incubated at 37°C overnight. Tryptic digests were

analyzed by LC-MS/MS using the QExactive mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with a RSLC nano HPLC (Ultimate 3000, Thermo Scientific, Bremen, Germany). Samples were concentrated on a 100 μm, 2 cm nanoviper pepmap100 trap column with loading buffer (2% Acetonitrile, 0.1% Formic acid) at a flow rate of 15 μl/minute. The peptides then eluted and separated with a Thermo RSLC 50 cm pepmap100, 75 μm id, 100°A pore size, reversed phase nano column with a 15 minute gradient of 90% buffer A (0.1% Formic acid) to 40% B (80% Acetonitrile 0.1% formic acid) and to 95% buffer B in 10 minutes, at a flow rate of 300 nl/minute. The eluant was nebulised and ionised using the Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of 1900V. Peptides were selected for MSMS analysis in Full MS/dd-MS2 (TopN) mode with the following parameter settings: TopN 10, resolution 17500, MSMS AGC target 1e5, 60 ms Max IT, NCE 27 and 3 m/z isolation window. Underfill ratio was at 10% and dynamic exclusion was set to 15 seconds.

Data from LCMSMS run was processed using proteome discoverer V1.4 (Thermo Fisher Scientific) and searched against a custom database comprising proteome datasets downloaded from the Uniprot web site (http://www.uniprot.org/) using the MS Amanda search engine. The following search parameters were used: missed cleavages, 1; peptide mass tolerance, ± 15 ppm; peptide fragment tolerance, ± 0.2 Da; peptide charge, 2+, 3+ and 4+; static modifications, carbamidomethyl; Dynamic modification, oxidation (Met). Low and medium confidence peptides were filtered with at least 0.02 FDR (high confidence)

2.12 Aβ peptide preparation and aggregation

Aβ1-40, Aβ1-42, ScrAβ1-40 and ScrAβ1-42 were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml, aliquotted in microcentrifuge tubes and stored at -20ºC. On the day of the experiment, an aliquot of Aβ1-40, Aβ1-42, ScrAβ1-40 and ScrAβ1-42 was diluted in anhydrous DMSO to 10 mM, which was then added to ice-cold DMEM/F12 medium with FCS and without phenol red to 0.5, 1.0 and 10 mM. Cells were incubated with these Aβ peptides over a 24 hr period.

2.13 Phospho-Thr555 CRMP-2 antibody detection

Each well of a 96-well plate was coated with 0.1, 1.0, 2.0, 4.0, 5.0 and 10.0 μg/ml Phospho-Thr555 CRMP-2 protein in 0.05 mol/L carbonate buffer (pH 9.6) overnight at 4°C. The wells were then washed three times with PBS (pH 7.4) and son specific binding sites were blocked using 10% FCS/PBS. Sera from immunised rabbits against the Phospho-Thr555 CRMP-2 peptide were diluted in 10% FCS/PBS (1:10,000) and added into the wells in triplicate. After 2 hrs incubation in RT, the serum was removed and wells were washed three times with PBS (pH 7.4). Peroxidase-conjugate sheep anti-human IgM/IgG antibody (Silenus Laboratory Melbourne) was prepared in 10% FCS/PBS then added into the wells then incubated for 2 hrs at RT. The loosely adherent antibodies were then washed off using PBS (x 3). Wells were then developed with 20 mg o-phenylene-diamine dihydrochloride (OPD; Sigma Chemial Company) for 30 min. The peroxidase-OPD reaction was finally halted with 25 μl of 3 mol/L HCL and the optical density of each well was measured at 490 nm using EIA microplate reader (Bio-Rad

Laboratory). Plate was placed into a plate reader and the optical density was measured for each well.

2.14 Sandwich ELISA for Aβ measurement

Each well of a 96-well plate was coated with 0.1, 1.0, 2.0, 4.0, 5.0 and 10.0 μg/ml Phospho-Thr555 CRMP-2 protein in 0.05 mol/L carbonate buffer (pH 9.6) overnight at 4°C. The wells were then washed three times with PBS (pH 7.4) and son specific binding sites were blocked using 10% FCS/PBS. Sera from immunised rabbits against the Phospho-Thr555 CRMP-2 peptide were diluted in 10% FCS/PBS (1:10,000) and added into the wells in triplicate. After 2 hrs incubation in RT, the serum was removed and wells were washed three times with PBS (pH 7.4). Peroxidase-conjugate sheep anti-human IgM/IgG antibody (Silenus Laboratory Melbourne) was prepared in 10% FCS/PBS then added into the wells then incubated for 2 hrs at RT. The loosely adherent antibodies were then washed off using PBS (x 3). Wells were then developed with 20 mg o-phenylene-diamine dihydrochloride (OPD; Sigma Chemial Company) for 30 min. The peroxidase-OPD reaction was finally halted with 25 μl of 3 mol/L HCL and the optical density of each well was measured at 490 nm using EIA microplate reader (Bio-Rad Laboratory). Plate was placed into a plate reader and the optical density was measured for each well.

2.15 Statistics

Data were analyzed using Graph Pad Prism v6.0 software. A two-tailed Student's *t* test or a one-way analysis of variance (ANOVA) with Tukey's post hoc test was used to determine statistical significance between groups that was set at $p < 0.05$ at a 95% confidence level for optical density levels obtained from immunoblot analysis.

CHAPTER 3: Aβ- dependent regulation of CRMP-2 and neurite function identified in a human neuroblastoma cell model

3.1 Introduction

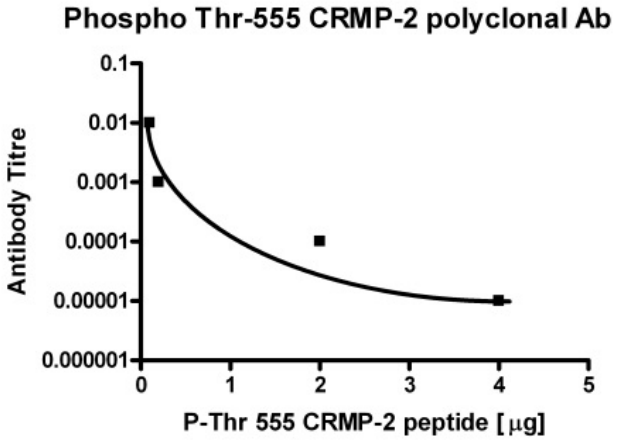
The involvement of protein kinases in the hyperphosphorylation of CRMP-2 makes the use of selective kinase inhibitors a potential therapeutic application in AD. Screening for promising kinase inhibitors requires the use of a well-characterised cell model. SH-SY5Y cells are a human neuroblastoma cell line commonly used in *in vitro* models of neuronal function and differentiation. SH-SY5Y cells proliferate through mitosis and differentiate by extending neurites. Proliferating cells may develop clusters of cells but specific treatments such as RA or BDNF can induce the cells to extend neurites and differentiate. Additionally, RA treatment inhibits cell growth and enhances production of noradrenaline from SH-SY5Y cells (Korecka et al., 2013). Once differentiated, these cells are ideal for the investigation of cell signalling pathways under a neurobiological experimental paradigm. It has been demonstrated that the differentiation of SH-SY5Y with RA increases tau content and phosphorylation (Jamsa et al., 2004). It has also been found that RA inhibits the transcription of CRMP-2 during SH-SY5Y neuroblastoma cell differentiation (Fontan-Gabas et al., 2007). Therefore, RA-treated SH-SY5Y cells seem an ideal cell model to define the most important kinases involved in Aβ-dependent phosphorylation of CRMP-2 and tau in the absence of altered transcription following the development of these cells. Petratos et al. (2008) have previously shown that Aβ treatment of SH-SY5Y cells induces phosphorylation of CRMP-2 at a threonine residue unrelated to the GSK-3β site (Petratos et al., 2008a). Importantly, the Aβ-induced threonine phosphorylation was shown to be reduced with the administration of the Rho kinase inhibitor, Y27632 (Petratos et al., 2008a). These data suggest that Aβ stimulation of SH-SY5Y cells elicits a Rho kinase-mediated phosphorylation of CRMP-2. The question that remains unanswered is whether Rho kinase-mediated phosphorylation of CRMP-2 is the prominent pathway leading to neurite outgrowth

and axonal transport inhibition or whether the other kinases (Cdk-5 and GSK-3β) also play a significant role in that mechanism. Recently it was shown that phosphorylation of CRMP-2 occurs at Ser522 in transgenic mouse models of AD and in AD brains (Cole et al., 2007). This phosphorylation of CRMP-2 with Cdk-5 makes it susceptible to be subsequently phosphorylated by GSK-3β. We have dissected out the Aβ-induced CRMP-2 phosphorylation events by designing constructs through site-directed mutagenesis. Since phosphorylation of CRMP-2 correlates with decreased tubulin and kinesin binding and neurite outgrowth inhibition, we have investigated these physiological outcomes in SH-SY5Y over-expressing CRMP-2 mutants in the presence of Aβ.

3.2 Phospho-Thr555 CRMP-2 antibody verification

Enzyme-Linked ImmunoSorbant Assay (ELISA) was used to determine phospho-Thr555 CRMP-2 peptide reactivity with the affinity purified polyclonal antibody. Using Tg2576 mice brain lysates, we found that immunoreactivity occurs at 1:10,000 dilution of the anti-Phospho-CRMP-2 antibody with the phospho-CRMP-2 peptide. Moreover, western blot analysis shows a ~62 kDa band with incubation of anti-phospho-CRMP-2 antibody which we were able to block by pre-incubation of the antibody with the phospho-Thr555 CRMP-2 peptide. Pre-incubation with the same CRMP-2 peptide without phosphorylation produces the same 62 kDa band. Incubation with the pre-bleed antiserum shows no reactivity with the 62 kDa band. The 62 kDa band was then analysed by mass spectrometry and confirmed as phosphorylated CRMP-2 (Figure. 5).

A


Phospho Thr-555 CRMP-2 polyclonal Ab
Antibody Titre
0.1
0.01
0.001
0.0001
0.00001
0.000001
0
1
2
3
4
5
P-Thr 555 CRMP-2 peptide [μg]

B

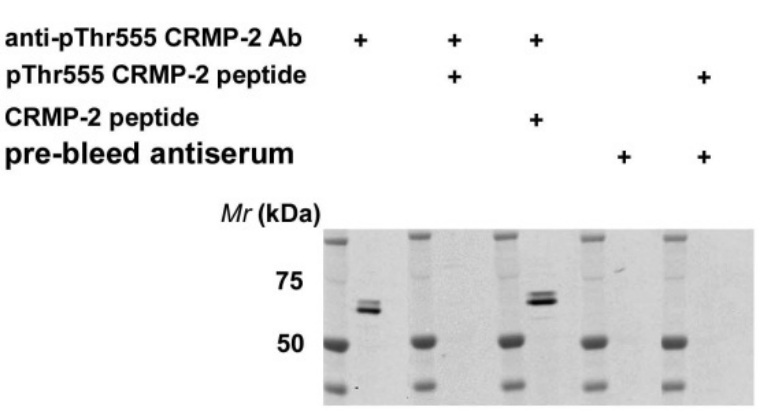

anti-pThr555 CRMP-2 Ab + + +
pThr555 CRMP-2 peptide + +
CRMP-2 peptide +
pre-bleed antiserum + +
Mr (kDa)
75
50

Figure 5: Characterisation of anti-Phospho-Thr555 CRMP-2 antibody.

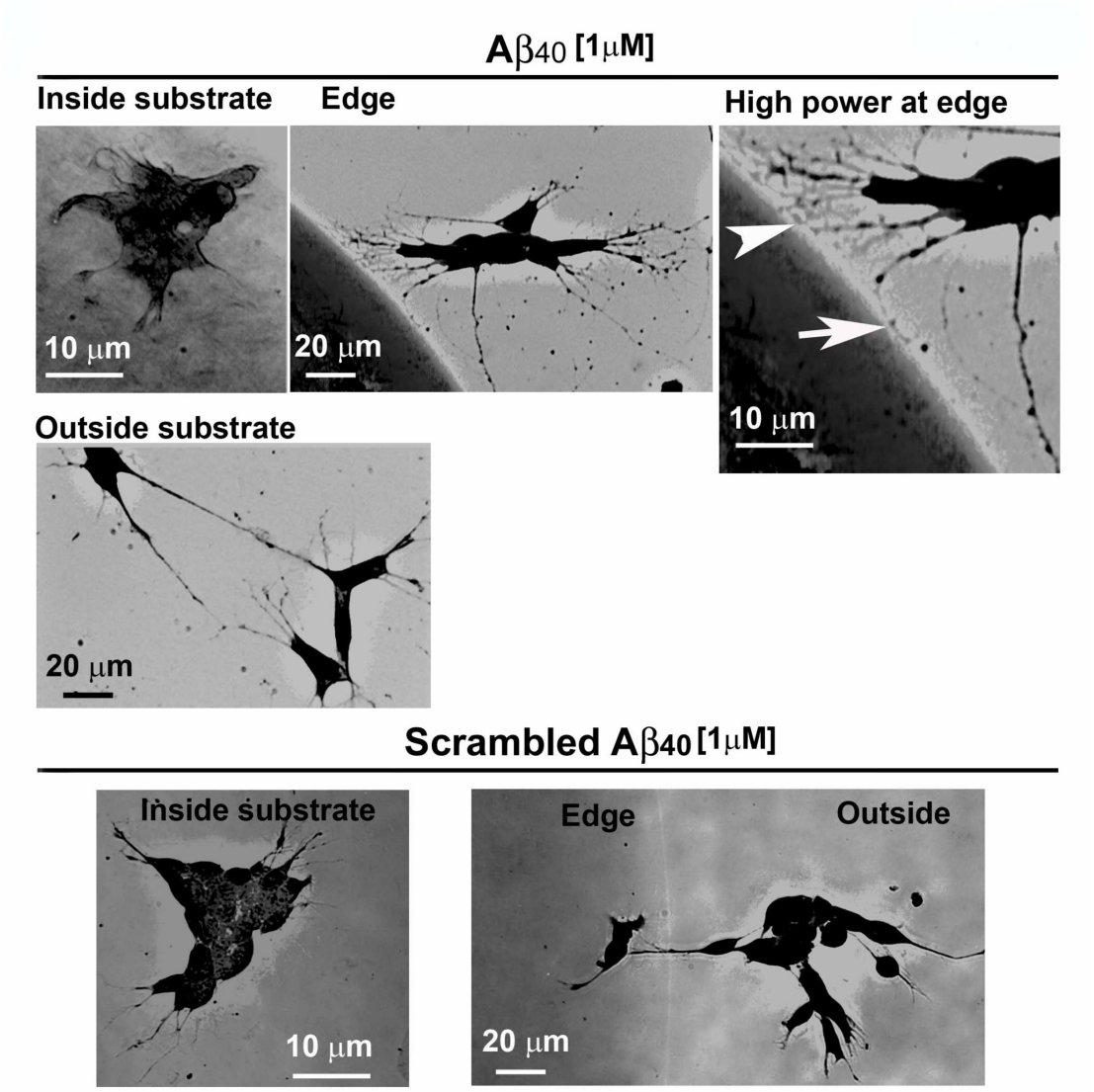
ELISA determining phospho-Thr555 CRMP-2 peptide reactivity with the affinity purified polyclonal antibody. **A)** Immunoreactivity occurs at 1:10,000 dilution of the anti-Phospho-CRMP-2 antibody with the phospho-CRMP-2 peptide. Tg2576 brain lysates react with the anti-phospho-CRMP-2 antibody by western blot. **B)** Lanes 1,3, 5, 7 and 9 represents the molecular weight marker. Lanes 2,4,6,8 and 10 represents a 12 old-of-age Tg2576 brain lysate. A ~62 kDa band appears with incubation of anti-phospho-CRMP-2 antibody alone and this immunoreactivity can be blocked by pre-incubation of the antibody with the phospho-Thr555 CRMP-2 peptide. Pre-incubation with the same CRMP-2 peptide without phosphorylation produces the same 62 kDa band. Incubation with the pre-bleed antiserum shows no reactivity with the 62 kDa band. The 62 kDa band was then analysed by mass spectrometry and confirmed as phosphorylated CRMP-2.

3.2 Altered neurite morphology in SH-SY5Y human neuroblastoma neurons develop near an Aβ-substrate in culture

In an attempt to investigate the effect of amyloid plaques on neurite elongation and CRMP-2 phosphorylation, we established SH-SY5Y cultures during their neurite outgrowth phase but grown on a centrally placed Aβ-peptide deposit (laboratory-made APs as a substrate) in a 24-well culture plate. The cells that attached and grew directly on the artificial 'AP' and the cells that grew adjacent to it displayed dystrophic neurites (Figure. 6A, arrowhead) with numerous varicosities (Figure. 6A, arrow). On the other hand, the axons of the cells that were growing away from the plaque grew in a more linear trajectory (Figure. 6A). This is very different from the cells attached to a scramble peptide artificial 'plaque', where the cells are visibly healthy and the axons grow straight through the edge of the artificial 'plaque' (Figure. 6A). These results suggest signalling deficits affecting the neuronal cytoskeleton effective at the active growth zones of neurites.

To examine the relationship between CRMP-2 phosphorylation at the T555 site and dystrophic neurites, immunocytochemical staining of SH-SY5Y cells with pT555 CRMP-2 and NF200 was performed. After adding the cells into wells containing an artificial 'amyloid plaque', cells that were attached to the plaque or growing near the plaque showed dystrophic neurites with substantial staining with pT555 CRMP-2 (Figure. 6B, arrow head & 6C). However, the cells that were growing away from the plaque showed neuritogenesis with less pT555 CRMP-2 staining at the neurites (Figure 6B & 6C). Similarly, the cells that were growing inside the scrambled peptide substrate-containing wells, showed neurito-genesis with vastly reduced pT555 CRMP-2 neuronal staining (Figure. 6B). These data illustrate that neuronal interactions with Aβ potentiate the phosphorylation of CRMP-2 at the T555 site.

A


Aβ40 [1μM]
Inside substrate
Edge
High power at edge
10 μm
20 μm
10 μm
Outside substrate
20 μm
Scrambled Aβ40 [1μM]
Inside substrate
10 μm
Edge
Outside
20 μm

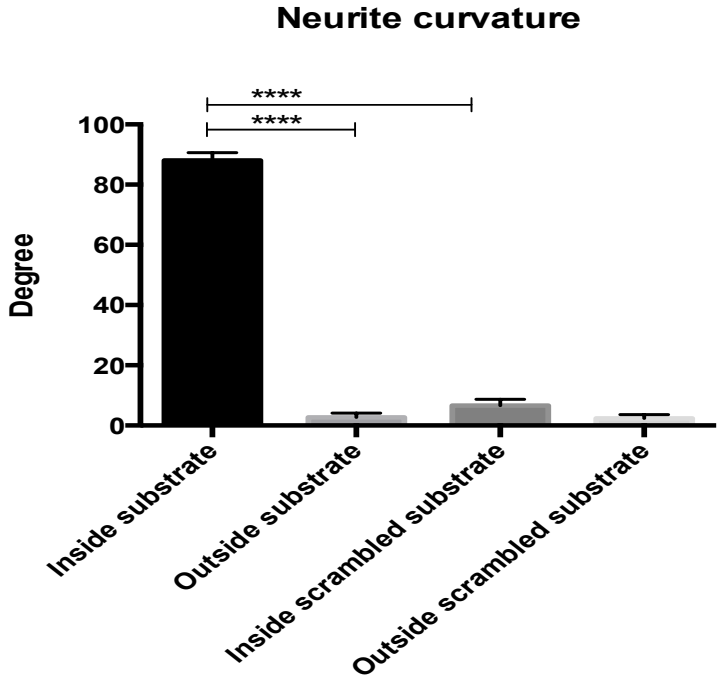
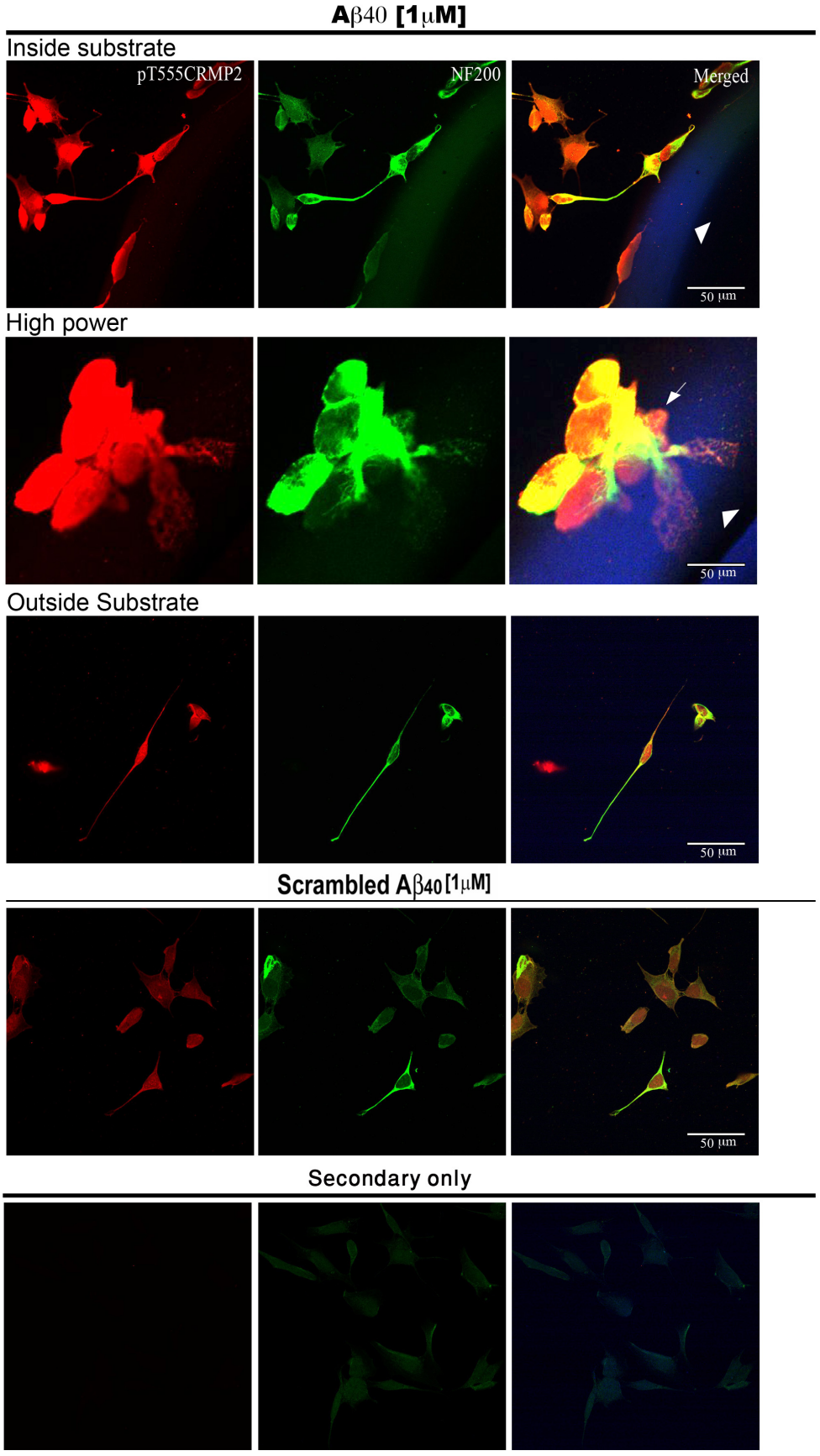

Neurite curvature
****
****
100
80
60
40
20
0
Degree
Inside substrate
Outside substrate
Inside scrambled substrate
Outside scrambled substrate

Figure 6: Altered neurite morphology in SH-SY5Y developed within or near amyloid plaques (AP).

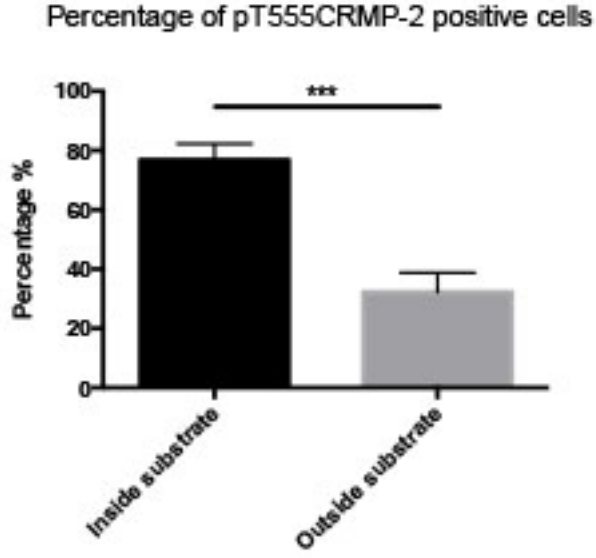
A) Represents cells that were growing on and around laboratory-made amyloid plaques in culture. Those cells that grow inside or near the substrate end up curving their axons, while the cells that were growing away from the substrate have straight axons. Cells that were growing within or near the scrambled peptide plaques are also illustrated. The cells look healthy and the axons are growing straight through the plaque. The graph shows significant difference in the degree of neurite curvature in cells that surround the artificial amyloid plaque, compared to the cells that are outside the amyloid plaque and also compared to the cells that are inside an amyloid plaque that was performed from a scrambled form of the peptide (**** $p<0.0001$) ($n=3$).

B


Aβ40 [1μM]
Inside substrate
pT555CRMP2
NF200
Merged
50 μm
High power
50 μm
Outside Substrate
50 μm
Scrambled Aβ40 [1μM]
50 μm
Secondary only

B) Cells grown on and around artificial amyloid plaques (arrowhead) in culture were immunostained with pT555 CRMP-2 and NF200. The cells that attached directly to the plaque or grew near the plaque showed substantial immunolabelling for pT555 CRMP-2 along with abnormal neurite growth (arrow). By contrast, the cells that were growing outside the substrate show straight elongated neurites with minimal pT555 CRMP2 immunostaining. Cells that were growing within scrambled peptide plaque show neuritogenesis with no pT555 CRMP-2 immunostaining.

C


Percentage of pT555CRMP-2 positive cells
***
Percentage %
100
80
60
40
20
0
Inside substrate
Outside substrate

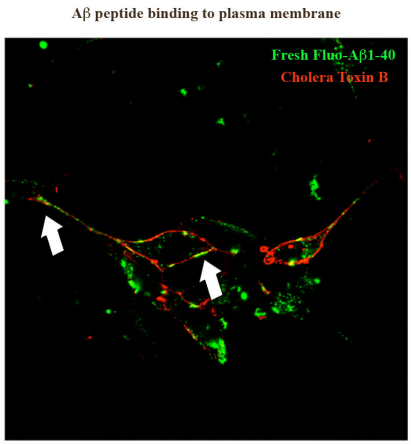
C) Percentage of pT555CRMP-2 positive SH-SY5Y cells inside vs outside the artificially made amyloid plaque. The graph shows significant difference in the percentage of pT555CRMP-2 stained SH-SY5Y cells that surround the artificial amyloid plaque, compared to the cells that are outside the amyloid plaque (*** $p<0.001$) (n=3).

3.3 Soluble Aβ preferentially increases CRMP-2 phosphorylation in SH-SY5Y neuroblastoma cells at the T555 site

Since it is well known that Aβ can bind to and disrupt the neuronal cell membrane, although the exact process is still controversial, we stained SH-SY5Y cells with fresh fluorescent Aβ1-40 after mapping the cells with cholera toxin B, which binds to gangliosides within phospholipids and lightens up the lipid raft (Miller et al., 2004). We found that there is colocalisation of cholera toxin B with Aβ1-40, which makes it plausible to suggest that Aβ1-40 also binds to the lipid raft and initiates a downstream signaling mechanism (Figure. 7A, arrow). Since generation of neurotoxic species of Aβ is characteristic of AD, we investigated the initial effects of Aβ1-40 and Aβ1-42 on phosphorylation of CRMP-2 in differentiated SH-SY5Y human neuroblastoma cells. We found that the phosphorylation of CRMP-2A (75 kDa) and CRMP-2B (62 kDa) at the T555 site was elevated, correlating with increases in the extracellular concentration of Aβ1-40 administred after a 24 hr period (Figure. 7B & 7C). However, we found decreased phosphorylation of CRMP-2A and CRMP-2B upon Aβ1-42 treatment of these cells under the same temperature and time of administration (Figure. 7B & 7C). More importantly, we identified a 55 kDa immunoreactive band that was only abundantly present upon Aβ1-42 treatment. We identified the band to be the C-terminal 55 kDa cleaved product of CRMP-2 as previously reported in Prion disease (Shinkai-Ouchi et al., 2010) and acquired brain injury samples (Zhang et al., 2007). These data support other putative post-translational modifications to CRMP-2 such as calpain cleavage, which has been reported as a major downstream event occurring in neuronal cell death (Zhang et al., 2007). On the other hand, despite readily detectable modifications in CRMP-2 phosphorylation at the T555 site, no differences were detected in the phosphorylation of CRMP-2 at the Thr514 and Ser522 sites (Figure. 7B, 7D & 7E). These data suggest that Aβ1-40 can modify CRMP-2 through phosphorylation at the putative Rho kinase site and that this is

not evident following Aβ1-42 administration. These results also suggest that Aβ1-42 can modulate CRMP-2 function by enhancing CRMP-2 cleavage rather than phosphorylation. This effect may be related to the neurotoxicity of this peptide, which is probably leading to cell death and CRMP-2 cleavage as a consequence. The results also demonstrated that the alternatively spliced form, namely CRMP-2A, predominantly increases with elevated extracellular levels of Aβ1-40 (Figure. 7B & 7C). Furthermore, we found increased levels of hyperphosphorylated tau (AT8) upon Aβ1-40 administration (Figure. 7B & 7F). On the other hand, treating the cells with the scrambled form of the Aβ peptide had no effect on the levels of CRMP-2 or tau phosphorylation (Figure. 7B & 7F). These data suggest that CRMP-2 phosphorylation at T555 site is an Aβ1-40-dependent mechanism.

A


Aβ peptide binding to plasma membrane
Fresh Fluo-Aβ1-40
Cholera Toxin B

B

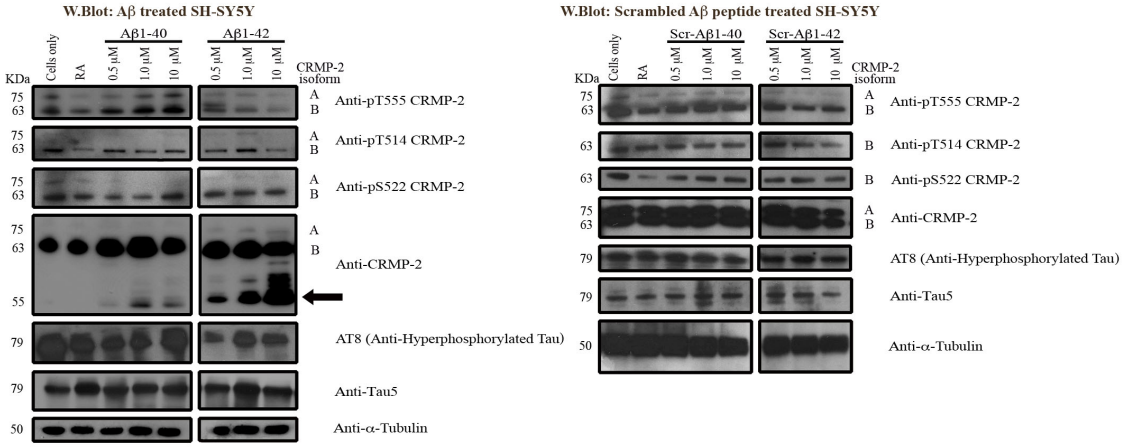
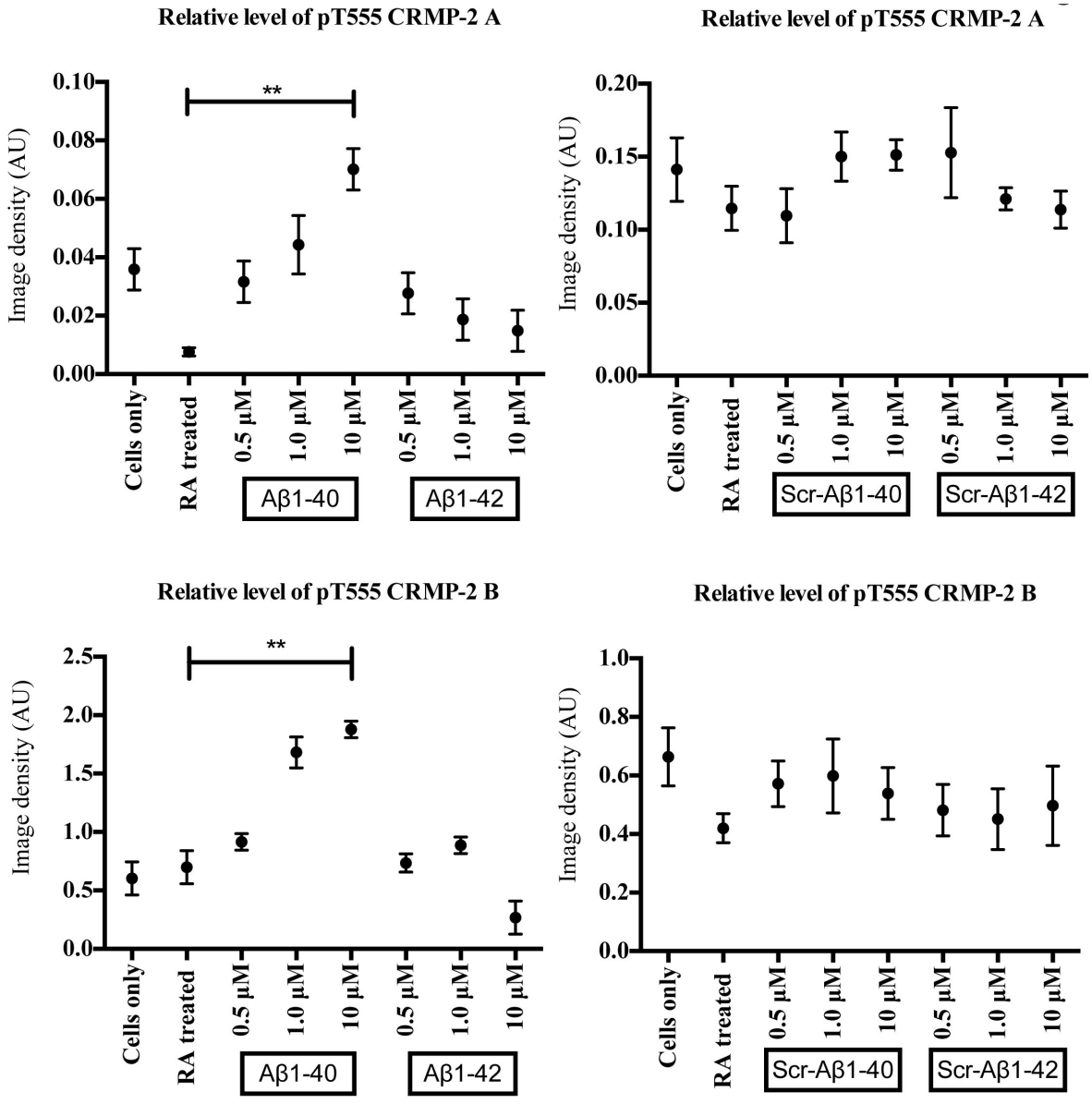

W.Blot: Aβ treated SH-SY5Y
Aβ1-40
Aβ1-42
Cells only
RA
0.5 μM
1.0 μM
10 μM
KDa
CRMP-2 isoform
Anti-pT555 CRMP-2
Anti-pT514 CRMP-2
Anti-pS522 CRMP-2
Anti-CRMP-2
AT8 (Anti-Hyperphosphorylated Tau)
Anti-Tau5
Anti-α-Tubulin
W.Blot: Scrambled Aβ peptide treated SH-SY5Y
Scr-Aβ1-40
Scr-Aβ1-42

Figure 7: Soluble Aβ preferentially increases CRMP-2 phosphorylation in SH-SY5Y neuroblastoma cells at the T555 site.

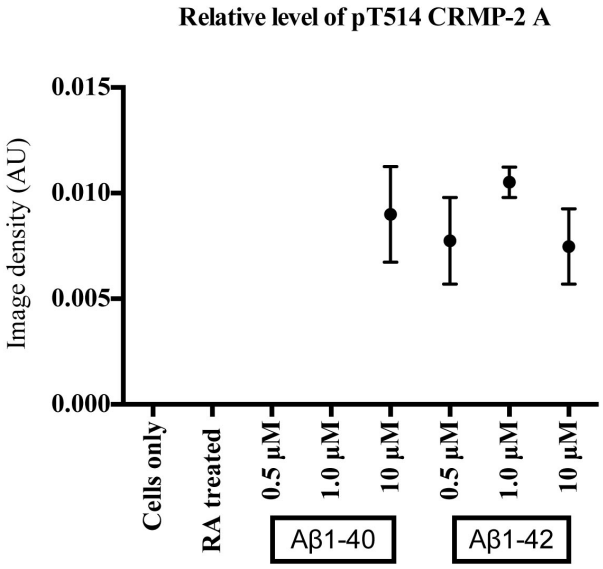
A) Aβ1-40 binds to the neuronal plasma membrane at cholera toxin B labelled domains (arrow). **B)** Shows western blotting results of SH-SY5Y cells that were treated with Aβ1-40, Aβ1-42, Scr-Aβ1-40 or Scr-Aβ1-42 for 24 hrs and stained with pT555 CRMP-2, pT514 CRMP-2, pS522 CRMP-2 and AT8. Staining was also performed using CRMP-2, Tau5 and α-Tubulin as a control. What is evident is the presence of approximately 55 KDa CRMP-2 cleavage product primarily in Aβ1-42 treated cells (n=3).

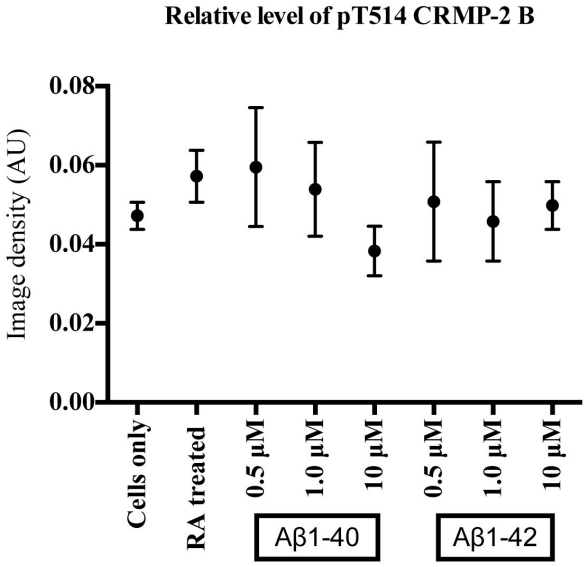
C

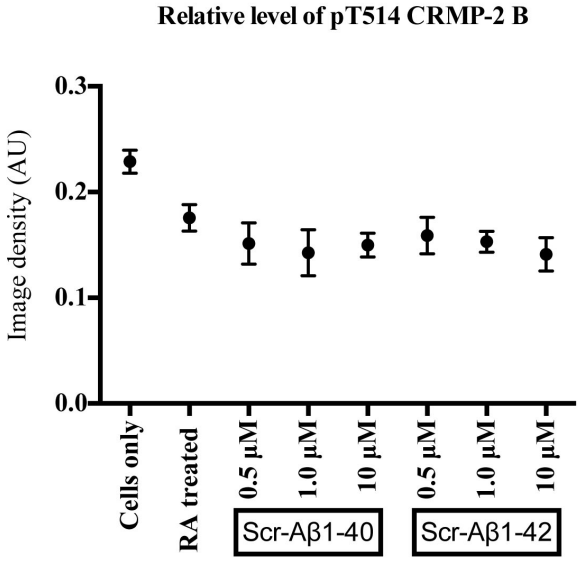

Relative level of pT555 CRMP-2 A
Image density (AU)
0.10
0.08
0.06
0.04
0.02
0.00
**
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Aβ1-40
Aβ1-42
Relative level of pT555 CRMP-2 A
Image density (AU)
0.20
0.15
0.10
0.05
0.00
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Scr-Aβ1-40
Scr-Aβ1-42
Relative level of pT555 CRMP-2 B
Image density (AU)
2.5
2.0
1.5
1.0
0.5
0.0
**
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Aβ1-40
Aβ1-42
Relative level of pT555 CRMP-2 B
Image density (AU)
1.0
0.8
0.6
0.4
0.2
0.0
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Scr-Aβ1-40
Scr-Aβ1-42

C) Aβ-induced phosphorylation of CRMP-2 at the T555 site increases with the increase in Aβ1-40 concentration administrated to the cells for 24 hours (* $p< 0.05$, ** $p<0.01$). Phosphorylation of both CRMP-2A and CRMP-2B alternatively spliced isoforms is potentiated after the administration of Aβ1-40, while the decrease in the phosphorylation of CRMP-2A and CRMP-2B at T555 site occurs after Aβ1-42 treatment (** $p<0.01$). Treating SH-SY5Y cells with different concentrations of scrambled Aβ1-40 and Aβ1-42 peptides shows no significant differences in the phosphorylation at T555 site in comparison to Aβ non-treated control cells (n=3).

D

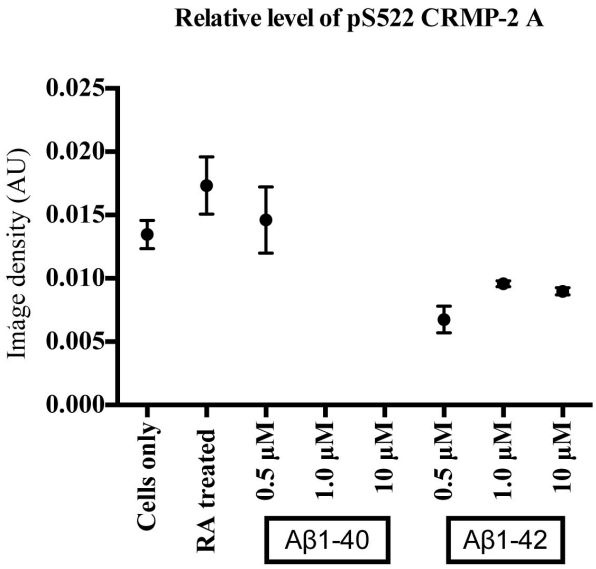

Relative level of pT514 CRMP-2 A
Image density (AU)
0.015
0.010
0.005
0.000
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Aβ1-40
Aβ1-42

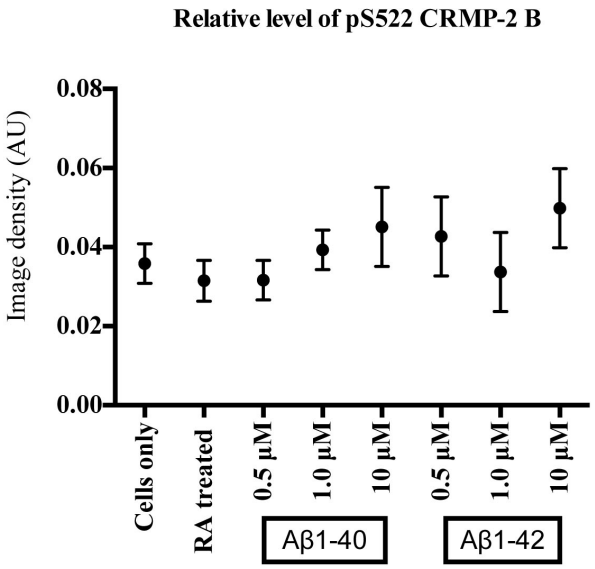

Relative level of pT514 CRMP-2 B
Image density (AU)
0.08
0.06
0.04
0.02
0.00
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Aβ1-40
Aβ1-42

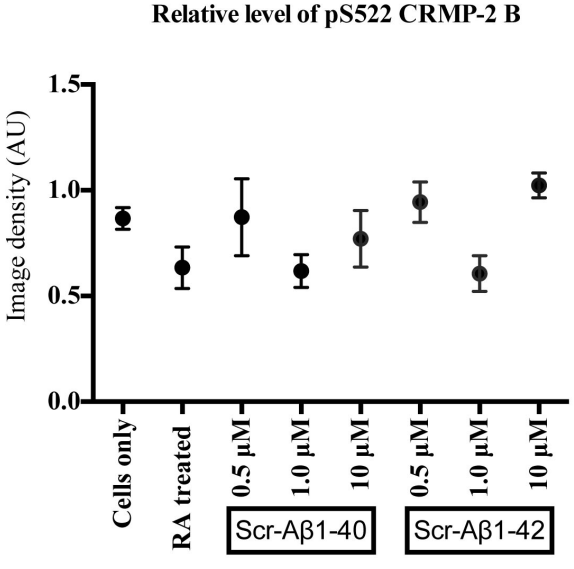

Relative level of pT514 CRMP-2 B
Image density (AU)
0.3
0.2
0.1
0.0
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Scr-Aβ1-40
Scr-Aβ1-42

D) Phosphorylation of CRMP-2B and CRMP-2A at the T514 site after Aβ1-40 and Aβ1-42 treatment shows no significant difference compared to untreated cells. Treating SH-SY5Y cells with different concentrations of scrambled Aβ1-40 and Aβ1-42 peptides also shows no significant differences in the phosphorylation at T514 site in comparison to Aβ non-treated control cells (n=3).

E

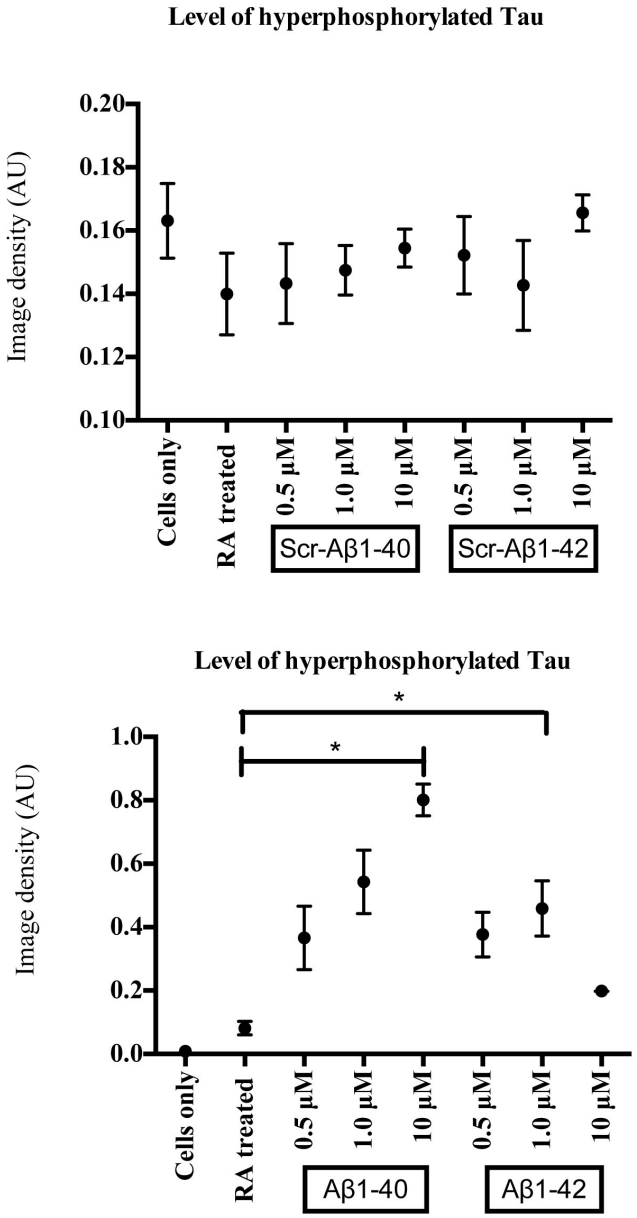

Relative level of pS522 CRMP-2 A
Image density (AU)
0.025
0.020
0.015
0.010
0.005
0.000
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Aβ1-40
Aβ1-42


Relative level of pS522 CRMP-2 B
Image density (AU)
0.08
0.06
0.04
0.02
0.00
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Aβ1-40
Aβ1-42


Relative level of pS522 CRMP-2 B
Image density (AU)
1.5
1.0
0.5
0.0
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Scr-Aβ1-40
Scr-Aβ1-42

E) Treating the cells with Aβ1-40 and Aβ1-42 shows no significant difference in CRMP-2B phosphorylation at the S522 site. Treating SH-SY5Y cells with different concentrations of scrambled Aβ1-40 and Aβ1-42 peptides also shows no significant differences in the phosphorylation at S522 in comparison to Aβ non-treated control cells (n=3).

F


Level of hyperphosphorylated Tau
Image density (AU)
0.20
0.18
0.16
0.14
0.12
0.10
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Scr-Aβ1-40
Scr-Aβ1-42
Level of hyperphosphorylated Tau
Image density (AU)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Cells only
RA treated
0.5 µM
1.0 µM
10 µM
0.5 µM
1.0 µM
10 µM
Aβ1-40
Aβ1-42

F) Hyperphosphorylated tau levels increase with the increase in Aβ1-40 concentration (* p< 0.05, ** p< 0.01). On the other hand, tau hyperphosphorylation shows non-significant increase with Aβ1-42 at 1.0 μM followed by a drop at 10.0 μM. Moreover, SH-SY5Y cells treated with different concentrations of scrambled Aβ1-40 and Aβ1-42 peptides show no significant difference in tau phosphorylation, compared to Aβ non-treated control cells (n=3).

3.4 Expression of the phospho-mutant T555A CRMP-2 construct in SH-SY5Y neuroblastoma cells promotes neurite elongation even in the presence of extracellular Aβ

To determine whether phosphorylation of CRMP-2 at the T555 site is the dominant molecular event responsible for Aβ-induced neurodegeneration, or whether alternative CRMP-2 phosphorylation events can also contribute to neurite outgrowth inhibition, SH-SY5Y cells were transiently transfected with Flag and myc-tagged CRMP-2 phospho-mutant constructs (Table 3) T555A (Rho-kinase site), T509A, T514A, S518A (GSK-3β sites) and S522A (Cdk5 site), then administered 10 μM of Aβ1-40 for 24 hrs. The cells were fixed and then immunostained with anti-NF200 and anti-Flag antibodies to demonstrate their ability for neurite outgrowth even in the presence of Aβ. Cells transfected with the T555A cDNA were able to form long thick neurites (Figure. 8A, arrow). By comparison, cells transfected with either Cdk5 or GSK-3β-specific phospho-site mutant CRMP-2 constructs exhibited stunted, retarded or deficient neurite growth (Figure. 8B - 8E, arrow). These results demonstrate that the inhibition of Aβ-dependent phosphorylation of CRMP-2 at the T555 site plays a central role in the preservation of neurites.

To interrogate whether the overexpression of mutant CRMP-2 constructs modified the phosphorylation of endogenous CRMP-2 at the T555 site in the presence of Aβ, we immunostained the transfected cells using our pT555CRMP-2 antibody. We found that phosphorylation of endogenous CRMP-2 at the T555 site could still be detected, even in the presence of over-expression of any of the phospho-mutant CRMP-2 constructs (Figure. 8A - 8E). These data suggest that despite transfection with these phospho-mutant constructs, Aβ can still elicit the phosphorylation of CRMP-2 at the T555 site. However, it was evident from these experiments that the overexpression of the T555A CRMP-2 mutant construct was able to

potentiate the growth of long neurites even in the presence of Aβ-dependent CRMP-2 phosphorylation. All other constructs failed to potentiate a neurite outgrowth effect in the presence of Aβ.

Table 3: cDNA of human CRMP-2

Construct	Mutation	Phosphorylation site abolished
CRMP-2 T555A	Thr-555 into Ala-555	ROCK2
CRMP-2 T509A	Thr-509 into Ala-509	GSK-3β
CRMP-2 T514A	Thr-514 into Ala-514	GSK-3β
CRMP-2 S518A	Ser-518 into Ala-518	GSK-3β
CRMP-2 S522A	Ser-522 into Ala-522	Cdk-5

A

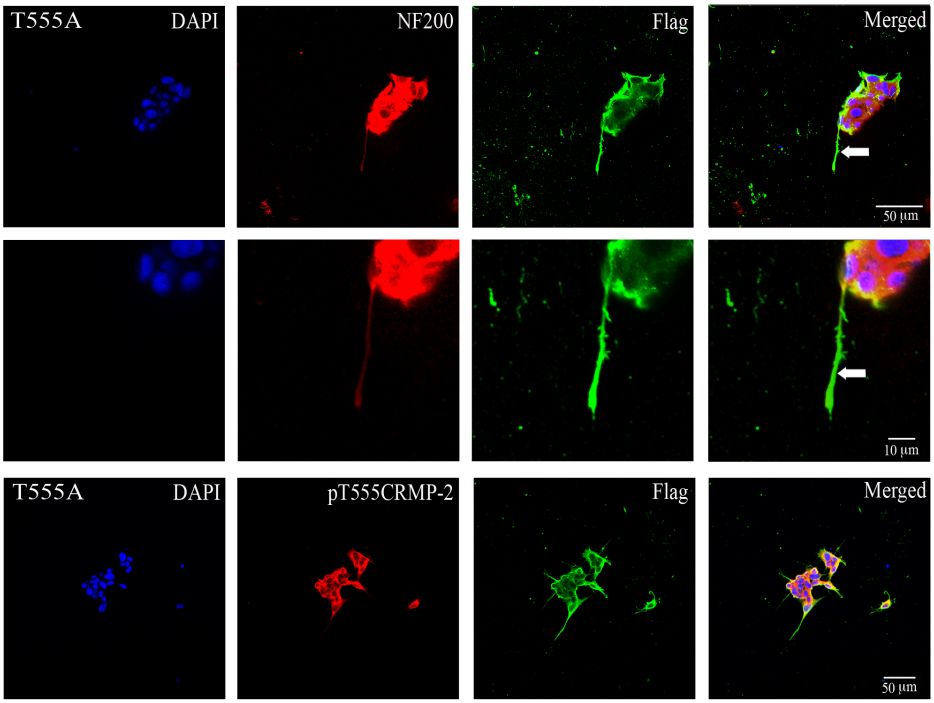
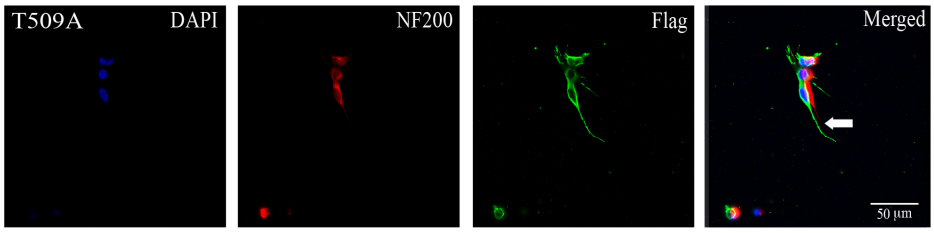

T555A
DAPI
NF200
Flag
Merged
50 µm
10 µm
T555A
DAPI
pT555CRMP-2
Flag
Merged
50 µm

Figure 8: Immunostaining of Aβ1-40 treated SH-SY5Y cells transfected with phospho-specific CRMP-2 mutant constructs using anti-Flag and anti-NF200 antibodies respectively.

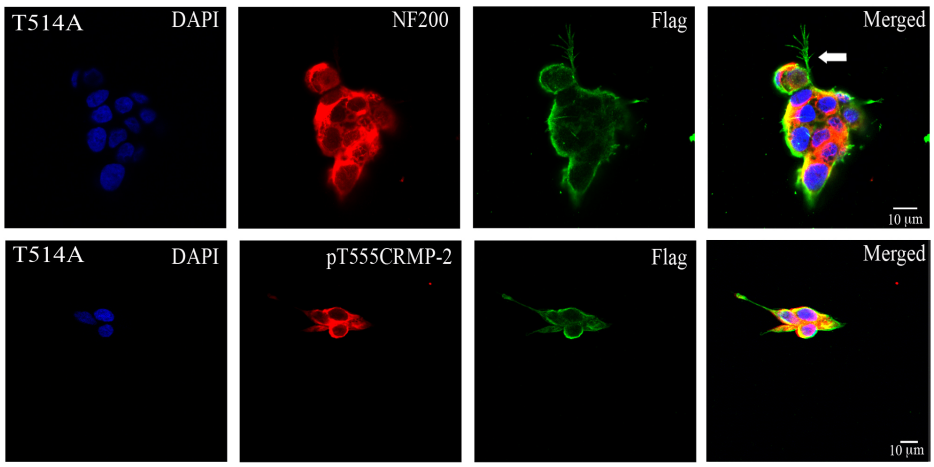
A) Represents cells that were transfected with the T555A CRMP-2 mutant construct and also treated with 10 μM of Aβ1-40 for 24 hrs in culture. First row represent cells stained with NF200, anti-Flag and DAPI with a higher magnification in the second row. Notice the anti-Flag positive labelling distributed within the axon and neuronal soma along with co-labelling for NF200 (neuronal soma). Importantly, those cells were able to produce long thick neurites (arrow). Third row represent cells stained with pT555 CRMP-2, anti-Flag and DAPI. Cells transfected with the T555A CRMP-2 mutant construct and also treated with 10 μM of Aβ1-40 for 24 hrs in culture still exhibited pT555 CRMP-2 immunostaining. Notice the anti-Flag positive labelling distributed within the axon and neuronal soma along with co-labelling for pThr555CRMP-2 (neuronal soma), which could be a result of endogenous phosphorylation of CRMP-2 after Aβ1-40 treatment.

B



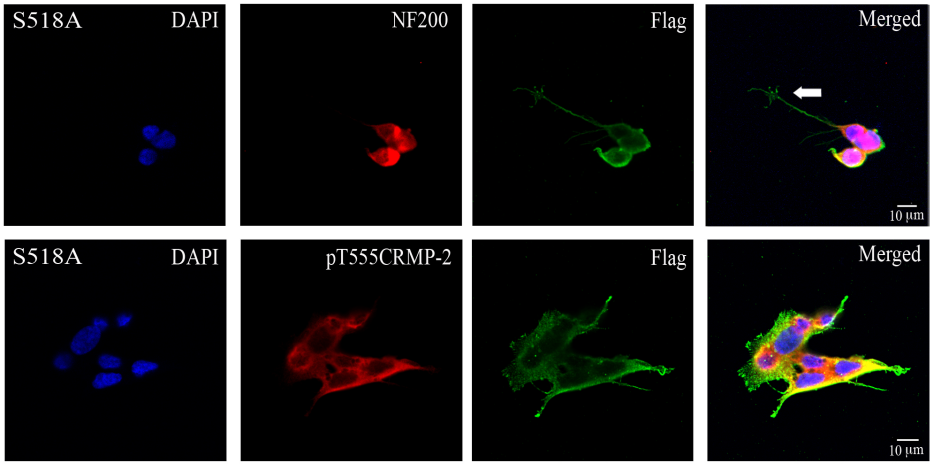
B) Shows cells that were transfected with the T509A CRMP-2 phospho-specific mutant construct. The anti-Flag labelling is distributed in the neurites and neuronal somas along with NF200 labelling that is distributed throughout the soma. These data demonstrate that in the presence of Aβ1-40, the neurites of these transfected cells are growth retarded (arrow).

C


T514A
DAPI
NF200
Flag
Merged
10 µm
T514A
DAPI
pT555CRMP-2
Flag
Merged
10 µm

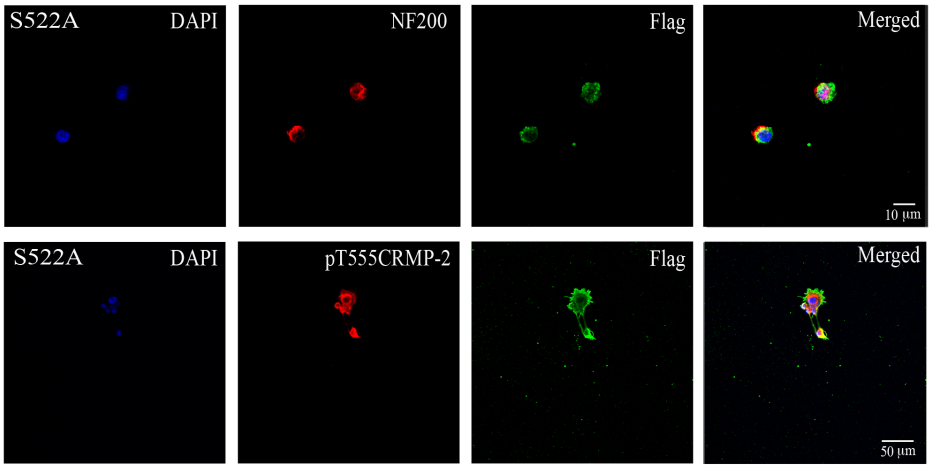
C) Represents those cells that were transfected with the T514A CRMP-2 mutant construct. First row represent cells stained with NF200, anti-Flag and DAPI. Again, the anti-Flag labelling is distributed along the axon and soma, with the anti-NF200 distrubution within the neuronal soma. Again, in the presence of Aβ1-40, the neurons display a branched neurites that are stunted in growth. Second row represent cells stained with pT555 CRMP-2, anti-Flag and DAPI. Cells that were transfected with the T514A CRMP-2 phospho-specific mutant construct also exhibit pT555 CRMP-2 immunostaining. The anti-Flag labelling is distributed in the neurites and neuronal soma along with pThr555CRMP-2 labelling that is distributed throughout the neuronal soma.

D


S518A
DAPI
NF200
Flag
Merged
10 μm
S518A
DAPI
pT555CRMP-2
Flag
Merged
10 μm

D) Illustrates cells that were transfected with the phospho-mutant S518A CRMP-2 construct. First row represent cells stained with NF200, anti-Flag and DAPI. Again, anti-Flag labelling is distributed in the neurites and cell body, along with NF200 that is distributed within the neuronal soma. Again in the presence of Aβ1-40, these transfected cells display short and branched patterns of neurite outgrowth (arrow). Second row represent cells stained with pThr555 CRMP-2, anti-Flag and DAPI. Cells transfected with the S518A CRMP-2 mutant construct again show the anti-Flag labelling distributed along the neurites and neuronal somas, with the pThr555 CRMP-2 distribution within the neuronal soma.

E


S522A
DAPI
NF200
Flag
Merged
10 μm
S522A
DAPI
pT555CRMP-2
Flag
Merged
50 μm

E) Shows cells that were transfected with the phospho-mutant S522A CRMP-2 construct. First row represent cells stained with NF200, anti-Flag and DAPI.The Flag and NF200 labelling is distributed throughout the neuronal soma with no neurite growth demonstrated in the presence of Aβ1-40. Second row represent cells stained with pThr555 CRMP-2, anti-Flag and DAPI. Cells that were transfected with the phospho-mutant S522A CRMP-2 construct show anti-Flag labelling distributed in the cell body, along with pThr555CRMP-2. These data demonstrate that endogenous phosphorylation of CRMP-2 in T555 site has been detected even after applying site-directed mutagenesis.

3.5 Expression of T555A CRMP-2 in SH-SY5Y neuroblastoma cells improves CRMP-2 association with kinesin and tubulin in the presence of Aβ

Important physiological roles that have been attributed to CRMP-2 include the binding of α- and β-tubulin heterodimers, to facilitate their transport to the plus-ends of microtubules as a means of promoting neurite extension (Fukata et al., 2002a). In addition, the association of CRMP-2 with kinesin-1, the microtubule motor protein complex (Szpankowski et al., 2012), can facilitate vesicular anterograde axonal transport (Kimura et al., 2005b). We transiently transfected SH-SY5Y cells with Flag- and myc-tagged CRMP-2 phospho-mutant constructs; T555A (Rho-kinase site), T509A, T514A, S518A (GSK-3β sites) and S522A (Cdk5 site). The cells were treated with Aβ1-40 or scrAβ1-40 [10 μM] for 24 hrs. CRMP-2 was immunoprecipitated using an anti-CRMP-2 monoclonal antibody (IBL), and then the levels of CRMP-2-bound tubulin and kinesin were determined by western blotting using either anti-α-tubulin or anti-KLC antibodies. We detected elevated levels of CRMP-2-bound kinesin in T555A transfected SH-SY5Y cells. Cells that were transfected with the other constructs exhibited reduction in kinesin-association that could be a result of endogenous phosphorylation of CRMP-2 at the T555 site following Aβ1-40 treatment (Figure. 9A & 9B). This phosphorylation was specifically illustrated immunocytochemically in transfected neurons with the phospho-mutant constructs (T509A, T514A, S518A, S522A and T555A) (Figure. 8A - 8E). We also found increased levels in CRMP-2-bound tubulin in T555A transfected SH-SY5Y cells (Figure. 9A & 9C). Cells that were transfected with the other constructs showed decreases in the levels of tubulin association (Figure. 9A & 9C), which again may well represent the endogenous phosphorylation of CRMP-2 at T555 site following Aβ1-40 treatment. It is important to note that the levels of tubulin and kinesin that were bound to CRMP-2 did not decrease in the presence of Aβ1-40 scrambled

peptide (control peptide administration experiment) (Figure. 7A - 7C). These data support the hypothesis that Aβ may cause a reduction in axonal transport through the phosphorylation of CRMP-2 at the T555 site, by reducing its capacity to bind tubulin and kinesin.

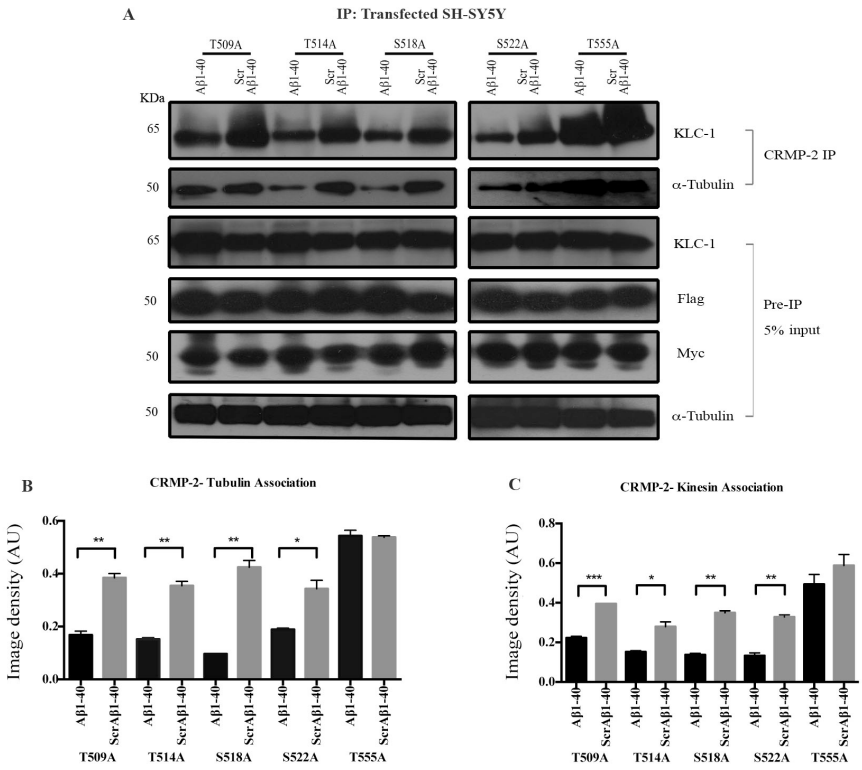

A
IP: Transfected SH-SY5Y
T509A
T514A
S518A
S522A
T555A
Aβ1-40
Scr Aβ1-40
KDa
65
50
KLC-1
α-Tubulin
CRMP-2 IP
Flag
Myc
Pre-IP
5% input
B
CRMP-2- Tubulin Association
Image density (AU)
C
CRMP-2- Kinesin Association

Figure 9: Expression of pT555A CRMP2 in SH-SY5Y neuroblastoma cells improves CRMP-2 associated kinesin and tubulin in the presence of Aβ.

A) Protein lysates from SH-SY5Y cells that were transfected with the CRMP-2 phospho-specific mutant constructs and treated with 10 μM of Aβ1-40 or scrAβ1-40 for 24 hrs in culture. Antibodies against the Flag and myc were successfully bound to purified protein from SH-SY5Y cells, which indicates the success in the transfection. **B)** Shows the increase in CRMP-2-bound kinesin in T555A transfected SHSY-5Y cells. Cells that were transfected with the other constructs show a decrease in kinesin association, which could be a result of endogenous phosphorylation of CRMP-2 at T555 site after Aβ1-40 treatment (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). **C)** Shows an increase CRMP-2-bound tubulin in T555A transfected SH-SY5Y cells. Cells that were transfected with the other constructs show a decrease in tubulin association which could be a result of endogenous phosphorylation of CRMP-2 at the T555 site after Aβ1-40 treatment (** $p<0.01$, * $p<0.05$).

3.6 Effect of Aβ on kinesin and tubulin binding to CRMP-2 in SH-SY5Y neuroblastoma cells

Since we determined that the major Aβ-dependent phosphorylation of CRMP-2 occurred at the T555 site in SH-SY5Y cells, we decided to investigate whether this CRMP-2 phosphorylation had the ability to dissociate kinesin and tubulin. We used undifferentiated SH-SY5Y cells (Control, no retinoic acid), differentiated SH-SY5Y cells treated with retinoic acid, SH-SY5Y cells treated with Aβ1-40 in three different concentrations [0.5, 1.0 and 10.0 μM], and finally SH-SY5Y cells treated with Aβ1-42 at three different concentrations [0.5, 1.0 and 10.0 μM]. To identify the interaction between the CRMP-2/Tubulin/Kinesin multimeric complex, we immunoprecipitated CRMP-2 from cell lysate samples following administration of the above-described Aβ concentrations and control experiments. Following western blotting, the membranes were probed with either monoclonal anti-KLC-1 or monoclonal anti-α-tubulin. We found that CRMP-2-bound kinesin and tubulin were reduced with the increase in the concentration of Aβ1-40 and Aβ1-42 administration, compared to untreated control cells and cells treated with scrambled peptide (Figure. 10). These data suggest that Aβ1-40 and Aβ1-42 can affect the ability of CRMP-2 to bind to tubulin heterodimers (soluble) and kinesin through CRMP-2 phosphorylation or CRMP-2 cleavage, respectively, as shown previously in chapter 3.3.

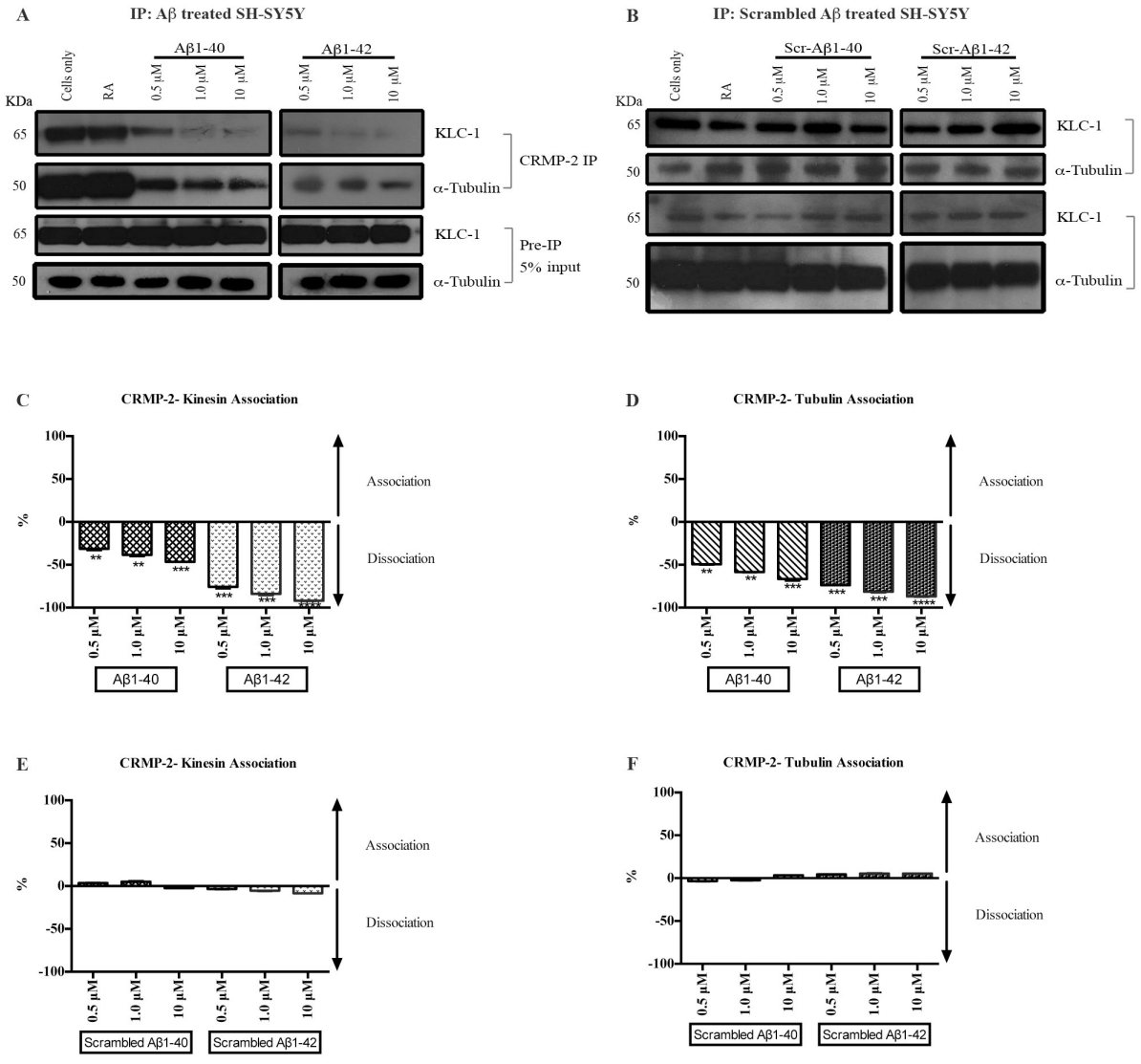

A
IP: Aβ treated SH-SY5Y
Aβ1-40
Aβ1-42
Cells only
RA
0.5 μM
1.0 μM
10 μM
KDa
65
50
KLC-1
α-Tubulin
CRMP-2 IP
Pre-IP
5% input
B
IP: Scrambled Aβ treated SH-SY5Y
Scr-Aβ1-40
Scr-Aβ1-42
C
CRMP-2- Kinesin Association
D
CRMP-2- Tubulin Association
Association
Dissociation
%
Aβ1-40
Aβ1-42
E
CRMP-2- Kinesin Association
F
CRMP-2- Tubulin Association
Scrambled Aβ1-40
Scrambled Aβ1-42

Figure 10: Decreased binding of kinesin and tubulin to CRMP-2 in Aβ1-40 and Aβ1-42 –treated SH-SY5Y human neuroblastoma cells.

CRMP-2 was immunoprecipitated with the polyclonal anti-CRMP-2 antibody and the immunoprecipitate was transferred onto a PVDF membrane and then probed for KLC-1 and α-tubulin to detect the level of kinesin and tubulin bound to CRMP-2, respectively. **A)** Shows the level of total kinesin and α-tubulin before and after immunoprecipitation of CRMP-2 from Aβ1-40 and Aβ1-42-treated SH-SY5Y. **B)** Shows the level of total kinesin and α-tubulin before and after immunoprecipitation of CRMP-2 from ScrAβ1-40 and ScrAβ1-42-treated SH-SY5Y. **C-D)** Aβ1-40-treated SH-SY5Y cells had approximately a 50% decrease in kinesin bound to CRMP-2, compared to control cells and cells treated with scrambled Aβ1-40 peptide (*** $p<0.001$, ** $p<0.01$). Aβ1-42-treated SH-SY5Y cells had approximately a 90% decrease in kinesin bound to CRMP-2, compared to control cells and cells treated with scrambled Aβ1-42 peptide (**** $p<0.0001$, *** $p<0.001$). **E-F)** Aβ1-40-treated SH-SY5Y cells had approximately a 60% decrease in α-tubulin bound to CRMP-2, compared to control cells and cells treated with scrambled Aβ1-40 peptide (*** $p<0.001$, ** $p<0.01$). Aβ1-42-treated SH-SY5Y cells had approximately an 80% decrease in α-tubulin bound to CRMP-2, compared to control cells and cells treated with scrambled Aβ1-42 peptide (**** $p<0.0001$, *** $p<0.001$) (n=3).

3.7 Transfection of primary cortical neurons from embryonic P7 rat with Flag-APP pDendra and Flag-BDNF pDendra to track Aβ-dependent axonal transport modifications in real time.

Since we defined the effect of Aβ-dependent phosphorylation of CRMP-2 on the dissociation from kinesin and tubulin, we decided to visualise this effect on primary cortical neurons using live cell imaging in an attempt to analyse this molecular dissociation with an axonal transport deficits. We transfected primary cortical neurons from P7 rat with Flag-BDNF and Flag-APP cDNA constructs inserted into the pDendra plasmid vector (Clontech). pDendra is a monomeric photoconvertible peptide that when it is co-expressed with either BDNF or APP, will be converted from a green to a red signal (normally photostable at 488 nm at low power intensity and upon excitation at 405 nm of intense blue light becomes converted to a red 543 nm signal). After complete photoconversion, the red fluorescence of Dendra2 increases 150–300 times, whereas the level of green fluorescence becomes 10–15 times lower. Thus, the increase in the red-to-green fluorescence ratio results in ~4000-fold contrast. This will provide us with a molecular tool to simultaneously track both the movement of the activated BDNF or APP protein and its replacement with the non-activated form. Primary neurons were successfully transfected with Flag-APP and Flag-BDNF pDendra with 62% and 57% efficiency respectively (Figure. 11).

A

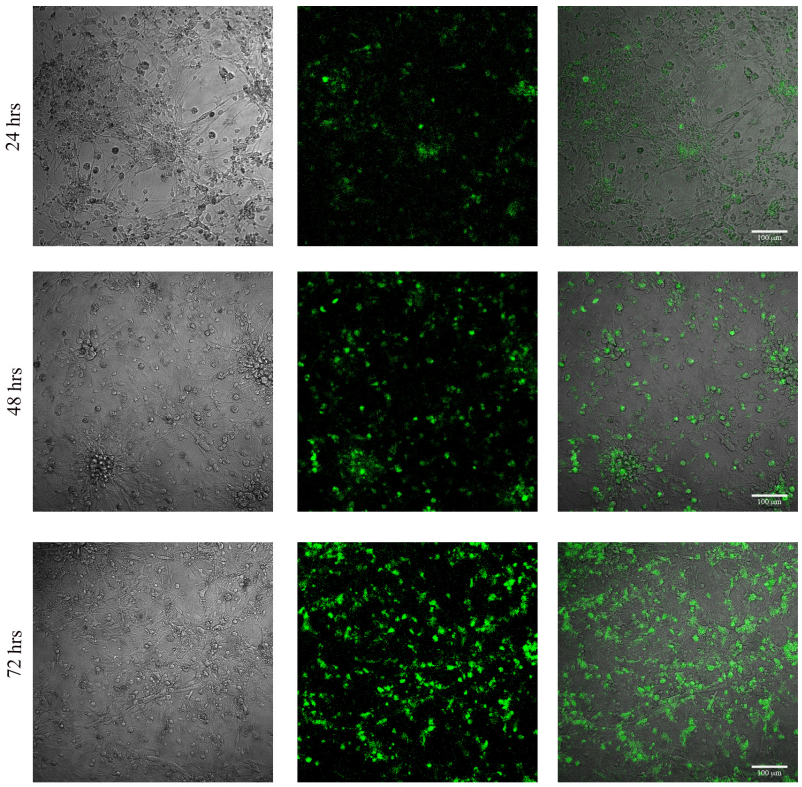
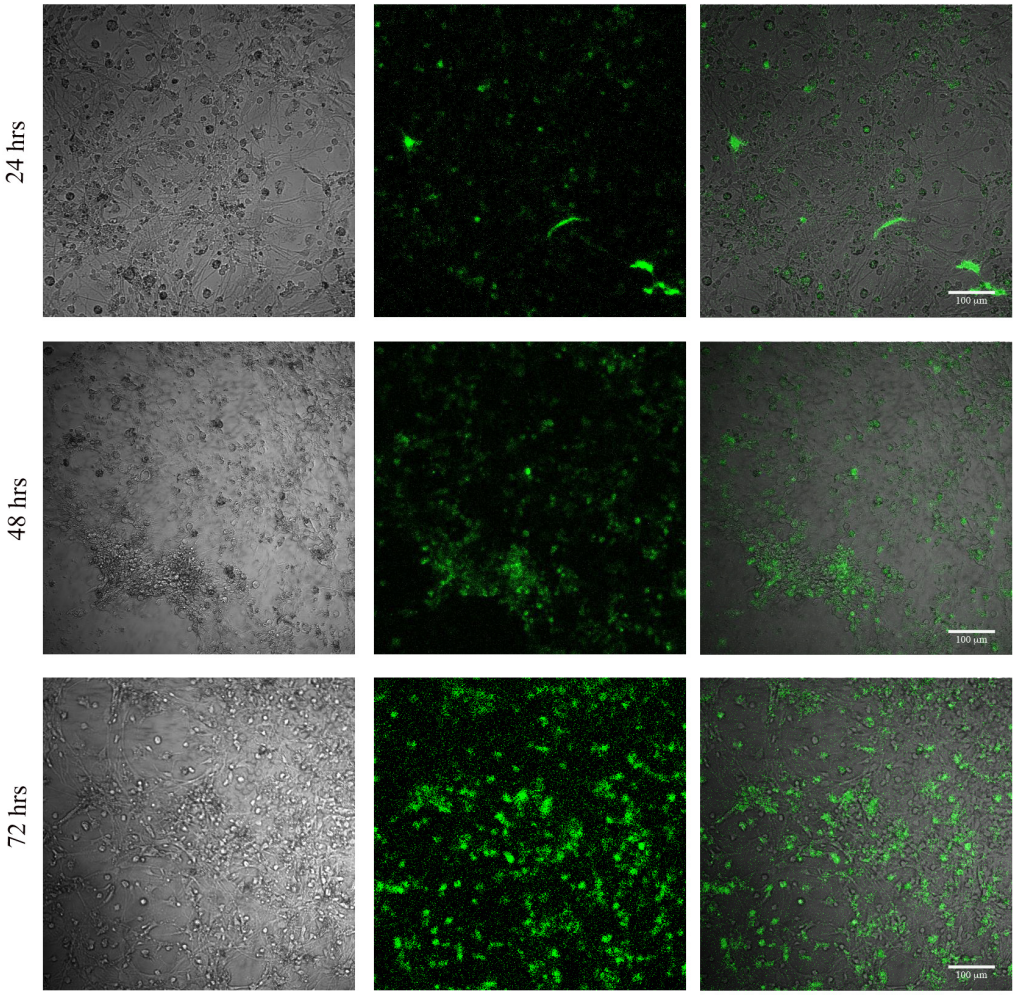


Figure 11: Transfection efficiency of primary cortical neurons from P7 rats with Flag-APP pDendra and Flag-BDNF pDendra

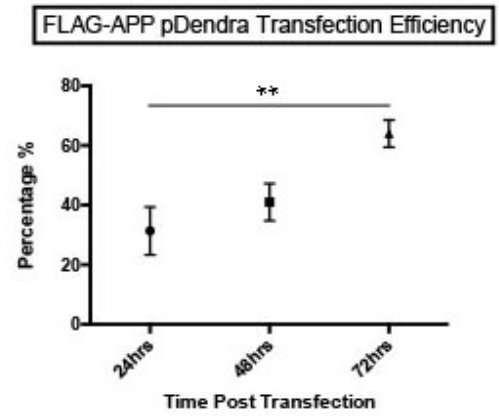
A) Lipofectamine transfection of primary cortical neurons from P7 rats with Flag-APP pDendra shows increased transfection efficiency after 24hrs.

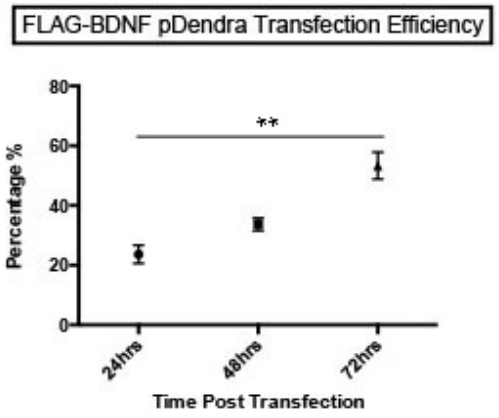
B



B) Lipofectamine transfection of primary cortical neurons from P7 rats with Flag-BDNF pDendra shows increased transfection efficiency after 24hrs.

C


FLAG-APP pDendra Transfection Efficiency
**
Percentage %
80
60
40
20
0
24hrs
48hrs
72hrs
Time Post Transfection


FLAG-BDNF pDendra Transfection Efficiency
**
Percentage %
80
60
40
20
0
24hrs
48hrs
72hrs
Time Post Transfection

C) Lipofectamine transfection of primary cortical neurons with Flag-APP pDendra and Flag-BDNF pDendra shows significant increase in the transfection efficiency at 72hrs post-transfection compared to 24hrs and 48hrs (** $p<0.01$) (n=3).

3.8 Discussion

This study aimed to define how Aβ regulates the phosphorylation of CRMP-2 during acute exposure possibly leading to neuritic dystrophy in AD. Recently, we found that Aβ inactivates CRMP-2 through a Rho kinase-dependent mechanism that causes alterations in the neuronal microtubule dynamics (Petratos et al., 2008a). In this project we aimed to investigate whether Aβ-dependent phosphorylation of CRMP-2 through Rho kinase is the major mechanism regulating neurite outgrowth and axonal transport or whether the other kinases also play a significant role in that mechanism. It has previously been suggested that elevated levels of GSK3b inhibit kinesin transport of membrane-bound organelles (MBOs) (Morfini et al., 2002). It has also been demonstrated that modulation of Cdk-5 activity can effect anterograde axonal transport (Ratner et al., 1998). Since both of these kinases have been reported to regulate the phosphorylation of CRMP-2 and its association with tubulin heterodimers during polymerization in neurons (Uchida et al., 2005a, Yoshimura et al., 2005a, Fukata et al., 2002a), we investigated whether the Aβ1-40-dependent phosphorylation of CRMP-2 was orchestrated by upstream activation of Cdk-5 and/or GSK-3β. Instead, our data illustrate that the Rho kinase-dependent phosphorylation of CRMP-2 was responsible for the dissociation of kinesin-1 and tubulin. Indeed, transfecting SH-SY5Y cells to overexpress the T555A mutant construct of CRMP-2 improves its association to kinesin-1 and tubulin, with prominent neurites formed in these transfected cells, even in the presence of extracellular Aβ. These results suggested that the CRMP-2/kinesin-1 transport mechanism may well be affected by increasing extracellular Aβ and its direct neuronal signalling.

In agreement with our results, Arimura et al. (2005) have reported that neurons transfected with the T555A mutant form of CRMP-2 did not retract their axons, in contrast with neurons transfected with either a myc-GST construct or with CRMP-2 containing a threonine to aspartate substitution (T555D), which is the phosphomimetic form of CRMP-2 specific for Rho kinase (Arimura et al., 2005a). However, this study was not conducted in the presence of extracellular Aβ and so does not define the aberrant signaling that can occur in neurons in the context of Aβ accumulation. Our results suggest that Aβ-dependent CRMP-2-mediated neurodegeneration can be overcome when specifically targeting phosphorylation at the threonine 555 site (Rho kinase specific site), and that this effect cannot be attributed to the other putative kinase sites, namely GSK-3β (T509/T514/S518) and Cdk-5 (S522).

It has been demonstrated that the motor protein kinesin-1 transports molecular cargo proteins into the synapse (Ferreira et al., 1992) and thus is important in axonogenesis (Baas, 1997, Terada et al., 2000). It has also been shown that CRMP-2 can directly interact with kinesin light chain (KLC) (Kimura et al., 2005b), and thus link KLC to the proteins that are required to be transported to the growth cone, thereby facilitating axon elongation (Nishimura et al., 2003, Kimura et al., 2005b, Fukata et al., 2002a). Hence, CRMP-2 functions as a cargo receptor for kinesin-1 and carries its interacting molecules such as tubulin heterodimers to the growth cone of the developing axon. This transport system is critical for the organization of the actin cytoskeleton and microtubule assembly in the distal end of the growing axon, thus enhancing axonal outgrowth (Kawano et al., 2005). In the present study, we found that the interaction of CRMP-2 with kinesin-1 decreases with the increase in Aβ1-40 concentration in SH-SY5Y cells. Since this interaction paralleled the increase in CRMP-2 dissociation from tubulin, we

hypothesized that the phosphorylation of CRMP-2 was regulating the dissociation of the key motor proteins and molecular cargo. We therefore overexpressed different phospho-mutant constructs, to abrogate the dissociation effects of phosphorylation of CRMP-2 driven by the increased concentration of extracellular Aβ. We found that by far the most effective phospho-mutant in abrogating Aβ-dependent kinesin-tubulin dissociation was the T555ACRMP2 mutant, previously utilized by our group to maintain axonal integrity in the mouse model of Multiple Sclerosis, experimental autoimmune encephalomyelitis (Petratos et al., 2012). These data make it tantalising to hypothesise that the Aβ1-40-dependent phosphorylation of CRMP-2 abrogates anterograde axonal transport, and thus axonal outgrowth.

In the CNS, degenerative axons are typically unable to regenerate due to the inhibitory surrounding environment and the deficiencies in neuronal-intrinsic regenerative mechanisms. However, it is feasible to improve the growth capacity of those axons. Pharmacological inhibition of GSK3β was shown to promote either axonal growth (Dill et al., 2008) or enhance axon outgrowth inhibition in the presence of myelin (Alabed et al., 2010). Liz et al. (2014) identified GSK3β as a key player in this process through its regulation of CRMP-2 and tau by phosphorylation (Liz et al., 2014). Using constitutively active GSK3β Ser9Ala knockin (KI) mice (McManus et al., 2005), GSK3β knockout heterozygous mice (Hoeflich et al., 2000), and mice with a neuron-specific deletion of GSK3β, these authors showed that improved microtubule dynamics in the growth cone are accomplished with the inactivation of the GSK3β-CRMP-2 pathway, stimulating axon regeneration. Furthermore, they demonstrated that CRMP-2 is the primary downstream target of GSK3β that mediates the regulation of microtubule dynamics in the growth cone demonstrated by reversion of elevated microtubule outgrowth speed of GSK3β-

deficient neurons upon overexpression of a phospho-mimetic T/D CRMP-2 mutant and attenuated inhibitory effects of myelin upon the overexpression of the phospho-resistant T/A CRMP-2 mutant in naïve DRG neurons. Taken together, these data may suggest that, irrespective of ROCK-mediated CRMP-2 phosphorylation at Thr555, inhibition of CRMP-2 phosphorylation at Thr514 by GSK3β is sufficient to reverse myelin inhibition in DRG neuron cultures.

Interestingly, it has been shown in a different study that repulsive guidance molecule A (RGMa) diminishes axonal outgrowth by inducing CRMP-2 phosphorylation via both ROCK2 and GSK3β signalling (Wang et al., 2013). However, in that study, the details on which the CRMP-2 phosphorylation site (Thr514 or Thr555) was assessed are missing. In the current study, we have tested whether it is possible to modify axonal outgrowth and transport in the presence of Aβ by overexpressing the T555ACRMP-2 mutant form. Our results, along with the previously described studies, point towards modulation of CRMP-2 activity as a therapeutic target to enhance axonal regeneration. Furthermore, our data may suggest that CRMP-2 is a key molecular target to design strategies to achieve Aβ inhibition.

Previously it has been shown that overexpression of the phospho-resistant mutant T555A-CRMP-2 (the Rho kinase phosphorylation site) counteracts the inhibitory effect of MAG on postnatal cerebellar neurons (Mimura et al., 2006). In addition, PP2A enhances axonal outgrowth by de-phosphorylation of CRMP-2 (Zhu et al., 2010). The involvement of CRMP-2 in many neurodegenerative disorders raised the requirement of developing therapeutic strategies targeting its activity. In fact, drugs controlling CRMP-2 levels and function are now being tested in the context of neurodegeneration (Khanna et al., 2012).

Previous studies have reported that dystrophic neurites are associated with amyloid plaques in AD tissue (Dickson et al., 1999, Geddes et al., 1986, Geddes et al., 1985, Masliah et al., 2003, Masliah et al., 1991, Su et al., 1993, Su et al., 1998) and have been considered to be a pathological cause of dementia in AD patients (Mirra et al., 1991). A study using the PS1M146L/APP751SL AD mouse model demonstrated that most (if not all) dystrophic neurites are associated with amyloid plaques (Torres et al., 2012). Additionally, several studies have shown plaque-associated axonal dystrophies in GABAergic and cholinergic neurons (neurons that do not express transgenic APP and do not accumulate intracellular Aβ) (Baglietto-Vargas et al., 2010, Moreno-Gonzalez et al., 2009, Ramos et al., 2006). Therefore, dystrophic neurite formation in such neurons could be stimulated by the presence of extracellular Aβ plaques. Moreover, the administration of Aβ antibodies to transgenic mice overexpressing mutant human APP (V717F, PDAPP mice) has resulted in linear and not curved or distorted neurites in a short period of time (Lombardo et al., 2003). However, there are currently no studies linking the functional deficit with the morphological abnormality. Here we report that neurons growing in the vicinity of a laboratory-made amyloid plaque display neuritic dystrophy. We have also shown that those neurites end up curving at the edge of the artificial amyloid plaque with substantial increases in phosphorylated CRMP-2 at the Thr555 site. Collectively, these data may suggest signaling deficits in the neuronal microtubule transport mechanism, rendering neurites dystrophic (Sanchez-Varo et al., 2012). In fact, Adalbert and colleagues (2009) have shown that dystrophic axons surrounding amyloid plaques remain connected to viable neuronal somata for quite a long period of time (Adalbert et al., 2009). Considering that those dystrophic neurites were able to regrow, developing a pharmaceutical therapy to target this stage of degeneration

would be promising. However, other studies have suggested that plaques correlate poorly with AD pathology in mouse models and AD patients (Herrup et al, 2010). They suggested that AβO is more neurotoxic and that AβOs interact with dendritic membrane receptors, such as NMDAR, leading to synaptic failure (Benilova et al, 2012, Ferreira et al, 2011). They have also suggested that abnormalities in KLC1vE-dependent transport enhances AβO production, which may thereby activate kinase signalling cascades, such as GSK3β, JNK, CK2, and Cdk5, leading to phosphorylation of APP, Aβ production, and disruption in motor protein activity and/or cargo binding.

It is established that dystrophic neurites occur in areas of synaptic loss in the hippocampal formation and neocortex (Tsai et al., 2004, Spires et al., 2005)**,** characteristically seen in AD brains at autopsy and typically associated with Aβ deposition (Naslund et al., 2000, Small and McLean, 1999). Aβ has been reported to induce neurite dystrophy in culture (Heredia et al., 2006) as well as in mutant mouse models of AD (Tsai et al., 2004, Spires et al., 2005). Recent evidence suggests a dynamic functional decline of the neuron in which Aβ causes progressive neuronal dystrophy and synaptic loss, occurring at an early stage, followed by a gradual decline in neuronal viability (Spires and Hyman, 2004). Neuronal dysfunction and cognitive decline in AD can be defined as a loss of neural networks through abnormal synaptic plasticity, a direct result of Aβ toxicity on neurites (Small, 2004). These neuritic changes in neurons of the frontal and temporal cortices manifest initially as mild cognitive impairment (MCI), followed by more severe memory loss as the disease progresses (Naslund et al., 2000). One definitive feature of Aβ-mediated neurite dystrophy is the reduction in length and calibre of the neurite seen both in

culture and *in vivo* (Tsai et al., 2004, Postuma et al., 2000), i.e. Aβ can mediate neurite outgrowth inhibition (Petratos et al., 2008a). Current therapeutics in AD target mechanisms to reduce the Aβ load in the brain thereby limiting the cognitive decline and neurodegenerative changes associated with AD. Compounds that can interfere with this adverse Aβ-dependent signalling pathway in AD are of potential therapeutic value as they may limit neuritic dystrophy and axonal transport dysfunction, thereby limiting cognitive decline. In this light, our findings suggest that this central role for Aβ-mediated phosphorylation of CRMP-2 in leading to neuritic dystrophy reveals a potential therapeutic target for AD.

CHAPTER 4: Neurodegenerative changes in the Tg2576 transgenic mouse model of AD is associated with increased phosphorylation of CRMP-2 with increasing age and amyloid load

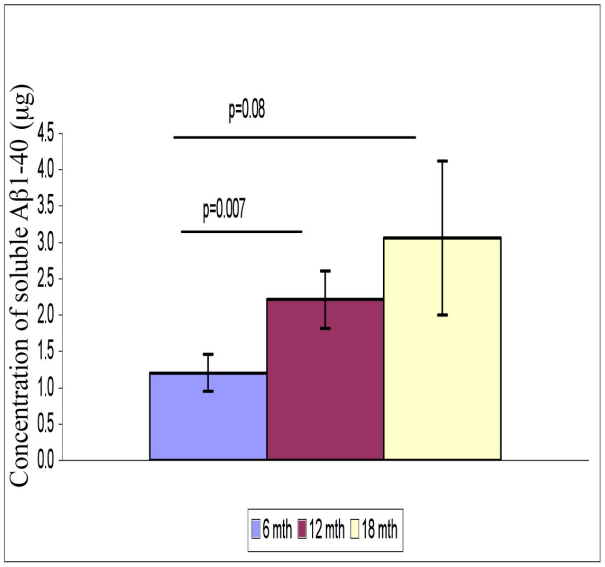
4.1 Introduction

Tg2576 mice overexpress a mutant form of human amyloid precursor protein (APP), bearing the Swedish mutation (APPK670/671L), which is linked to early-onset familial Alzheimer's disease (AD). This model was originally developed by Karen Hsiao Ashe. These transgenic mice develop amyloid plaques and progressive cognitive deficits (Hsiao et al., 1996), which makes them an excellent model for the examination of the relationship between increasing Aβ load in the CNS and memory deficits. Tg2576 mice demonstrate a relatively rapid rise in cortical and hippocampal Aβ levels beginning at 6 months, followed by amyloid plaque deposits starting at 9-12 months of age (Kawarabayashi et al., 2001). Different forms of Aβ, distinguishable by their solubility properties, are present in varying amounts during the lifetime of Tg2576 mice (Kawarabayashi et al., 2001). Detergent-soluble Aβ is present throughout life, whereas insoluble Aβ is absent until 6 months. Tg2576 mice show impaired cognitive functions in spatial tasks, working memory, and in the contextual fear conditioning test at less than six months-of-age. However, other studies have demonstrated normal cognition at this age with progressive impairment beginning at 12 months-of-age. Dendritic spine loss has also been reported by 4.5 months in the CA1 region of the hippocampus with no neurofibrillary tangle pathology. Our data showed increased phospho-Thr555 CRMP-2 levels in the 12 month-old Tg2576 mouse brain (a time when there exist elevated soluble forms of Aβ in this animal model (Fodero et al., 2002)) (Figure. 12A & 12B). What we need to ascertain now is whether this increase in the amyloid load correlates to the phosphorylation states of CRMP-2 at the T555 site and whether it coexist in the APs from Tg2576 mice.

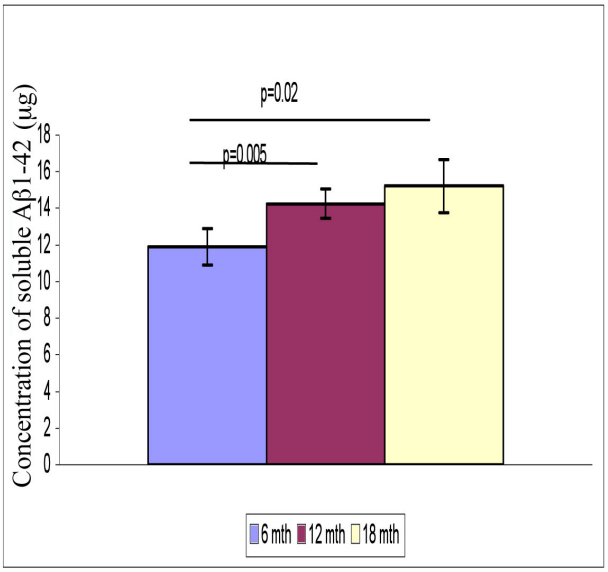
4.2 Increased levels of pT555-CRMP-2 are observed in neurons undergoing degeneration in the cortex and hippocampus of Tg2576 transgenic mice

We have previously reported that the phosphorylation of CRMP-2 occurs in the brains of Tg2576 mice as they age, correlating with the increase in Rho-A-GTP levels, a consequence of decreased Rac1-GTP activity (Petratos et al., 2008a). We now posed the question of whether this phosphorylated form of CRMP-2 was specific to neurons that were degenerating in the transgenic mouse brain. At 6 months-of-age (when initial cognitive deficits have been reported to appear (Westerman et al., 2002)), and 12 months-of-age (when substantial amyloid plaques along with cognitive decline are present), we found increased labelling of pT555-CRMP-2 localised to neurons exhibiting abnormal hyperphosphorylated tau. This was observed specifically in degenerating neuronal somata and neurites within the cortices and hippocampi of Tg2576 mice (Figure. 13). Occasionally, we also observed double-labelled cortical neurons for pT555-CRMP-2 and AT8 in Tg2576 mice at 6 months-of-age (n=5) (Figure. 13). We have also found that there is a significant increase in the pT555 CRMP-2 positive amyloid plaques in the Tg2576 mice at 12 month-of-age (Figure. 12). These data demonstrate that pT555 CRMP-2 levels were increased in AT8-positive degenerative cortical and hippocampal neurons of the APP (Swe) mutant Tg2576 mice under increased Aβ load in the brain (Figure. 13).

A


Concentration of soluble Aβ1-40 (μg)
p=0.08
p=0.007
6 mth
12 mth
18 mth

B


Concentration of soluble Aβ1-42 (μg)
p=0.02
p=0.005
6 mth
12 mth
18 mth

C

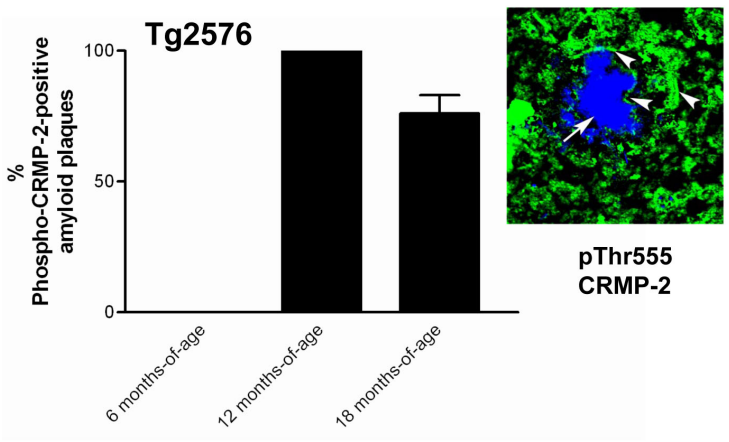

Tg2576
% Phospho-CRMP-2-positive amyloid plaques
6 months-of-age
12 months-of-age
18 months-of-age
pThr555 CRMP-2

Figure 12: Level of Aβ in the brain of Tg2576 transgenic mice.

A) Shows significant increase in Aβ1-40 concentration with the increase in the age of Tg2576 mice. **B)** Shows significant increase in Aβ1-42 concentration with the increase in the age of Tg2576 mice. **C)** Shows the percentage of pCRMP-2 positive amyloid plaques in Tg2576 mice (Arrows: AP (Thioflavin T); arrow heads: pThr555 +ve neurites).

A

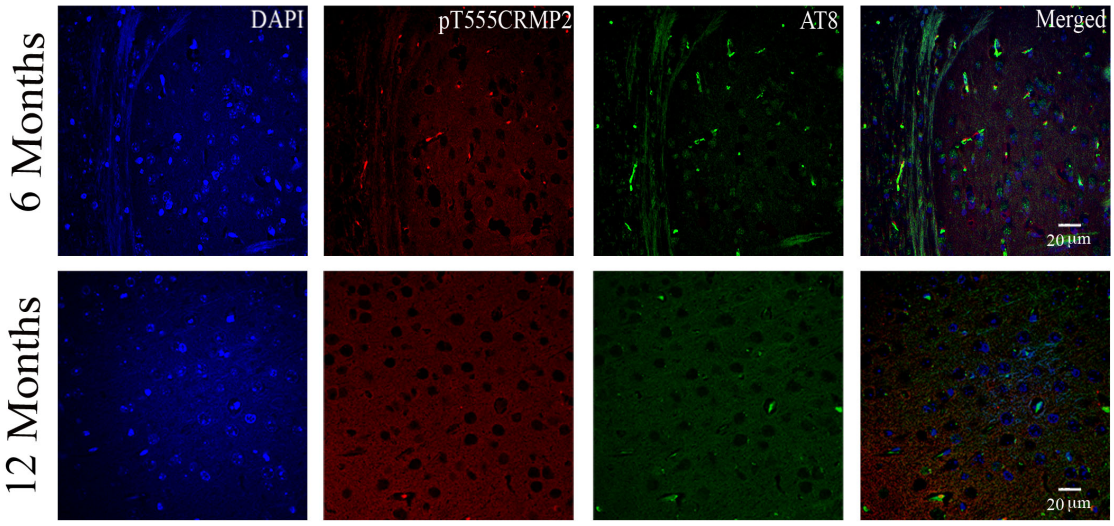
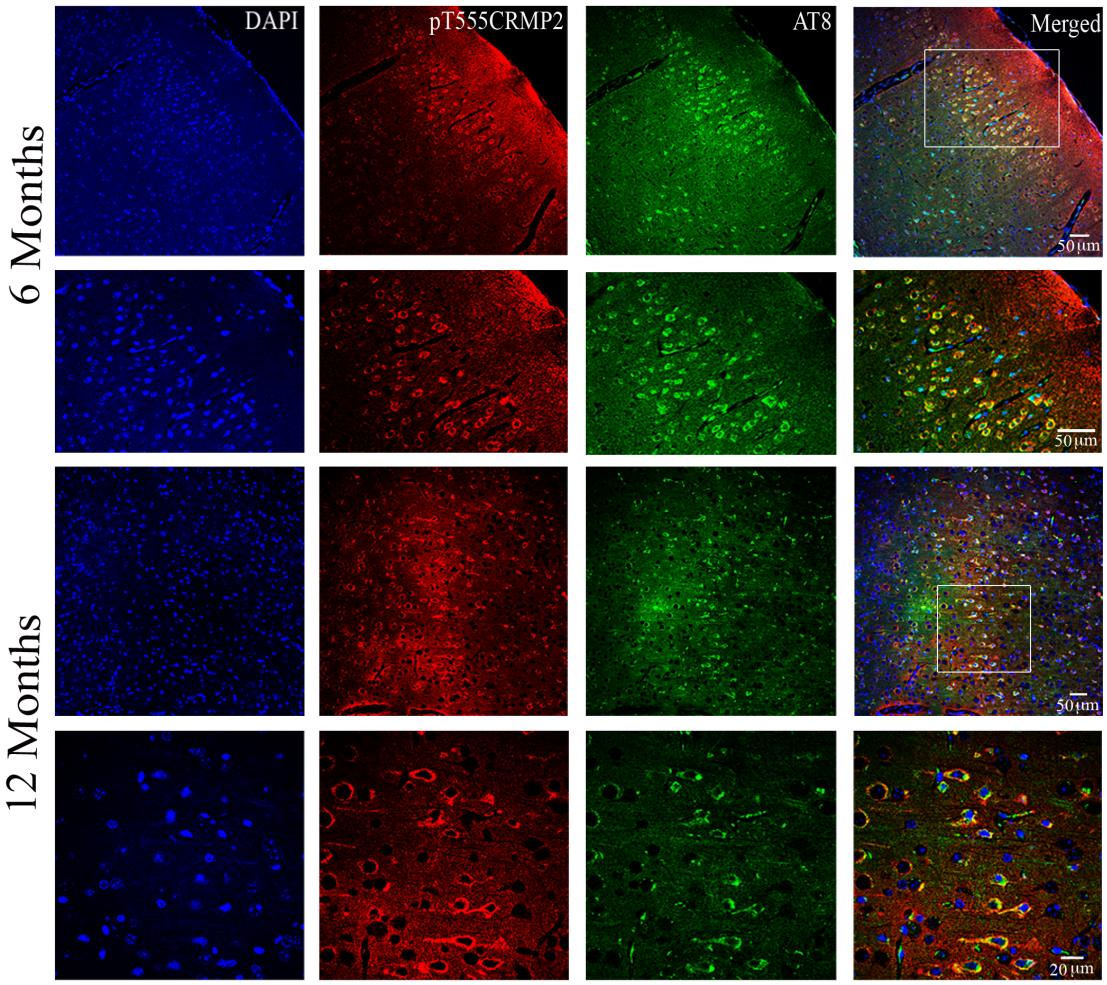


Figure 13: Immunostaining of paraffin sections from Tg2576 mouse model at 6 months and 12 months of age along with their littermates with pThr555CRMP-2 and AT8.

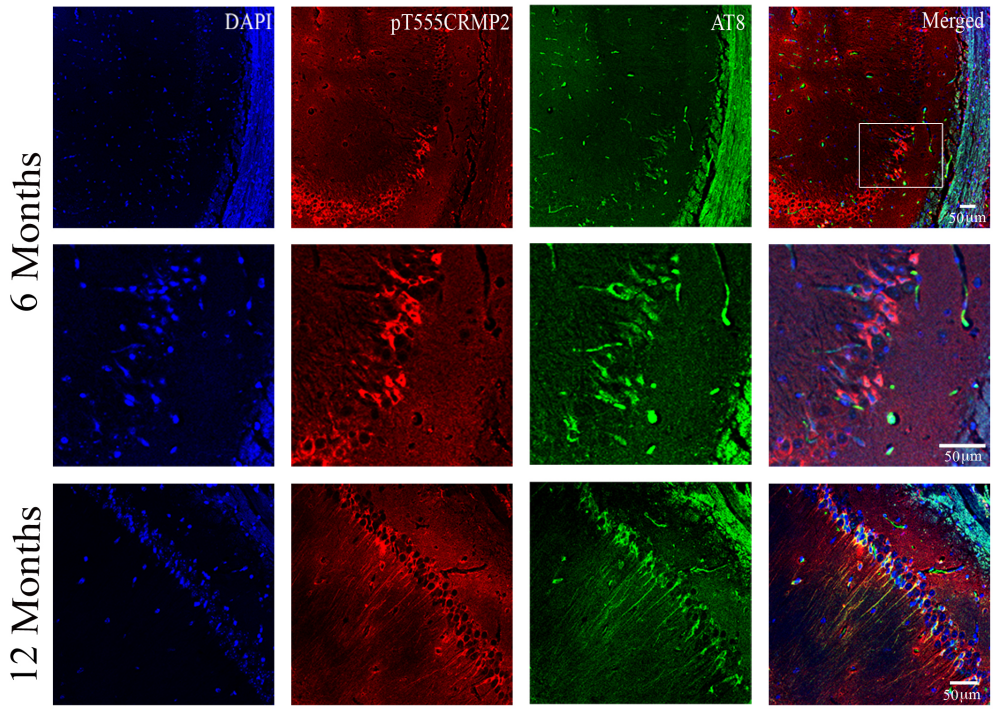
A) Neither polyclonal anti-PThr555-CRMP-2 antibody nor anti-AT8 reactivity was demonstrated in cortical neurons by double immunofluorescence staining on 10 μm coronal parraffin sections of brain from 6-month-old and 12-month-old control mice.

B


DAPI
pT555CRMP2
AT8
Merged
6 Months
12 Months
50 µm
50 µm
50 µm
20 µm

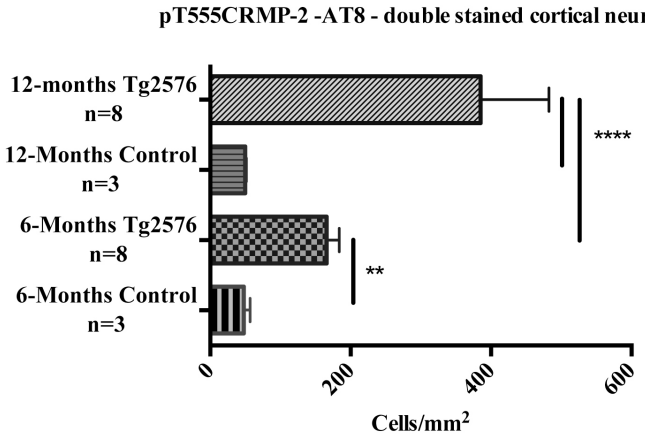
B) Polyclonal anti-PThr555-CRMP-2 antibody reactivity was demonstrated by double immunofluorescence staining on 10 μm coronal parraffin sections of brain showing co-localisation with AT8-positive cortical degenerative neuronal somata and axons of 6-month-old and 12-month-old Tg2576 mice. DAPI nuclear counterstain is shown under the UV emission spectrum.

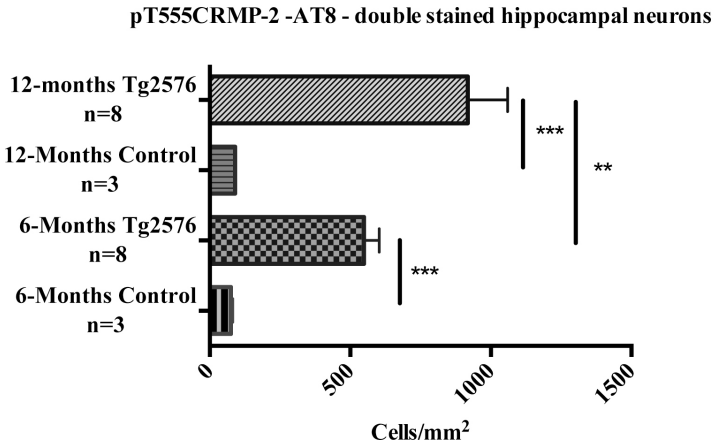
C


DAPI
pT555CRMP2
AT8
Merged
6 Months
12 Months
50μm
50μm
50μm

C) Polyclonal anti-PThr555-CRMP-2 antibody reactivity was demonstrated by double immunofluorescence staining on 10 μm coronal parraffin sections of brain, showing co-localisation with AT8-positive hippocampal degenerative neuronal somata and axons of 12-month-old Tg2576 mice. DAPI nuclear counterstain shows under the UV emission spectrum.

D


pT555CRMP-2 -AT8 - double stained cortical neurons
12-months Tg2576
n=8
12-Months Control
n=3
6-Months Tg2576
n=8
6-Months Control
n=3
****
**
0
200
400
600
Cells/mm2


pT555CRMP-2 -AT8 - double stained hippocampal neurons
12-months Tg2576
n=8
12-Months Control
n=3
6-Months Tg2576
n=8
6-Months Control
n=3
***
**
***
0
500
1000
1500
Cells/mm2

D) Quantification of double stained cortical neurons shows an approximately 2-fold increase in the double stained neurons with the increase in the age of the mice (**** $p<0.0001$, ** $p<0.01$). Quantification of double stained hippocampal neurons shows an approximately 2-fold increase in the double stained neurons with the increase in the age of the mice (*** $p<0.001$, ** $p<0.01$).

4.3 Discussion

Both the phosphorylation of CRMP-2 and the hyperphosphorylation of tau were found to occur in degenerating neurons of the hippocampus and cortex in the aging Tg2576 mouse brain when the Aβ load in the brain was highest. Together these findings are consistent with the notion that tau hyperphosphorylation and CRMP-2 phosphorylation may indeed involve Aβ-dependent mechanisms governing neurodegeneration, possibly by abrogating key anterograde axonal transport mechanisms as described in the previous chapter, however, definitive evidence for this needs to be physiologically demonstrated.

Vesicular transport requires an intact microtubule cytoskeleton (Boland et al., 2008, Kochl et al., 2006, Kovacs et al., 1982). Microtubules are stabilized by the tau protein, which has been shown to dissociate from microtubules in AD and FTD (Gustke et al., 1992). Tau hyperphosphorylation is a pathological hallmark seen in AD and other neurodegenerative disorders. Oligomeric Aβ has been shown to disrupt the microtubule cytoskeleton and cause neuritic dystrophy (Zempel et al., 2010) through hyperphosphorylation of tau, limiting its association to microtubules and causing its aggregation into NFTs (De Felice et al., 2008, Morris et al., 2011). This effect appears to be mediated by Aβ-dependent hyperphosphorylation of tau at Ser202/Thr205 (AT8 epitope) (Jin et al., 2011). It has been reported that Aβ oligomers enhance tau cleavage by calpains and caspases, forming fragments that aggregate independently of hyperphosphorylation (Reifert et al., 2011). In this study, we have demonstrated significant increases in tau hyperphosphorylation with elevated extracellular Aβ1-40 concentrations, specifically corresponding to the elevation of CRMP-2 phosphorylation along with tubulin and kinesin-1 dissociation. Both the phosphorylation of CRMP-2 and the hyperphosphorylation of tau were found to occur in

degenerating neurons of the hippocampus and cortex in the aging Tg2576 mouse brain when the Aβ load in the brain was highest. Together these findings are consistent with the notion that tau hyperphosphorylation and CRMP-2 phosphorylation are Aβ-dependent mechanisms leading to neurodegeneration, probably by abrogating anterograde axonal transport machinery.

Since the generation of the first AD transgenic mouse model, now over two decades ago (Quon et al., 1991), studies of these mice have generated fundamental information about the pathogenesis of AD. In the present study, we have demonstrated that the increase in Aβ load in the brain is consistent with the elevation in the phosphorylation of CRMP-2 at the T555 site. Although Aβ involvement in cognitive decline in Tg2576 and other APP transgenic mice has been well established, studies regarding the onset of cognitive impairment in Tg2576 mice and the form of Aβ that is responsible are in fact inconclusive. Studies have demonstrated deficits appearing as early as 3 months, as late as 15 months, and even at intermediate ages (Chapman et al., 1999, Hsiao et al., 1996, King et al., 1999, Morgan et al., 2000), implicating soluble forms of Aβ for earlier deficits (King et al., 1999), whereas other studies have shown onsets at 9–11 or 15 months, invoking insoluble deposits of Aβ as the initiators of cognitive decline (Hsiao et al., 1996, Morgan et al., 2000). Studies using two lines of transgenic mice expressing similar levels of wild-type and mutant APP have found that the earliest memory loss coincided with the appearance of insoluble Aβ and occurred at 6 months. Genetically accelerating the appearance of insoluble Aβ using the PS1 mutant mouse model resulted in an earlier onset of memory decline. Studies using the TgCRND8 mice, which exhibit a rapid rise in insoluble Aβ at 10 weeks, have demonstrated spatial memory deficits at 11 weeks (Chishti et al., 2001). King et al. (1999) have demonstrated that soluble Aβ caused memory loss occurring at 3 months in Tg2576 (King et al.,

1999). However, studies by Westerman et al (2002) showed that before 6 months, behavioural abnormalities are age-independent and related to APP overexpression, and importantly also that soluble Aβ had no deleterious effect on memory (Westerman et al., 2002). Inverse correlations between memory and amyloid load have been shown in both PDAPP mice (Chen et al., 2000a) and PS1/APP mice (Gordon et al., 2001), suggesting a relationship between memory loss and plaque deposition. In contrast, Westerman et al. (2002) found no correlation between memory and insoluble Aβ and therefore concluded that the major cause of memory loss in Tg2576 is not insoluble Aβ (Westerman et al., 2002).

Several studies have been performed to define the relationship between amyloid load and memory in AD. Early studies showed little or no correlation between amyloid load and dementia (Berg et al., 1993, Arriagada et al., 1992, Terry et al., 1991). Afterwards, more sensitive antibody-based methods were used and identified a highly significant relationship (Bartoo et al., 1997, Cummings et al., 1996, Naslund et al., 2000). Despite these improvements, explaining why some individuals with high plaque loads are cognitively normal is still unanswered (Dickson et al., 1992, Katzman et al., 1988, Delaere et al., 1990). A similar phenomenon exists in some old Tg2576 mice. Westerman et al. (2002) suggested that if cognitive decline is caused by small Aβ assemblies formed during the conversion of detergent-soluble to insoluble Aβ, then some individuals with low levels of these Aβ could be cognitively intact but would accumulate large amounts of deposits or insoluble Aβ over time (Westerman et al., 2002). Studies have suggested that Aβ oligomers cause neuronal toxicity and that they are linked to the conversion of detergent-soluble to insoluble Aβ formation (Hartley et al., 1999, Hsia et al., 1999, Lambert et al., 1998, Mucke et al., 2000, Roher et al., 1996, Wang et al., 1999).

CHAPTER 5: Increased CRMP-2 phosphorylation and kinesin dissociation in human degenerative autopsy tissue

5.1 Introduction

To conduct this study, we sourced frozen human brain tissue from the VBBN following postmortem of patients who had been diagnosed with either AD or other neurological diseases, or had in fact died of complications without neurological disease for comparison (Table 1). We aimed to determine whether there is a common molecular pathway occurring in the neurological disorders in which axonal dystrophy and transport deficits are predominant. FTD is the second-most common form of dementia and is primarily characterised by behavioural, language and cognitive problems rather than memory deficits (Boxer and Miller, 2005, Goedert et al., 2012, Rascovsky et al., 2011). FTD is characterised by cortical degeneration in the frontal and temporal lobes (Boxer and Miller, 2005, Goedert et al., 2012). FTD is a common tauopathy lacking the amyloid pathology, with up to 20% of patients carrying mutations in the MAPT gene located on chromosome 17q21, which encodes the microtubule-associated protein tau (Goedert and Spillantini, 2011). HD is an autosomal dominant neurodegenerative disorder characterised by motor, behavioural, and cognitive abnormalities (for review see (Frank, 2014)). HD pathology involves early and prominent loss of striatal medium spiny neurons and eventually more degeneration of cortical, thalamic, hippocampal and hypothalamic neurons and general loss of cerebral tissues (Cepeda et al., 2014). HD is caused by expansion in the CAG triple repeats within the *huntingtin* gene, resulting in the formation of mutant form of the huntingtin protein (for review see (Frank, 2014)). Multiple Sclerosis (MS) is an immune-mediated inflammatory disease affecting the CNS resulting in destruction of oligodendrocytes with nerve demyelination and axonal damage. MS patients experience neurologic dysfunction resulting in autonomic, sensory, visual, and/or motor deficits (Carta et al., 2014, Chwastiak et al., 2002, Pompili et al., 2012). A direct comparison was performed between the phosphorylation status of CRMP-2 in

these neurodegenerative diseases with either inflammation, tauapathy or amyloidopathy and tauapathy. In accordance with these data, the association of kinesin to CRMP-2 was also assessed amongst the brain samples in an attempt to define potential effects on axonal transport in the context of the neuropathological hallmarks.

5.2 CRMP-2 phosphorylation is increased in human brain lysates from VBBN donor individuals with AD

To define the specific CRMP-2 phosphorylation events occurring in post-mortem AD samples, western blot analysis was performed on AD temporal and frontal lobe cortical samples, then compared with other non-inflammatory and inflammatory neurodegenerative diseases that included HD (caudate nucleus samples), secondary progressive MS (periventricular white matter lesion samples), FTD (cortical samples), along with brain samples from non-neurological disease controls (acute cardiac arrest). The data show that in AD, as well as in the other neurodegenerative diseases of FTD, HD and MS, all brain lysate samples exhibited phosphorylation at the pT555 of the major isoform, CRMP-2B (62 kDa) (Figure. 14A & 14C). However, the CRMP-2A alternatively spliced variant (75 kDa) was only increased within the AD, HD and MS brain samples (Figure. 14A & 14B). We also found significant increases in the other putative phosphorylation sites for CRMP-2 in AD, FTD, HD and MS patients, namely, pT514CRMP-2A, pT514CRMP-2B, pS522CRMP-2A and pS522CRMP-2B (Figure. 14A, Figure. 14D - 14G). These data may suggest that CRMP-2 undergoes hyperphosphorylation during neurodegeneration regardless of whether peripheral inflammatory mechanisms are operative.

Intriguingly, we identified a 55 kDa immunoreactive band that was only abundantly present in the FTD and MS brain lysates. We subsequently immunoprecipitated CRMP-2 using the monoclonal antibody that detected this band, and after in-gel digestion, processed this band for identification through mass spectrometry (LCMS). We identified the band to be the C-terminal 55 kDa cleaved product of CRMP-2, as previously reported in Prion disease (Shinkai-Ouchi et al., 2010) and acquired brain injury samples (Zhang et al., 2007) (Table 4). More importantly,

these data support the previously identified role of Aβ1-42 in CRMP-2 cleavage, which has been indicated upon SH-SY5Y treatment with different concentration of Aβ1-42 (Chapter 3).

Table 4: List of identified protiens from mass spectrometric analysis showing that the 55 kDa band is a cleaved form of CRMP-2

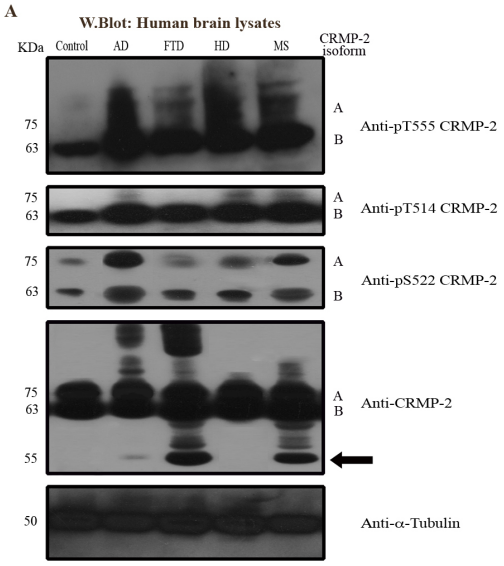
Table lists full names including abbreviations, fraction(s) proteins were identified from, molecular mass in Dalton (Da) and peptide sequence and sequence coverage (%) used to identify each protein using mass spectrometry.

Protein name	Abbreviation	Accession number	Da	Fraction(s)	Mascot search score	Sequence coverage (%)	Total peptides matched
14-3-3 **ε** (match to mouse protein)	1433**ε**	NP_033562	29170	1,2,3	156	46	11
14-3-3 γ	1433γ	XP_001378951	28271	1,2,3	465	36	7
14-3-3 **ζ**	1433**ζ**	XP_001380146	34634	1,2,3	477	35	10
Aconitase 2	Aco2	XP_001378711	87034	10	76	5	4
Actin (β) isoform 1	ACTA1	XP_001362898	41710	4,5	449	39	12
Actin (γ)	ACTAG2	XP_001370868	41766	4	448	36	11
Albumin	ALB	XP_001364858	68048	3,4,5,6,7,8,9,11	436	24	16
α-enolase (2-phospho-D-glycerate hydrolase)	ENO1	XP_001362200	47061	6,7,8,10,12	237	27	12
Annexin A2	ANXA2	XP_001374196	46964	8	82	10	4
Adenosine Triphosphate synthase α-subunit	ATP5A1	XP_001364704	59709	8,9	296	23	12
Adenosine Triphosphate Synthase β-subunit (mitochondrial)	ATP5B	XP_001364069	55874	1,2,4	489	45	16
Casein-α-1 match to Bovine protein	CASEIN	NP_851372	24513	9	79	21	3
Chaperonin containing-t-complex polypeptide 1, β subunit	CCT2	XP_001369473	72938	6	120	5	3
Cofilin-1 match to sheep protein	CFL1	NP_001009484	18507	8,9,10,11,12	174	22	3
Chaperonin 10	HSP10	XP_001379358	27222	11	80	13	3
Collapsin response mediator protein 2A	CRMP2A	XP_001371325	73683	6	83	17	8
Destrin	DSTN	XP_001374193	18462	9,10	131	24	4

Dihydropyrimidinase like 3	DPYSL3	XP_001378663	74174	6	69	4	2
Elongation factor 1	EEF1A1	XP_001365660.1	50152	8,10,12	63	4	2
Fatty acid binding protein (Brain type)	FABP7	XP_001369599	14925	5	83	33	4
Fatty acid binding protein (Heart Type)	FABP3	XP_001381724	14806	5,6	108	23	3
Fructose-bisphosphate aldolase C by homolgy	ALDOC	XP_001368691	39344	7,8	439	54	13
Guanosine diphosphate dissociation inhibitor 1	GDI1	XP_001362742.1	50584		45	3	1
General transcription factor II I Isoform 4	GTFII-I	NP_001074217	107921	1	46	21	14
Glial Fribrillary Acidic Protein	GFAP	XP_001368268	49654	4	117	13	6
Glucose regulated protein 78	GRP78	XP_001365714	72408	2,3	194	19	9
Glyceraldehyde 3 phosphate dehydrogenase	GAPDH	XP_001364734	35961	9,10,11,12	165	23	7
Hemoglobin-α	HBA1	NP_001028158	15316	10	165	54	7
Hemoglobin embryonic-β chain	HBG2	XP_001365409	15978	10	49	23	3
Hemoglobin-**ε**	HBE	XP_001365336	16111	8,10,11,12	499	55	7
Heterogenous nuclear ribonucleoprotein A2/B1	HNRNPAB	NP_872591.1	32440	10,11,12	96	48	12
HSP90	HSP90	XP_001367371	84485	2	45	1	1
HSP1β match to mouse protein	HSP1	NP_032328	83229	9,10	58	4	3
HSP60 (mitochondrial)	HSP60	XP_001370003	61099	4	48	12	4
HSP84β	HSP84	XP_001367493	66226	3	154	24	9
Internexin neuronal intermediate filament-α	INA	XP_001369078	55274	6	77	6	3

Lactate dehydrogenase	LDHB	NP_001028150	36548	5	99	14	4
Lactoglobulin match to bovine protein	LGB	NP_776354	19870	9	66	21	3
Malate dehydrogenase 2 , Nicotinamide adenine dinucleotide (mitochondrial)	MDH1	XP_001366592	35602	9,10,11	204	29	9
Neurofilament L subunit	Nefl	XP_001372723	77043	2	187	20	15
Peptidylprolyl isomerase A-like	PPIAL	XP_001377979	17919	8,9	98	39	7
Peptidylprolyl isomerase B	PPIB	XP_001366685	23758	12	65	4	1
Pol polyprotein	HBZ	XP_001372225	31211	10	47	15	4
Profilin	PFN1	XP_001366082	14996	11	74	11	1
Pyruvate dehydrogenase (PDH)	PDH	XP_001368538	117405	8	67	1	1
Pyruvate kinase (muscle)	PKM2	XP_001371268	57926	7,8	297	26	13
Transketolase	TKT	XP_001379400	67914	7	152	14	8
Triosephosphate isomerase	TPI1	XP_001370168	26732	8	50	11	2
Tropomyosin 2 (fibroblast rat isoform 2)	TPM1	XP_001365835	32676	8	74	33	10
Tropomyosin 3 gamma isoform 8	TPM3	XP_001365838	29013	3	66	35	9
Tubulin-α	TUBα	XP_001363356	50120	1,2,3,4	594	44	15
Tubulin-β	TUBβ	XP_001368231	49875	1,2,3	351	34	11
Ubiquitin	Ub	XP_001375230	17953	8	55	31	5
Vacuolar adenosine triphosphate synthase subunit A	vATP5A1	XP_001362881	68259	4	111	22	11
Voltage dependent	VDAC3	XP_00137	3060	11	128	13	3

anion channel 3		3131	3				
Voltage dependant anion selective channel protein 1	VDAC1	XP_001365887	30698	9,10,11	343	34	7


A
W.Blot: Human brain lysates
KDa
Control
AD
FTD
HD
MS
CRMP-2 isoform
75
63
A
B
Anti-pT555 CRMP-2
Anti-pT514 CRMP-2
Anti-pS522 CRMP-2
Anti-CRMP-2
55
50
Anti-α-Tubulin

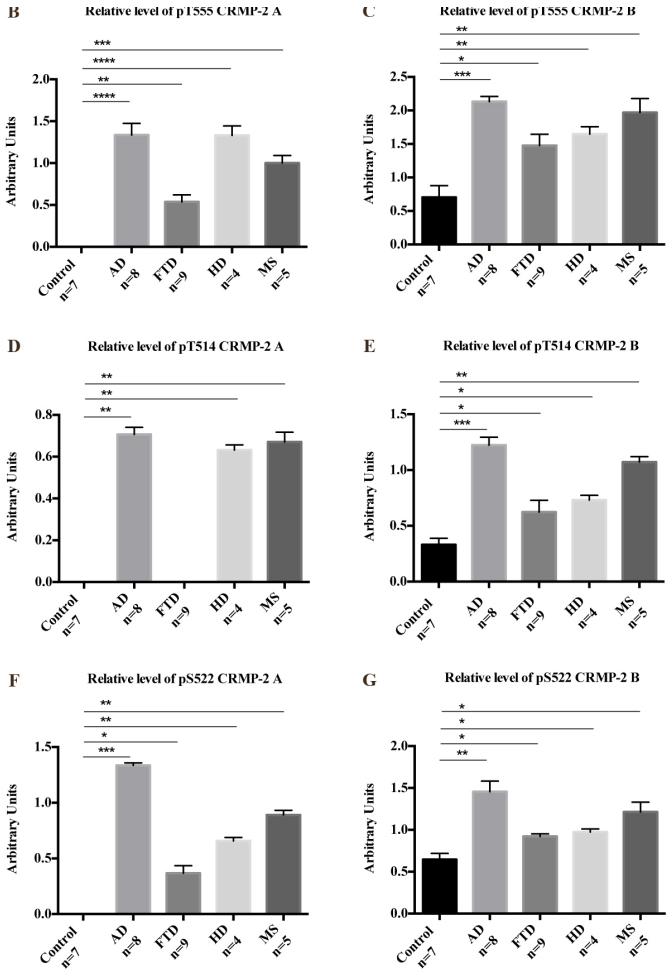

B
Relative level of pT555 CRMP-2 A
C
Relative level of pT555 CRMP-2 B
D
Relative level of pT514 CRMP-2 A
E
Relative level of pT514 CRMP-2 B
F
Relative level of pS522 CRMP-2 A
G
Relative level of pS522 CRMP-2 B
Arbitrary Units
Control n=7
AD n=8
FTD n=9
HD n=4
MS n=5

Figure 14: CRMP-2 phosphorylation at T555, T514 and S522 sites in AD, FTD, HD, MS and NNDC brain lysates.

A) The results show increase in the phosphorylation of CRMP-2 at T555, T514 and S522 sites in AD, FTD, HD and MS brain lysates compared to non-neurological controls. What is evident is the presence of approximately 55 KDa CRMP-2 cleavage product, primarily in FTD and MS brain lysates (arrow). **B)** Shows an increase in the levels of phosphorylated CRMP-2A at the Thr555 site in AD, FTD, HD and MS brain lysates (**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$). **C)** Shows an increase in the levels of phosphorylated CRMP-2B at the Thr555 site in AD, FTD, HD and MS brain lysates (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). **D)** Shows an increase in the levels of phosphorylated CRMP-2A at the Thr514 site in AD, HD and MS brain lysates ($p<0.01$). **E)** Shows an increase in the levels of phosphorylated CRMP-2B at the Thr514 site in AD, HD and MS brain lysates (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). **F**) Shows an increase in the levels of phosphorylated CRMP-2A at the Ser522 site in AD, HD and MS brain lysates (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). **G)** Shows an increase in the levels of phosphorylated CRMP-2B at the Ser522 site in AD, FTD, HD and MS brain lysates (** $p<0.01$, * $p<0.05$).

5.3 CRMP-2-Kinesin and CRMP-2-Tubulin association are decreased in AD, FTD, HD and MS brain lysates

We finally correlated the levels of phosphorylation of CRMP-2 present in AD and other neurological disorders with its association to tubulin and kinesin-1 in order to identify the possible molecular events in axonal transport during neurodegeneration. We found that there were approximately 90%, 85%, 80% and 70% decrease in the percentage of kinesin bound to CRMP-2 in AD, MS, FTD and HD samples respectively, compared to normal controls (Figure. 15A & 15B). Furthermore, we identified an approximate 80%, 40%, 60% and 50% reduction in tubulin-bound CRMP-2 in AD, MS, FTD and HD brain lysates, respectively, compared to the normal brain lysate control samples (Figure. 15A & 15C). These data suggest that tubulin and kinesin dissociation from CRMP-2 could be central to the neurodegeneration and deafferentation in different non-inflammatory and inflammatory neurological disorders, and may implicate extracellular Aβ as a dominant mechanism. Moreover, targeting dissociation of this multimeric complex may represent a potential therapeutic strategy for AD in an effort to limit neurodegeneration.

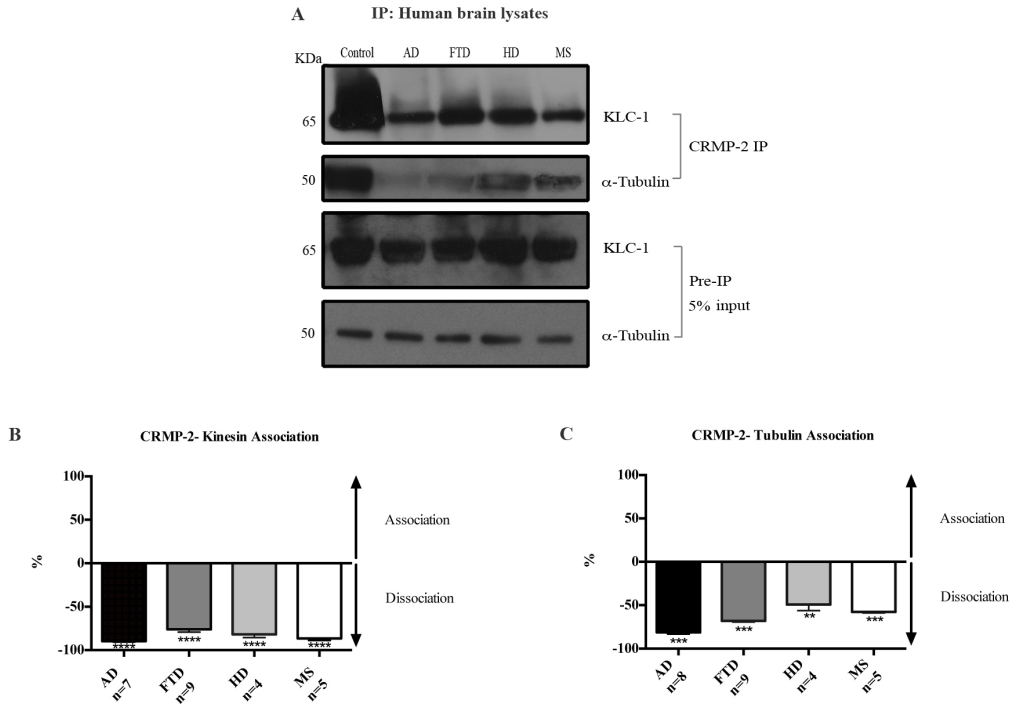

A
IP: Human brain lysates
KDa
Control
AD
FTD
HD
MS
65
KLC-1
CRMP-2 IP
50
α-Tubulin
65
KLC-1
Pre-IP
5% input
50
α-Tubulin
B
CRMP-2- Kinesin Association
100
50
0
-50
-100
%
Association
Dissociation
****
AD n=7
FTD n=9
HD n=4
MS n=5
C
CRMP-2- Tubulin Association
***
**
AD n=8
FTD n=9
HD n=4
MS n=5

Figure 15: Decreased binding of kinesin and tubulin to CRMP-2 in postmortem AD, FTD, HD and MS brain lysates.

CRMP-2 was immunoprecipitated with the polyclonal anti-CRMP-2 antibody, the immunoprecipitate was transferred onto a PVDF membrane and then probed for KLC-1 and α-tubulin to detect the level of kinesin and tubulin bound to CRMP-2, respectively. **A)** Shows the level of total kinesin and α-tubulin before and after immunoprecipitation. **B)** AD, MS, HD and FTD lysates have approximately 90%, 85%, 80% and 70% decrease in the percentage of kinesin bound to CRMP-2 compared to control, respectively (**** $p<0.0001$). **C)** AD, FTD, MS and HD lysates have approximately 80%, 60%, 50% and 40% decrease in the percentage of a-tubulin bound to CRMP-2 compared to control, respectively (*** $p<0.001$, ** $p<0.01$).

5.4 Double immuno-labelling of degenerative neurons from patients with AD, FTD, MS and HD with pT555-CRMP-2 and AT8

To confirm that the phosphorylated form of CRMP-2 was colocalised with hyperphosphorylated tau within degenerative neurons, immunohistochemical staining of frozen sections was performed for the AD, FTD, MS, HD and NNDC paired brain samples obtained from the western blot experiments (Figure. 14). We found that increased labelling of pT555-CRMP-2 was localised to degenerative neurons exhibiting abnormal hyperphosphorylated tau (Figure. 16). Quantification of the double stained neurons shows significant increases in pT555CRMP-2-tau stained degenerative neurons in AD, FTD and HD cortex as compared to NNDC. Also, quantification of the double stained neurons shows a significant increase in pT555CRMP-2-tau stained degenerative neurons in MS white matter compared to NNDC. This finding provides evidence that neurodegeneration exhibits common molecular pathways that drive neuronal dysfunction and axonal dystrophy. Introducing novel inhibitors of CRMP-2 phosphorylation might not aid only in the treatment of AD patients but also in the treatment of patients exhibiting other neurological disorders.

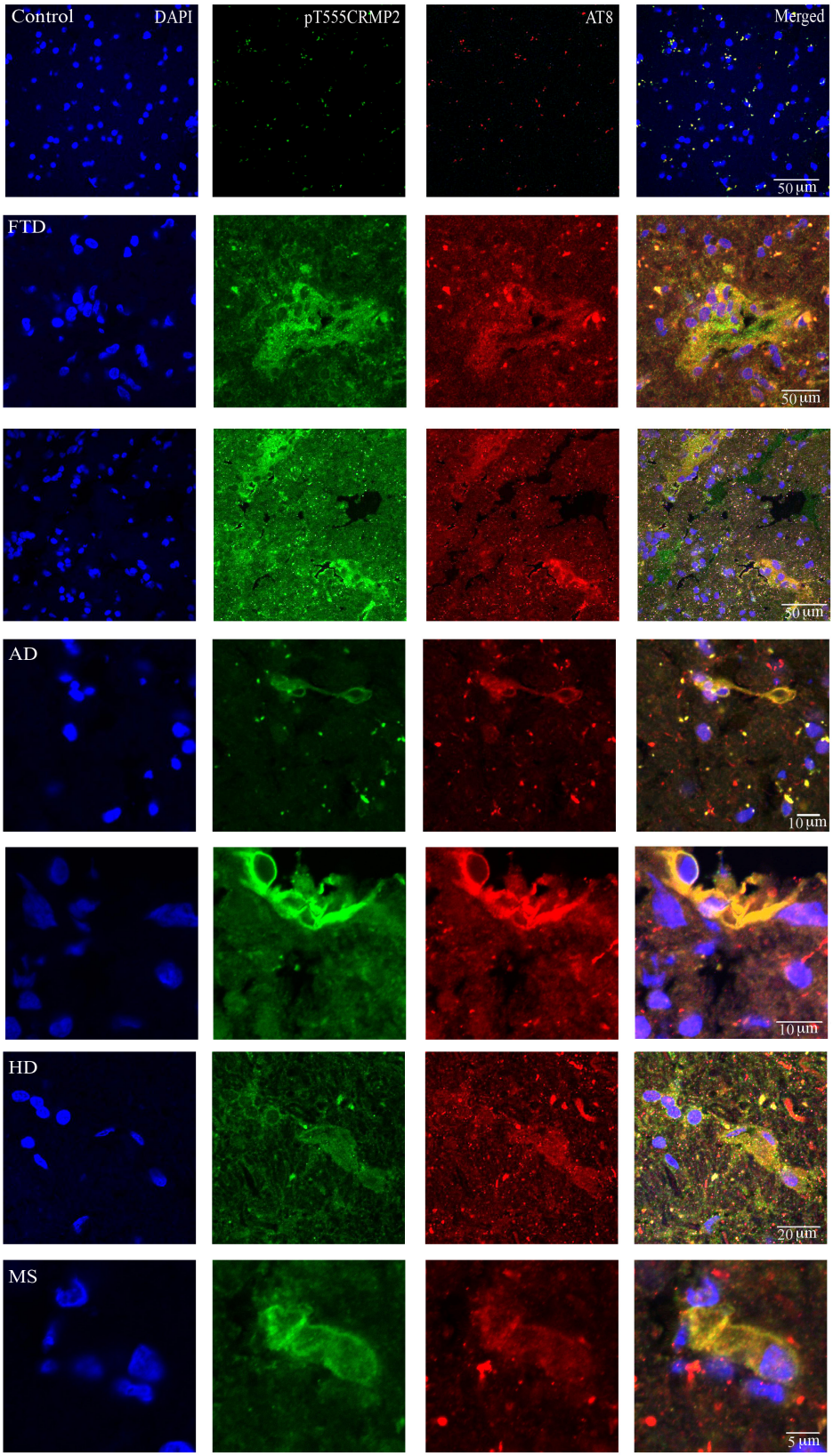
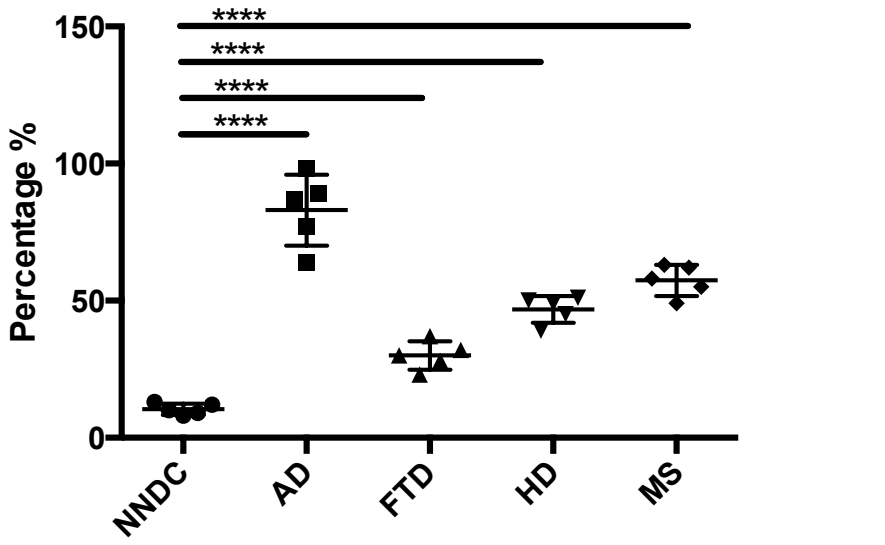

Control
DAPI
pT555CRMP2
AT8
Merged
50 µm
FTD
50 µm
50 µm
AD
10 µm
10 µm
HD
20 µm
MS
5 µm

Figure 16: Immunostaining of frozen sections from donor individuals with AD, FTD, HD and MS with pThr555CRMP-2 and AT

A) Neither polyclonal anti-PThr555-CRMP-2 antibody nor anti-AT8 reactivity were demonstrated in cortical neurons by double immunofluorescence staining on 10 μm frozen sections of brain from non-neurological disease control individuals. Polyclonal anti-PThr555-CRMP-2 antibody reactivity was demonstrated by double immunofluorescence staining on 10 μm frozen sections of brain showing co-localisation with AT8-positive cortical degenerative neuronal somata and axons of individuals with FTD, AD, HD and MS. DAPI nuclear counterstain is shown under the UV emission spectrum


pT555CRMP-2 and AT8 double stained neurons
Percentage %
150
100
50
0
****
****
****
****
NNDC
AD
FTD
HD
MS

B) Percentage of pT555CRMP-2-AT8 double-labelled neurons. The graph shows significant differences in the percentage of pT555CRMP-2-AT8 double stained neurons of frozen sections obtained from AD, FTD, HD and MS patients compared to non neurological disease control (****$p<0.0001$).

5.5 Discussion

The most compelling data implicating a pathological correlation with aberrant CRMP-2 in the present study is from the analysis of the human brain lysates. We detected a significant increase in the phosphorylation of CRMP-2 at the T555 site in AD cortical lysates as well as those from other neurodegenerative disorders (FTD, HD and MS) when compared with brain lysates from non-neurological disease controls. However, we also detected a significant increase in the phosphorylation of CRMP-2 at the T514 and S522 sites in AD, FTD, HD and MS brain lysate samples suggesting that hyperphosphorylation of this protein occurs during inflammatory and non-inflammatory neurodegeneration, irrespective of the Aβ load in the brain. Nevertheless, as we observed in culture and in the Tg2576 mouse model, the relationship between phosphorylation of CRMP-2 at the GSK-3β and Cdk-5 sites and Aβ-dependent signalling remains weak, since we did not find a significant change in these phospho-specific CRMP-2 sites with increasing extracellular Aβ1-40 (Chapters 3 and 4). Hence, the direct effect of Aβ-dependent phosphorylation of CRMP-2 at the reported Rho-kinase site (Thr555) would seem to be the crucial signal causing catastrophic dissociation between the key axonal transport motor protein kinesin and its cargo tubulin, possibly leading to axonal dystrophy. Our data indicated that the key microtubule-associated protein CRMP-2 is incapable of associating with tubulin or the anterograde transport protein, kinesin-1 in AD cortical lysates as well as those from other neurodegenerative disorders (FTD, HD and MS) when compared with brain lysates from non-neurological disease controls. In contrast, Williamson et al. (2011) suggested that CRMP2 hyperphosphorylation is characteristic of AD and is not a feature common to other neurodegenerative diseases (Williamson et al., 2011).

However, in agreement with our data, Petratos et al (2012) have shown previously that pThr555CRMP-2 is abundant in the degenerating spinal cord neurons and axons of EAE-induced mice and active multiple sclerosis lesions. Moreover, the introduction of a site-specific T555A mutation in CRMP-2 through a recombinant adenoassociated virus 2 (rAAV2) delivery system to retinal ganglion cells, limits axonal degeneration in the optic nerve during the peak stage of EAE (Petratos et al., 2012).

The accumulation of organelles and various proteins in the axons and cell bodies of neurons has been shown in a number of neurodegenerative diseases (for review see (Coleman, 2005)). Interruption in axonal transport is thought to underlie the formation of these lesions in many of these diseases (Morfini et al., 2009a). Substantial evidence has suggested that defective axonal transport machinery can contribute to the initiation or progression of pathologies in some neurodegenerative diseases such as AD, HD, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (for review see (Goldstein, 2012, Millecamps and Julien, 2013). For instance, axonal swellings containing accumulations of vesicles, mitochondria, motors, and cytoskeletal components indicative of transport defects can be found in virtually all neurodegenerative pathologies (for review see (Coleman, 2005)). Using immunoblotting, it has been demonstrated that a significant reduction in the levels of KLCs and dynein intermediate chain (DIC) in the frontal cortex, but not in the cerebellar cortex, of AD patients occurs when compared with normal controls (Morel et al., 2012). The same study found a reduction of KLC1 immunoreactivity in neurons in most AD patients (Morel et al., 2012). Other studies have demonstrated decreases in kinesin mRNA and protein in the grey matter of MS patients compared to normal

controls. Moreover, they have shown a positive correlation between the levels of kinesin and disease duration (Hares et al., 2013). These studies highlight the disconnect that exists between physiological axonal transport and degenerative change during neuropathology.

Recently, it has been reported that PS reduction stimulates anterograde and retrograde transport of axonal APP *in vivo*, suggesting that PS plays an important role in the regulation of APP vesicle motility by kinesin-1 and dynein motor proteins, respectively (Gunawardena et al, 2013). Some studies have suggested that PS can influence GSK3β activity to affect kinesin-1 mediated axonal transport (De Strooper et al, 2010, Pigino et al, 2003, Lazarov et al, 2007). However, the extrapolation from our data would point to evidence that overexpression of the phospho-mutant from of CRMP-2, where Rho kinase phosphorylation of CRMP-2 is abrogated, was the only way to improve the re-association of kinesin and by extrapolation, axonal transport. Taken together, the data suggest that axonal transport defects induced by loss of PS-mediated regulatory effects on APP vesicle motility could be a result of PS processing of APP and Aβ-dependent phosphorylation of CRMP-2 and inhibition of CRMP-2 accociation to kinesin-1, leading to the neuronal and synaptic defects observed in AD patients. Thus, our work may highlight a potential novel therapeutic pathway for early intervention, prior to neuronal loss and clinical manifestation of disease with elevated Aβ load in the brain.

CHAPTER 6: General discussion, clinical interpretation and future directions

6.1 General Discussion

It is well known that cognitive decline occurring in both familial and sporadic Alzheimer's disease (AD) is brought about through the aberrant processing of APP, which seeds neurotoxic oligomeric species of Aβ that can promote deafferentation of neurons. Recently, a receptor for oligomeric Aβ has been defined as the PrP^C with a tangible signal transduction mechanism proposed to play a role in excitotoxic damage (Um et al., 2012a). In the studied described above, we have focused on how extracellular Aβ can disrupt important neuronal anterograde transport machinery to amplify the neurodegenerative process. Our findings indicate that Aβ1-40 can modulate the key microtubule-associated protein CRMP-2 through direct phosphorylation at the Threonine555 site, rendering this molecule incapable of associating with the anterograde transport protein kinesin-1 and tubulin. This specific dissociation of CRMP-2 and kinesin-1 could be re-established in the presence of Aβ, but only if the SH-SY5Y neuroblastoma cells were transfected to overexpress a form of CRMP-2 that was incapable of phosphorylation at the Thr555 site. Moreover, we found CRMP-2 phosphorylation and its dissociation from kinesin and tubulin in AD brain samples suggests that an anterograde transport deficit may exist in neurons during neurodegenerative change in the AD brain.

Previous studies have suggested that abnormal forms of PS1, APP, Aβ, and tau can disrupt axonal transport during AD at both early and late stages of cognitive decline. Early transport defects occur before morphological abnormalities take places (For review see (Goldstein, 2012)) while late defects are believed to arise from axonal dystrophy, microtubule dissolution, tau aggregation, and tau-induced kinase activation

(Mandelkow et al., 2003, Morfini et al., 2009a, Stokin and Goldstein, 2006). Transport of brain-derived neurotrophic factor or BDNF is also impaired, independent of tau (Ramser et al., 2013). Studies of cells in culture from mice expressing mutations in APP or β- and γ-secretase demonstrated FAT deficits significantly before amyloid plaque and NFT accumulation (Lazarov et al., 2007, Pigino et al., 2003, Rodrigues et al., 2012b, Stokin et al., 2008). These findings were confirmed using MRI *in vivo* (Minoshima and Cross, 2008). Additionally, downregulation of kinesin light chain-1 or KLC-1 can cause axonal swellings to appear and enhances Aβ overproduction and accumulation (Stokin et al., 2005a). These findings suggest that early FAT deficits may enhance Aβ-dependent neurotoxicity. Genetic screening has identified KLC1vE as a regulator of Aβ overproduction in mice (Morihara et al., 2014). Furthermore, KLC1vE levels were significantly higher in AD patients than normal controls. These results suggest that intracellular trafficking may be a causative molecular event governing cognitive decline during AD. Our study suggested that the intracellular trafficking mechanism can be potentiated by Aβ-dependent regulation of CRMP-2 phosphorylation and association to tubulin and kinesin-1 through the activation of Rho kinase. These data were confirmed using SH-SY5Y cells, primary neurons and human lysates. Therefore, manipulating Aβ-dependent CRMP-2 modifications may affect AD progression. A previous study has reported that a brain sulfur amino acid metabolite, lanthionine ketimine (LK), can bind to CRMP-2 and alters its capability to interact with proteins (Hensley et al., 2010a). They have also reported that a cell-penetrating ester derivative (LKE) demonstrates neuroprotective, neurotrophic, and antineuroinflammatory properties (Hensley et al., 2010a, Hensley et al., 2010b). Additionally, a study using a triple transgenic AD (3_ Tg-AD) mouse model has administered LKE to the mice and tested their cognitive function and

brains histology and biochemistry (Hensley et al., 2013). They have demonstrated that KLE diminished the three hallmarks of AD including Aβ and Tau accumulation, cognitive deficits and microglial activation. They have also reported limited Aβ production in SH-SY5Y cells without associated neurotoxicity (Hensley et al., 2013). Other studies suggested that LK derivatives limited the neuropathology in mouse models of MS and cerebral ischemia (Dupree et al., 2015, Nada et al., 2012). These studies suggest that LK derivatives may have potential for treating different neurodegenerative pathologies including AD. Finding a novel therapy that may target these particular molecules and re-establish axonal outgrowth and anterograde transport is absolutely crucial and might be a promising treatment for AD patients.

6.2 Clinical interpretation

Both ROCK1 and ROCK2 are well-characterised downstream effector kinases of the small GTPases. Besides their importance in regulating actin cytoskeleton reorganisation, they play a critical role in cell migration, chemotaxis, adhesion, reactive oxygen species formation, APP processing, and apoptosis (Doe et al., 2007). In this study we found that inhibition of the phosphorylation of CRMP-2 at the putative ROCK2 site enhances CRMP-2 association to tubulin and kinesin in SH-SY5Y cells. Previous work has identified that nonsteroidal anti-inflammatory drugs limit the incidence of AD by inhibiting ROCK activity (Ding et al., 2010). It has also been determined that a ROCK inhibitor can improve cognitive function in rats (Huentelman et al., 2009). Additionally, it has previously been demonstrated that NSAIDs can reduce Aβ production through the inhibition of Rho-GTPases and their principal downstream effector, ROCK1 and ROCK2 (Zhou et al., 2003). Furthermore, evidence suggests that inhibition of ROCK can promote neurite growth and reduce the Aβ secretion induced by Nogo-P4 stimulation of cultured cortical neurons (Xiao et al., 2012). Also, the ROCK1 and ROCK2 inhibitor Y-27632 (Uehata et al., 1997) has previously been shown to reduce brain levels of Aβ1--42 in an AD mouse model, with no significant effect on total soluble Aβ (Zhou et al., 2003). Moreover, Herskowitz et al. (2013) demonstrated that introducing an inhibitor of ROCK2 limited APP processing in cellular and animal models of AD (Herskowitz et al., 2013). These investigations demonstrated that the inhibition of ROCK2 function using the small molecule SR3677 limits sAPPβ production and Aβ levels by suppression of BACE1 activity, disrupting BACE1 endocytic trafficking and modifying APP phosphorylation at the T654 site in neurons and in the 5X FAD mouse brain. They showed that SR3677 promotes APP trafficking to LAMP1-positive compartments suggesting that

the decrease in APP may be due to increased APP recycling and subsequent processing by α-secretase, enhanced APP traffic to degradative pathways, or both. However, inhibiting both ROCK1 and ROCK2 with RhoI or Y-27632 showed opposed changes on Aβ levels, suggesting that the net result of inhibiting both kinases has a limited effect. It has been shown that RNAi depletion of ROCK1 in human neuroblastoma cells enhances Aβ1-40 production. At present, fasudil is the only clinically trialed ROCK inhibitor. Song et al. (2013) demonstrated that intracerebroventricular injection of male Wistar rats with Aβ1-42 enhances neuronal death in the hippocampus and memory deficits, and that fasudil treatment significantly improved spatial learning and memory impairment, neuronal loss, and neuronal injury (Song et al., 2013). Other studies have demonstrated that fasudil can improve learning and memory deficits in aged rats (Huentelman et al., 2009), and that it has neuroprotective effects on cerebral ischaemia-induced neuronal injury in animal models (Koumura et al., 2011). However, fasudil lacks selectivity to ROCK1 or ROCK2 (Shibuya et al., 2005). A comparison of fasudil with SR3677 indicated that SR3677 has an approximately eightfold selectivity of ROCK2 over ROCK1 (Herskowitz et al., 2013). Clearly, finding a novel and selective ROCK inhibitor may be more effective in AD by inhibiting both Aβ production and Aβ-dependent phosphorylation of CRMP-2 through the T555 site, and its consequent inhibition of axonal growth and transport machinery.

6.3 Study limitations and future directions

In this project, we aimed to demonstrate the effect of Aβ-dependent phosphorylation of CRMP-2 on anterograde axonal transport. Co-transfection of primary neurons with the phospho-mutant CRMP-2 constructs along with APP-FLAG pDendra or BDNF-FLAG pDendra followed by live cell imaging to track the movement of APP and BDNF along the axon would provide us with a molecular tool to identify the most important phosphorylation site in the context of axonal transport. The purpose of using transfected BDNF and APP is to improve the detection capacity, allowing us to demonstrate anterograde transport of these molecules from the neuronal soma down the axonal microtubule system. In this project, transfection of the primary neurons with APP-FLAG pDendra and BDNF-FLAG pDendra using Lipofectamine 3000 was successful with high transfection efficiency (Figure. 10). However, co-transfection of the primary neurons with the phospho-mutant CRMP-2 constructs, along with APP-FLAG pDendra or BDNF-FLAG pDendra using Lipofectamine 3000, appeared to be very toxic and led to substantial cell death at different concentrations. Unfortuantely, due to a shortage of both time and funds, we were not able to repeat the experiment using a different transfection method. Indeed, co-transfection of primary neurons using the Neucleofector system should be carried out. The transfected neurons should be plated into the side (somal) compartment of a microfluidic chamber (Xona Microfluidics), with axons allowed to grow into the second and third compartments. Then, the third compartment (distal axons) should be administered with Aβ40 [1 μM] or scrambled Aβ40 peptide [1 μM] control, and the level of axonally transported Flag-tagged BDNF and APP should be assayed. The levels of phosphorylated CRMP-2 in these compartments, along with tubulin and kinesin-1, should also be assayed using western blotting and immunoprecipitation after cell lysis. Visualisation of the velocity

of photoconverted Dendra2-BDNF or APP, as active anterograde transport along the axon occurs, should also be assessed after Aβ stimulation through live cell imaging. If successful, this proposed experiment would provide evidence for the kinase(s) operative in Aβ-mediated CRMP-2 phosphorylation. These experiments may for the first time demonstrate how Aβ-dependent kinase phosphorylation of CRMP-2 can disrupt the anterograde axonal vesicular transport of APP and BDNF along microtubules.

Tg2576 mice overexpress a mutant form of human amyloid precursor protein (APP), and as a result develop cortical and hippocampal atrophy and progressive cognitive deficits (Hsiao et al., 1996). Tg2576 mice show a rapid rise in cortical and hippocampal Aβ levels, beginning at 6 months and followed by amyloid plaque deposits starting at 9-12 month-of-age (Kawarabayashi et al., 2001). We have demonstrated that Aβ may regulate the key microtubule-associated protein CRMP-2 through direct phosphorylation at the Threonine555 site, making CRMP-2 unable to associate with tubulin and the anterograde transport protein kinesin-1. This dissociation of CRMP-2 from tubulin and kinesin-1 was re-established in SH-SY5Y neuroblastoma cells by overexpressing a mutant form of CRMP-2 that was incapable of phosphorylation at the Thr555 site, even in the presence of Aβ. As previously mentioned, the introduction of a site-specific T555A mutation in CRMP-2 through a recombinant adenoassociated virus 2 (rAAV2) delivery system to retinal ganglion cells limits axonal degeneration in the optic nerve during the peak stage of EAE (Petratos et al., 2012). Intrahippocampal injection of Tg2576 mice with hippocampal neurons overexpressing this mutant form of CRMP-2 can be performed to demonstrate whether the mutant CRMP-2 can compensate the endogenous phosphorylation occurring and limit the axonal degeneration in those mice. The level

of CRMP-2 bound tubulin and kinesin-1 should be measured using immunoprecepitation. Moreover, immunohistochemical staining of the neurofilament should reveal whether there is any improvement in the axonal outgrowth. More importantly, live *in vivo* imaging of hippocampal axons can be monitored to identify axonal transport during Aβ accumulation in the CNS. Furthermore, primary neurons from Tg2576 mice can be transfected with Flag-APP pDendra or Flag-BDNF pDendra to track axonal transport using live cell imaging. In addition, as Tg2576 mice show impaired cognitive functions in spatial tasks, working memory and in the contextual fear conditioning test (Corcoran et al., 2002), testing the improvement in cognitive function can also be valuable. If successful, these experiments may provide a promising therapeutic target for the treatment of AD patients, as well as patients with other neurological diseases.

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APPENDIX

Appendix I

Reagents and media preparation:

1- **10x Kreb's stock**

36.25g Nacl

2.0g KCL

0.7g $NaH_2PO_4.H_2O$

13.0g D-glucose

0.05g phenol red

29.7g Hepes acid

450 ml MilliQ water

pH to 7.4

Filter sterlise @ 0.22 μm

2- **1x Kreb's stock**

50 ml 10x Kreps stock

446 ml dH_2O

1.5g BSA

4 ml 3.85% $MgSO_4$

pH to 7.4

Filter sterlise @ 0.22 μm

3- **Primary neurons plating media**

500 ml DMEM high glucose

0.5 ml gentamycin

50 ml heat inactivated FCS

25 ml heat inactivated horse serum

4- Neurobasal culture media

50 ml neurobasal media

1 ml B27 supplement

50 µl Gentamycin

125 µl 200 mM glutamine

5- DMEM/F12 media

445 ml DMEM/F12

50 ml FCS

5 ml penicillin/streptomycin antibiotics

6- 3.85% $MgSO_4$ stock

1.54g $MgSO_4.7H_2O$

50 ml dH_2O

Filter sterlise

7- Poly-D-lysine stock

25 ml poly-D-lysine (Sigma P-0899)

50 ml water

8- Trypsin

25 mg Trypsin (Sigma T-9003)

10 ml 1x kreps

Filter sterilise

9- DNase/SBTI

8 mg DNase (Sigma D-5025)

26 mg SBTI (Sigma T-9003)

10 ml 1x Kreps

Filter sterilise

10- 10x Tris Buffered Saline Tween (TBST)

12.1g Tris

88g Sodium Chloride (Nacl)

5ml Tween-20

1L MilliQ water

11- Western blot running buffer

47.5 ml Nupage MOPS SDS running buffer (Life technology)

2.4 ml Nupage Antioxidant (Life technology)

52.5 ml dH_2O

12- Western blot blocking buffer (5% Skim milk)

5g Skim milk powder

100ml 1x TBST

pH to 7.4

13- 10x Western Transfer Buffer

30g Tris

148g Glycine

1L MilliQ water

14- 1X Phosphate Buffered Saline (PBS)

8g of NaCl

0.2g of KCl

1.44g of Na_2HPO_4

0.24g of KH_2PO_4

pH to 7.4 with HCl

1L dH_2O

15- IHC blocking buffer

10% FBS

0.3% Triton X-100

10 ml PBS

16- LB broth

10g Nacl

10g Tryptone

5g yeast

1L dH_2O

pH to 7.0

Autoclave

17- LB agar

10g Nacl

10g Tryptone

5g yeast

20g agar

1L dH_2O

pH to 7.0

Autoclave

18- 10% agarose gel

10g agarose powder

100 ml TAE

Appendix II

1- APP-FLAG pDendra plasmid map

GCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTT
ACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAA
TGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACA
AATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTG
CATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGGCGTAAAT
TGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAG
CTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAA
AAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGT
CCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTA
TCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGG
GGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCG
ATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGG
AAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCA
CGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGC
GCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTA
TTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGA
TAAATGCTTCAATAATATTGAAAAAGGAAGAGTCCTGAGGCGGAAAGAA
CCAGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCC
CAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGG
TGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCA
TCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCC
CCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTT
TTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAA
GTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAGATCGATCAA
GAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGC
AGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCAC
AACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAG
GGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAA
CTGCAAGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCC
TTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGC
TATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTG
CCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTT
GATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCG
AGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACG
AAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCG
AGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTT
GCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTG
GCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGT
GATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCT
TTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCT
TGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGC
GACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGA
AAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCC
AGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCTAGGGGGAGGCTA
ACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGG
CAATAAAAAGACAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAA

CGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCACCGAGACC
CCATTGGGGCCAATACGCCCGCGTTTCTTCCTTTTCCCCACCCCACCCCCC
AAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCGGGGCGGCAGGC
CCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATTTAAAACTT
CATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATG
ACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTA
GAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGC
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AGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCA
AGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAG
TGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGA
CGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTG
CACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTAC
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CAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAG
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CTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTA
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GTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
CCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGAC
GTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGT
GGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATA
TGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGG
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Xho1 site Purple: Dendra2 Blue text: Signal peptide Underlined text: Flag

Grey highlighted text: linker Red text: APP

2- BDNF-FLAG pDendra plasmid map

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Xho1 site Purple: Dendra2 Blue text: Signal peptide Underlined text: Flag

Grey highlighted text: linker Red text: BDNF