From Chromatin to Protein Synthesis: the role of Glutamate, Amyloid Beta and Tau in Alzheimer's disease

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Dedication

This thesis dedicated to;

i. The memory of my late mother – Kolo Bukar Maina. I miss her very much. I would have loved for her to see what I came to achieve as her last born;

ii. My father; who has been incredibly supportive of my career – Dad, thank you for showing us love and guidance as we grew up, and for supporting us in all our quest for scholarship since birth.

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Prophet Muhammad (peace and blessings of Allah be upon him) said:

"There is no disease that Allah has created, except that He also has created its treatment."

Sahih Bukhari.

The above statement continues to drive my passion in the field of Medicine, with the hope that someday, I will contribute to finding treatments or cure for diseases, such as Alzheimer's disease.

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Abbreviations

Aβ	-	Amyloid beta
AD	-	Alzheimer's disease
AGEs	-	Advanced glycation end products
AICD	-	APP intracellular cytoplasmic domain
AMPA	-	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APH-1	-	Anterior pharynx-defective 1
APOE	-	Apolipoprotein
APP	-	Amyloid precursor protein
APPsβ	-	BACE1-generated APP soluble extracellular fragment
APP-CTFβ	-	BACE1-generated APP cell membrane fragment
APP-CTFα	-	α secretase-generated APP cell membrane fragment
APPsα	-	α secretase-generated APP soluble extracellular fragment
BACE 1	-	β-site APP cleaving enzyme 1
BDNF	-	Brain-derived neurotrophic factor
CaMKII	-	Ca2+ /calmodulin-dependent protein kinase II
CENP-A	-	Centromere protein A
CENP-B	-	Centromere protein B
CDK	-	Cyclin-dependent kinase
CHO	-	Chinese hamster ovary cells
CK	-	Casein kinase
CNS	-	Central nervous system
CSF	-	Cerebrospinal fluid
DNMTs	-	DNA methyltransferases
dsDNA	-	Double stranded DNA
fAD	-	familial AD
FTD	-	Frontotemporal dementia
FTDP-17	-	FTD with Parkinsonism linked to chromosome 17
GSK	-	Glycogen synthase kinase
GWAS	-	Genome-wide associated studies
HMGA	-	High motility group A proteins
HMGB1	-	High mobility group box 1 protein
HPG	-	Homopropargylglycine

HP1	-	Heterochromatin protein 1
H4K20	-	Histone H4 lysine 20 methylation
H3K9	-	Histone H3 lysine 9 methylation
Kaps	-	β-karyopherin
KAP1	-	Kruppel-associated Box (KRAB)-associated Co-repressor
LTP	-	Long term potentiation
MAPT	-	Microtubule-associated protein
MBD	-	Tau protein microtubule-binding domain
MBN	-	Magnocellular nucleus basalis
MCI	-	Mild cognitive impairment
NADs	-	Nucleolar-associated chromatin domains
NCLK	-	Neuronal cdc2-like kinase
NLS	-	Nuclear localisation signal
NMDAR	-	N-methyl-D-aspartate receptor
nNOS	-	Neuronal nitric oxide synthase
NoRC	-	Nucleolar remodelling complex
NORs	-	Nucleolar organiser regions
NPC	-	Nuclear pore complex
nP-Tau	-	Non-phosphorylated tau identified by Tau 1 antibody
NR2B	-	NMDAR subtype 2B
NTRs	-	Nuclear transport receptors
PEN-2	-	Presenilin enhancer 2
PERK	-	Pancreatic endoplasmic reticulum kinase
PDPK	-	Proline-directed protein kinases
PET	-	Positron emission tomography
PFA	-	Paraformaldehyde
PHD	-	Plant homeodomain
PHF	-	Paired helical filaments
PKA	-	Protein kinase A
Plaques	-	Amyloid plaques
PNS	-	Peripheral nervous system
PP	-	Protein phosphatase
PRD	-	Tau protein proline-rich domain

PS	-	Presenilin
P-Tau	-	Phosphorylated tau antibody against phosphor T231
RAGE	-	Receptor for AGEs
RIPA	-	Radio immunoprecipitation assay
rDNA	-	ribosomal DNA
ROS	-	Reactive oxygen species
rRNA	-	ribosomal RNA
Snf2H	-	Sucrose nonfermenting protein 2 homolog
ssDNA	-	Single stranded DNA
Tangles	-	Neurofibrillary tangles
TBP	-	TATA-binding protein
TIP5	-	Transcription Termination Factor I-Interacting Protein 5
TREM2	-	Triggering receptor expressed on myeloid cells 2 gene
UBF	-	Upstream binding factor
UPR	-	Unfolded protein synthesis response
5-mC	-	5-methylcytosine

Abstract

Alzheimer's disease (AD) is the most common form of dementia, which is characterised by extracellular Aβ plaques and intracellular neurofibrillary tangles, comprised of fibrils of Aβ42 and tau protein, respectively. A species of tau protein localised to the nucleus has been discovered, but its role in AD is still unclear. Glutamate excitotoxicity, oxidative stress, DNA damage, alteration of the chromatin and nucleolar stress are key features of AD. The early stages of the disease are characterised by minimal neurodegeneration and altered protein synthesis machinery. The culprit (s) and molecular link between these changes and the role of nuclear tau are unclear. This work utilised glutamate stress and Aβ42 oligomers to investigate the involvement of nuclear tau in the chromatin alteration, nucleolar dysfunction, and downstream protein synthesis impairment that occurs in AD. This revealed that glutamate stress in SHSY5Y neuroblastoma cells results in oxidative stress, a nuclear upsurge of phosphorylated tau and delocalisation of nucleolar tau, alongside, DNA damage, heterochromatin loss, nucleolar stress and protein synthesis inhibition, partly through eIF2α phosphorylation. Likewise, short incubation of SHSY5Y cells with Aβ42 oligomers led to significant oxidative stress, with gradual accumulation of nucleolar stress, which resulted in altered transcription and processing of 45S pre-rRNA and decrease in protein synthesis, without DNA damage. Although both glutamate and Aβ ultimately decreased protein synthesis, Aβ incubation led to an increase in heterochromatin formation and a reduction in RNA synthesis without DNA damage, pointing to a different mechanism of toxicity by the Aβ and glutamate stress. To characterise a nuclear role for tau, this work localised

tau in the nucleolus and heterochromatin in the SHSY5Y cells and the human brain, where it associates with TIP5 – a key player in heterochromatin formation. Accordingly, tau knockdown destabilises the heterochromatin and increases rDNA transcription, indicating that tau is essential for silencing of the rDNA and heterochromatin stability, similar to TIP5. Overall, this thesis provides evidence that implicates glutamate and Aβ toxicity in some of the changes that occur in the disease and specifically implicates Aβ42 as a key culprit that drives changes in the early stage of the disease. It also reveals a new role for tau in the nucleus and points to its pathological involvement in AD.

Publications and Submitted Articles

The following published articles and abstracts contain results described in this thesis:

Maina, M. B., Al-Hilaly, Y. K, Serpell, L. C. 2016. Nuclear Tau and Its Potential Role in Alzheimer's disease. *Biomolecules*. 6(1):9.

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Maina, M. B, Bailey, L. J., Wagih, S., Biasetti, L., Pollack, S., Thorpe, J. R., Doherty, A. J. and Serpell, L. C. 2017. Tau Protein: Novel interacting partner and role in the nucleolus. In: *1st Euro Tau Meeting, Lille***,** France, April 27-28, page 74.

Maina, M. B, Bailey, L. J., Doherty, A. J. and Serpell, L. C. 2016. A Molecular link between oligomeric Aβ and Tau in neuronal damage in Alzheimer's disease. In: *CNRS Conference Jacques Monod,* Protein misfolding in disease - Toxic aggregation-prone proteins in aging and age-related diseases: from structure to pathology and spreading, Roscoff (Brittany), France, September 12-16, page 61.

The following are submitted articles from the results described in this thesis:

Maina, M. B, Bailey, L. J., Wagih, S., Biasetti, L., Pollack, S., Thorpe, J. R., Doherty, A. J. and Serpell, L. C. 2017. Nuclear Tau regulates heterochromatin stability and rDNA transcriptional silencing. *Submitted.*

Maina, M. B, Bailey, L. J., Doherty, A. J. and Serpell, L. C. 2017. Amyloid β oligomers induce nucleolar stress and changes in nucleolar tau distribution leading to protein synthesis dysfunction in human neuroblastomas. *Submitted*

Chapter one

1. General Introduction

Alzheimer's disease (AD), first described 110 years ago by Alois Alzheimer, is the most common form of dementia (Alzheimer et al., 1995). There is currently no disease-modifying treatments for the disease, and about 5% of cases are early-onset, affecting people in their 30s, 40s, and 50s, while the majority of cases are late-onset, affecting people above 65 years (Perkins, 2016). Globally, over 47 million people currently live with dementia, mostly AD, and as the population ages, this figure is estimated to double every 20 years, such that about 131 million people would become affected by 2050 (Prince et al., 2016). In the UK, about 850, 000 people currently live with dementia, costing over £24 billion to the UK economy (Mitchell et al., 2016). Altogether, this cost around $818 billion to the economy on a global scale (Prince et al., 2016).

In his 1907 article describing the disease, Alzheimer reported various abnormalities in his patient – Auguste Deter, which included, memory loss, hallucinations, and disorientation. Postmortem analysis revealed cortical atrophy without macroscopic focal degeneration; arteriosclerotic vascular changes, and microscopically, disintegrated neurons, intracellular neurofibrillary tangles (henceforth called tangles) and "minute miliary foci" deposited extracellularly as amyloid plaques (henceforth called plaques) (Fig. 1.1) (Alzheimer et al., 1995). Six decades after this report, Blessed et al. found that the number of plaques in the cerebral gray matter were associated with the aberration of intellectual and personality functions in old age and this is associated with an increased risk of dementia (Blessed et al., 1968). This provided early evidence for the prevalence of AD among the elderly. Later on, it was found that cortical deficits in the cholinergic system which is important for learning and memory

(Deiana et al., 2011), and cognitive dysfunction were found to show a close link with plaque burden (Perry et al., 1978). However, several studies subsequently established that the burden of plaques does not correlate with the severity of dementia (Serrano-Pozo et al., 2011). However, a positive correlation between the burden of tangles and the extent of dementia in the disease was found (Arriagada et al., 1992, Samuel et al., 1991). Altogether, these findings triggered strong interest in understanding the biochemistry of these plaques and tangles. This led to the discovery that the plaques are predominantly made up of an amino acid peptide of about 40-42 residues and 4.2 kDa, now called amyloid beta (Aβ) (Glenner and Wong, 1984). While the tangles are comprised of hyperphosphorylated form of the microtubule-associated protein tau (Wood et al., 1986, Grundke-Iqbal et al., 1986).

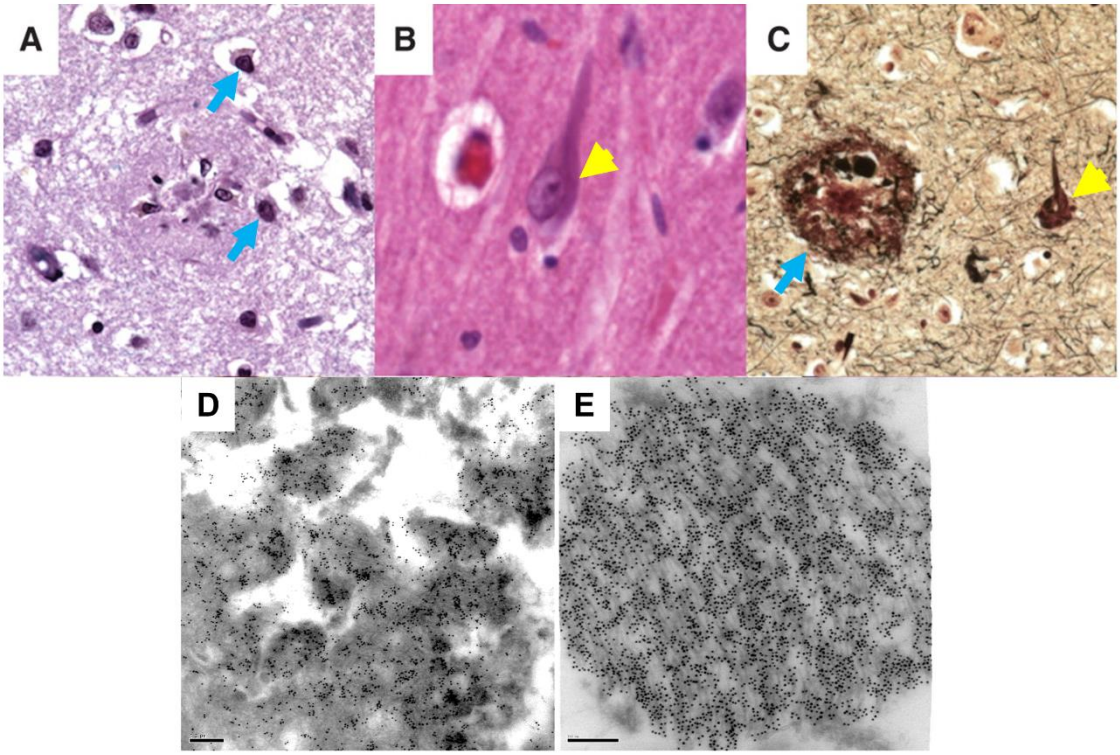

Figure 1.1 The key hallmarks of Alzheimer's disease
(A) Haematoxylin and Eosin (H&E) stained frontal cortex tissue from an AD patient showing plaques (blue arrows). (B) H&E stained hippocampal pyramidal neurons showing tangle (yellow arrow). (C) Silver-stained AD brain section showing both plaque and tangle. Immunogold labelling with anti-Aβ42 antibody showing amyloid plaque (Scale bar, 200 nm) (D) and anti-tau antibody showing tangle (scale bar, 100 nm) (E). A, B & C taken from (Serrano-Pozo et al., 2011) and D & E kindly provided by Dr Youssra Al-Hilaly in the Serpell Lab.

1.1 Amyloid beta and Alzheimer's disease

Aβ peptide is synthesised from the processing of a single-pass integral membrane protein called amyloid precursor protein (APP) encoded by a gene located on chromosome 21, which has 18 exons, of which, exon 16 and 17 encode the Aβ peptide (Yoshikai et al., 1990). Through alternate splicing, the APP transcript yields about eight different isoforms of various lengths, all having a large N-terminal extracellular domain, three most common of these being a 695 amino acid isoform that exclusively localises to the central nervous system (CNS), 751 and 770 amino acid isoforms that are distributed ubiquitously (O'Brien and Wong, 2011). The cleavage of APP on the

aspartate residue at the beginning of the APP Aβ sequence by a transmembrane enzyme called β secretase 1 or β-site APP cleaving enzyme 1 (BACE 1) generates a soluble extracellular fragment called APPsβ and a 99 residue cell membrane-bound fragment (APP-CTFβ/C99) through a process called "ectodomain shedding" (Kang et al., 1987). Subsequent cleavage of the APP-CTFβ by an integral membrane multi-subunit enzyme complex called γ-secretase yields APP intracellular cytoplasmic domain (AICD) and species of Aβ peptide (Fig. 1.2).

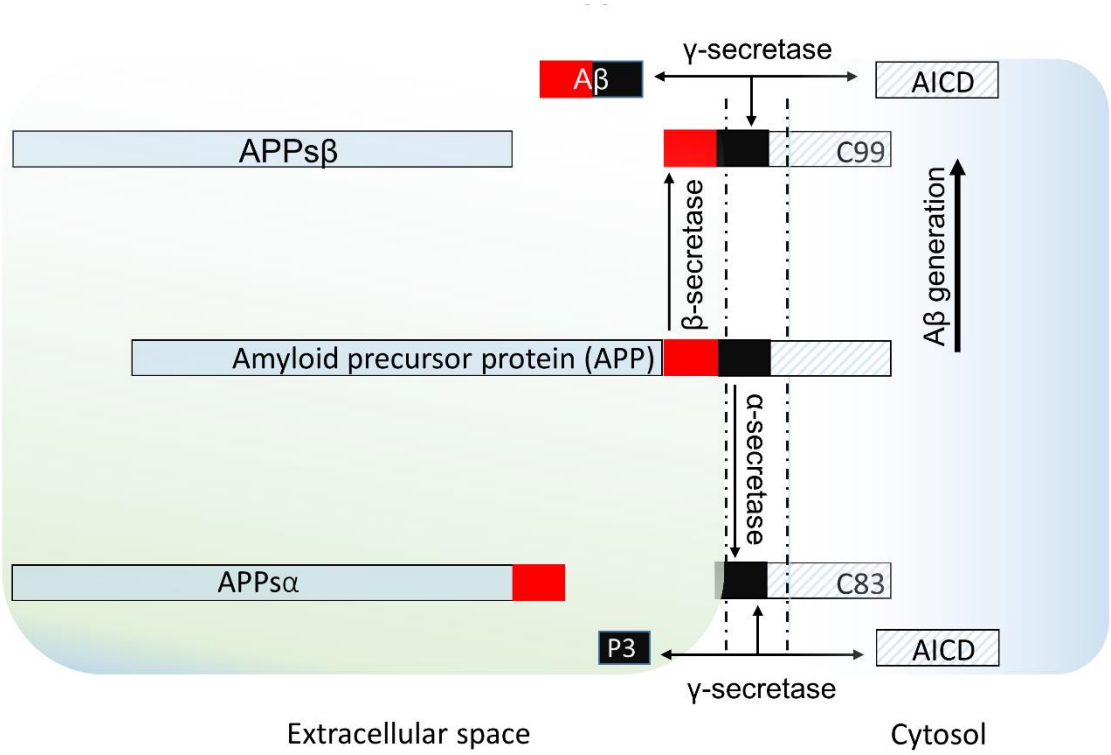

Figure 2.2 Proteolytic processing of APP.
Adapted from Dries and Yu 2008. (A) Two pathways process APP; non-amyloidogenic and amyloidogenic pathways. In the non-amyloidogenic pathway, α-secretase cleaves APP within the Aβ domain to generate APPsα and C83 membrane-bound residue, which subsequently becomes cleaved by γ-secretase to produce ~3 kDa peptide (p3) and AICD. In the amyloidogenic pathway, BACE 1 cleaves APP at the beginning of the N-terminal portion of Aβ domain to generate APPsβ and C99 membrane-bound residue, subsequent cleavage by γ-secretase lead to the generation of Aβ and AICD.

The γ-secretase complex is comprised of nicastrin, anterior pharynx-defective 1 (APH-1), presenilin enhancer 2 (PEN-2) and the catalytic unit - presenilin (PS) (Kang et al., 1987, Dries and Yu, 2008). Depending on the site on the APP-CTFβ, cleavage by PS

leads to the production of Aβ peptide species of varying lengths resulting to Aβ37 through Aβ49 (Fig. 1.3). The production of Aβ is however precluded when a BACE 1 competing proteolytic enzyme called α-secretase cleaves APP within the Aβ domain, between Lysine 16 and Leucine 17, leading to the generation of a large soluble extracellular fragment (APPsα) and an 83 residue membrane-bound transmembrane fragment (APP-CTFα/C83). The subsequent cleavage of the APP-CTFα fragment by γ-secretase leads to the generation of a non-amyloidogenic ~3 kDa p3 fragment and AICD (Kang et al., 1987, Dries and Yu, 2008). Thus, it is the combination of BACE 1 and γ-secretase that drives APP through the amyloidogenic pathway of Aβ synthesis. Interestingly, some APP molecules that escape cleavage at the plasma membrane have been reported to undergo cleavage by BACE 1 and γ-secretase upon internalisation into the endosomal system, leading to the generation of some fraction of Aβ (Kamenetz et al., 2003).

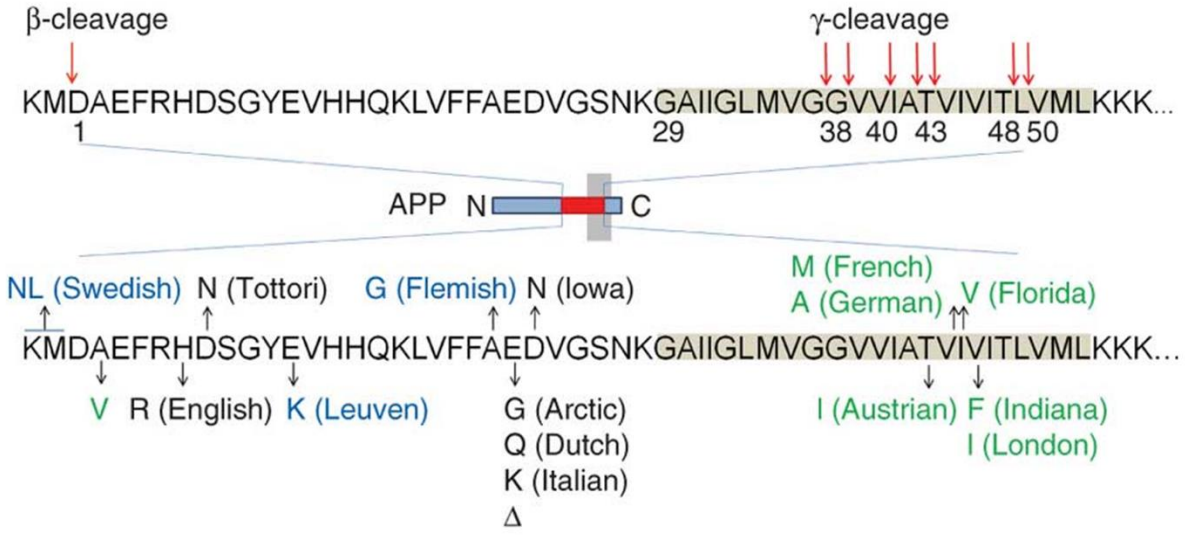

Figure 3.3 Alzheimer's disease - causing APP mutations.
Taken from Benilova et al. 2012. Different mutations from position 670 to 717 of the APP gene that causes familial AD. Swedish mutation (KM670/671NL) is a double mutation that results from a substitution of two amino acids K and M at position 670-671 to N and L. Leuven mutation (E682K) results from the substitution of E to K at position 682. English mutation (H677R) results from the substitution of H to R at position 677. Tottori mutation (D678N) resulting from the substitution of D to N at position 678 of APP gene. Flemish mutation (A692G) resulting from the substitution of A to G at position 692. Iowa mutation (D694N) results from the substitution of D to N at position 694. Three mutations arise from substitutions at the same region (693) - Arctic (E693G) substitution of E to G, Dutch (E693Q) substitution from E to Q and Italian (E693K) substitution from E to K. French (V715M) and German (V715A) mutations arise from substitution of the V715 to M and A, respectively. While Austrian mutation (T714I) results from the substitution of T to I, just before the V715. Florida mutation (I716V) results from the substitution of I to V at position 716. While Indiana (V717F) and London (V717I) mutations arise from a substitution of V717 to F and I, respectively. Most of these mutations lead to the increase production of Aβ or its aggregation. Symbols: Lysine (K), methionine (M), asparagine (N), leucine (L), histidine (H), arginine (R), glutamic acid (E), aspartic acid (D), glycine (G), alanine (A), glutamine (Q), isoleucine (I), valine (V), threonine (T), phenylalanine (F).

Many mutations in the APP gene directly causing familial forms of AD (fAD) have been discovered (Fig. 1.3), and usually resulting in an increase in the levels of Aβ species, mostly Aβ40 and Aβ42 (Benilova et al., 2012). Aβ40 is the major species of Aβ found in biological fluids (Chartier-Harlin et al., 1991) and constitutes about 90% of the Aβ peptides found in non-AD condition, compared to the 5 – 10% of Aβ42 found in unaffected individuals (Kang et al., 1987). In fAD, the Aβ42:Aβ40 ratio of 1:9 shifts to a ratio of 3:7, and this has been shown to be essential for the toxicity of Aβ (Kuperstein et al., 2010). While both Aβ42 and Aβ40 are components of the AD plaques, Aβ42

appears to be an exclusive component of the developing plaques (Van Broeckhoven et al., 1990) and has higher aggregation propensity (Levy et al., 1990). It was subsequently demonstrated that the level of Aβ42 could serve as an important indicator of AD, where the soluble Aβ42 species level becomes specifically low in the cerebrospinal fluid (CSF) of AD patients, compared to the control. This in turn is correlated with high plaque burden in the AD brain, suggesting that its CSF reduction is due to its accumulation in the plaques (Jack et al., 2013; Tapiola et al., 2009). The difference in the Aβ species in AD and non-AD conditions allows for the quantification of plaque burden using imaging (e.g., positron emission tomography (PET) and CSF soluble Aβ values, as a diagnostic tool for AD. For instance, the CSF level of Aβ42 and tau serve as a biomarker to delineate AD from non-AD condition (Tapiola et al., 2009).

1.1.1 Amyloid cascade hypothesis

With the identification of Aβ as the special substance that accumulates in plaques (Glenner and Wong, 1984) and the cloning of its parent protein – APP (Kang et al., 1987), the idea among scientists that Aβ is important in AD, became even more convincing. This was partly because the majority of Down syndrome (Trisomy 21) cases result from the acquisition of three copies of the APP gene, suggesting that excess Aβ contributes to the disease (Head et al., 2012). Indeed, a series of discoveries were made on some of the mutations directly causing fAD, mostly linked to Aβ generation, deposition or aggregation propensity (Fig. 1.3) (Hardy, 1991, Chartier-Harlin et al., 1991, Goate et al., 1991, Van Broeckhoven et al., 1990, Levy et al., 1990). Although the majority of AD cases are late onset and sporadic, this led to the formulation of the "amyloid cascade hypothesis", which argues that genetic and other causes of AD act to induce the deposition of plaques, which subsequently drives downstream changes, such as tau phosphorylation, cell loss and dementia (Hardy and Higgins, 1992). The

hypothesis posits that APP mutation or other causative factors (e.g. head injury) induce the deposition of Aβ, leading to the cascade of deleterious events, including the disruption of Ca^{2+} homeostasis that could result in increased intraneuronal Ca^{2+} concentration, alteration of cellular homeostasis, tau hyperphosphorylation and tangle formation, cell loss, vascular damage and dementia (Hardy and Higgins, 1992). Around the same time, Dennis Selkoe proposed that a decrease in the clearance of Aβ and its overload could contribute to the pathology seen in AD, in line with the amyloid cascade hypothesis (Selkoe, 1991). Further evidence supporting the cascade hypothesis was found based on missense mutations of the PS gene, which affects the proteolytic cleavage of APP, leading to an excessive production of Aβ42, resulting in an aggressive form of early-onset AD (Selkoe, 1999). The amyloid cascade hypothesis has been supported by biomarker studies which show that changes in CSF levels of Aβ and its deposition into plaques appear decades before the onset of dementia (Jack et al., 2013). A reformulated version of the cascade hypothesis suggested that the formation of plaques due to increasing production and deposition of Aβ leads to microglial and astrocytic activation and accumulation of oxidative injury. Subsequently, these directly cause neuronal dysfunction and neurotransmission deficit. Alternatively, these changes alter the balance of kinases and phosphatases, resulting in abnormal tau phosphorylation, tangle formation, neuronal dysfunction and cognitive impairment (Selkoe, 2000, Selkoe and Hardy, 2016).

The plethora of studies that accumulated since the inception of the amyloid cascade hypothesis now revealed that the plaques might not be toxic after all. These data show that plaque burden does not correlate with the severity of dementia (Serrano-Pozo et al., 2011). Many patients with severe cognitive decline show no plaque deposits, and paradoxically some normal individuals show plaque deposits in their brain (reviewed

in Pimplikar, 2009). Moreover, cognitive decline in AD mouse models (e.g., 3xTg) appear before plaques (Billings et al., 2005). Studies now show that instead of acting extracellularly, Aβ could exert its deleterious effects intracellularly in a soluble form (Zhang et al., 2002, Soura et al., 2012). Some studies demonstrate that it could exert its effects through membrane interaction, such as through the receptor for advanced glycation end products (AGEs) (Reddy and Beal, 2008), cellular prion protein receptor (Schonheit et al., 2004) or the formation of ion channels (Arispe et al., 2007). Recently, it was shown that one mechanism of Aβ toxicity occurs via Tau/FYN kinase interaction at the membrane that leads to excitotoxicity – a signature found in the AD brain (Ittner et al., 2010, Ittner et al., 2016).

1.1.2 Glutamate excitotoxicity

Glutamate is a non-essential amino acid and one of the principal excitatory neurotransmitters in the vertebrate brain. Most of the excitatory neurons in the CNS are glutamatergic. However, glutamate hardly crosses the brain-blood barrier, as such, it is synthesised locally in the CNS, stored in the neuronal synapse and release upon depolarization of a neuron (Daikhin and Yudkoff, 2000). Once released by presynaptic neurons, glutamate mainly acts either via glutamate metabotropic receptors, in which case it acts via a second messenger system to stimulate the postsynaptic neuron, or via three families of ionotropic receptors on the postsynaptic neuron, namely N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and Kainite (KAR), leading to the influx of positive ions (mainly Na^+ and Ca^{2+}) into postsynaptic neuron (Mark et al., 2001). The response caused in the postsynaptic neuron is essential for many brain functions, such as synaptic plasticity, learning and memory, and maintenance of consciousness. However, an excessive level of glutamate or hyperpersensivity of glutamate receptors equally plays a neurotoxic role in ageing

and neurodegenerative diseases. Normally, in the synaptic vesicles, glutamate can reach a concentration of ~ 100 mM/L, while upon release, its concentration can go as high as 5 mM in the synaptic cleft, which becomes cleared within milliseconds (Featherstone, 2010). The abnormal rise or decrease in clearance of glutamate in the synaptic cleft leads to hyper excitation of postsynaptic neurons, which can result to neurotoxicity.

This toxic effect of glutamate became known in 1950, based on the observation that the administration of Sodium L-glutamate, popularly used in Chinese restaurants, causes the death of inner retinal cells in mice within few hours, indicating that in excess concentration, glutamate can be neurotoxic (Lucas and Newhouse, 1957). Monosodium glutamate was subsequently found to cause neuronal degeneration in the neurons of the peripheral and central nervous system in mice, rats, rabbits, and rhesus monkeys, which can be mimicked by other excitatory amino acids, but not non-excitatory amino acids, hence the term "excitotoxicity" (Lai et al., 2014). Interestingly, decades of research has also provided substantial evidence to indicate that excitotoxicity could contribute to neurological damage in both acute (e.g., stroke and head trauma) and chronic neurological diseases (e.g., amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease and AD) (reviewed in Lai et al., 2014, Lewerenz and Maher, 2015).

Glutamate is normally kept intracellular or cleared from the synaptic cleft following its release. However, spillage of glutamate from injured neurons, reduction in its clearance from the synaptic cleft, glutamate receptor abnormalities, and other factors cause exaggerated activation of glutamate receptors, causing neurotoxicity in many of such diseases (Nishizawa, 2001, Kritis et al., 2015). Years of research has deciphered

the molecular mechanism causing this toxicity. It occurs due to an exaggerated influx and intracellular accumulation of Ca^{2+} due to the effect of glutamate on post-synaptic neurons, by acting on both ionotropic and metabotropic glutamate receptors, with initial insult mediated by the ionotropic receptors. The Ca^{2+} storm causes the breakdown of cellular homeostasis by the activation of proteases which leads to the degradation of cellular substrates, such as cytoskeletal proteins and metabolic enzymes. It also results in the aberrant activation of kinases, induction of lipid peroxidation, mitochondrial dysfunction, upregulation of neuronal nitric oxide synthase (nNOS), oxidative stress, lysosomal leakage, and aberration of several other signal cascades that culminates to distorted molecular harmony, leading to DNA damage and cell death (Fig. 1.4) (Didier et al., 1996, Mattson, 2003, Mark et al., 2001, Kritis et al., 2015). An excessive amount of glutamate could cause oxidative stress by inhibiting the uptake of cysteine through the glutamate/cystine antiporter, which is an essential component of the antioxidant defence system required for the synthesis of glutathione, this leads to the decrease in intracellular glutathione and sustained oxidative stress which in turn could make neurons more vulnerable (Sato et al., 2005, Murphy et al., 1989).

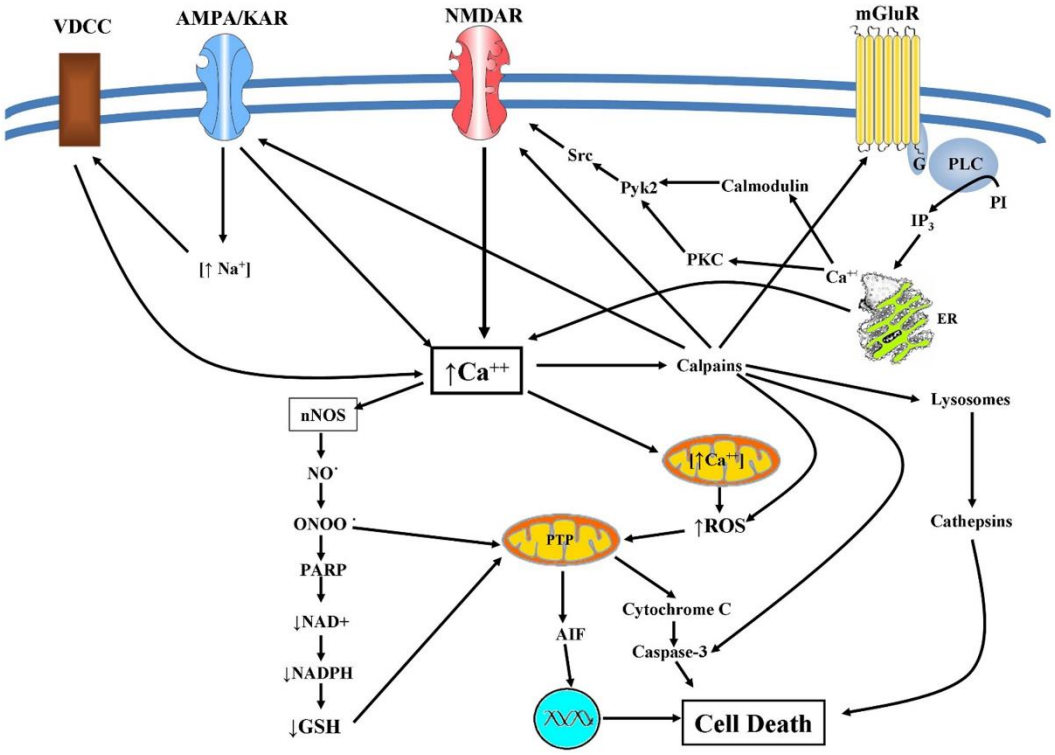

Figure 4.4 Mechanism of glutamate excitotoxicity.
Taken from (Kritis et al., 2015). The increase in Ca^{2+} induced by glutamate's impact on its receptors induces nitric oxide (NO) production, mitochondrial dysfunction, PARP activation, nicotinamide adenine dinucleotide (NAD) depletion and downregulation of antioxidant enzymes such as Glutathione (GSH). This insult also leads to the production of peroxynitrite, leading to lipid peroxidation, activation of proteases which leads to the degradation of cellular substrates, such as cytoskeletal proteins and metabolic enzymes and protein dysfunction. Oxidative stress and sustained increase in mitochondrial Ca^{2+} can both induce mitochondrial permeability transition pore (PTP) opening, loss of ionic homeostasis, matrix swelling, cytochrome c release and caspase activation leading to cell death. The cell death could also occur via a different mechanism involving apoptosis-inducing factor (AIF) translocation to the nucleus to induce DNA fragmentation. Accumulation of misfolded proteins and depletion of endoplasmic reticulum (ER) Ca^{2+} storage can result in ER stress and activation of unfolded protein response (UPR), which can also drive cell death via protein synthesis inhibition. The Ca^{2+} overload can also lead to the activation of Calpains, resulting to both calpain dependent and cathepsin-dependent cell death. Glutamate also impacts on metabotropic glutamate receptor leading to the modulation of NMDARs through via a Src-dependent mechanism in a PKC or calmodulin-dependent manner.

Many reports have revealed that the AD brain shows the signature of excitotoxic neurodegeneration (Ong et al., 2013). The human APP mouse model of AD shows premature death partly due to excitotoxicity (Roberson et al., 2007). Aβ toxicity, rather than plaque deposition has been implicated in excitotoxicity in the TgCRND8 AD mouse model which carries the APP Swedish and Indiana mutations (Del Vecchio et al., 2004). Indeed, different studies indicated that Aβ oligomers could induce

excitotoxicity through different pathways, including the stimulation of glutamate release or inhibiting its uptake and alteration of signalling pathways related to activation of glutamatergic receptors. For instance, Aβ oligomers have been shown to stimulate glutamate release and its extracellular accumulation in both primary neurons (Brito-Moreira et al., 2011) and astrocytes (Talantova et al., 2013). The infusion of soluble Aβ to the rat magnocellular nucleus basalis (MBN) was shown to compromise the neurons of the MBN and the cholinergic fibres to the neocortex through a mechanism that involved extracellular glutamate accumulation, NMDAR activation, astroglial depolarization and an intracellular Ca^{2+} overload leading to cell death (Harkany et al., 2000). Ittner et al. (Ittner et al., 2010) showed that one mechanism of the Aβ-induced excitotoxicity is via the interaction and subsequent anchorage of FYN kinase by tau protein to the postsynaptic density, enhancing the phosphorylation of the NMDARs to cause downstream excitotoxicity. All these show that glutamate excitotoxicity is one pathway through which Aβ could perform its atrocities in AD.

1.1.3 Amyloid beta oligomers as the neurotoxic species

The current hypothesis is that soluble Aβ species, especially oligomers, rather than plaques, are the culprit behind the toxicity of Aβ in AD, even though a consensus is lacking about the exact chemical nature and specification of these soluble species (Benilova et al., 2012). Along this line, in cultured neurons and the mouse brain, oligomeric Aβ has been shown to perform many atrocities, including the induction of dystrophic neurites, dendritic simplification, and dendritic spine loss via the activation of calcineurin and nuclear factor of activated T cells (Wu et al., 2010). Oligomeric species of Aβ have been found to induce excitotoxicity (Fuchsberger et al., 2016), to be involved in the impairment of LTP via an excessive activation of extrasynaptic NR2B-

containing NMDARs (Li et al., 2011), and implicated in synaptic dysfunction and mitochondrial aberration (Reddy and Beal, 2008). In the double-transgenic APP^{swe}-Tau mouse, neuronal loss and activated astrocytes in the entorhinal cortex and the CA1 hippocampal subfield were found to correlate with the burden of Aβ oligomers (DaRocha-Souto et al., 2011). In human AD, soluble Aβ also correlates positively with the severity of dementia (McLean et al., 1999, Walsh and Selkoe, 2007). With the onset of the accumulation of Aβ oligomers, the novel AD mouse model - PS1V97L-Tg expressing the human PS gene with the V97L mutation, show synaptic alteration, tau hyperphosphorylation, and glial activation, hence supporting an early role for this Aβ species and their role in neurotoxicity (Zhang et al., 2014). In trying to understand further mechanisms of Aβ toxicity, work from the Serpell group had demonstrated how oligomeric Aβ42 become internalised to cause lysosomal leakage in neuroblastoma cells, alter synaptic function in primary hippocampal neurons and upon addition, impair learning and memory in the pond snail *Lymnaea stagnalis* (Soura et al., 2012, Marshall et al., 2016). These all indicate the deleterious role of soluble Aβ, rather than plaques, in confirmation of its position in the revised amyloid cascade hypothesis (Zhu et al., 2011, Selkoe and Hardy, 2016). Although the plaques may not be completely non-toxic, they may contain around them an equilibrium of both toxic oligomers and inert fibrils which may "spill over" to surrounding tissues to cause neuronal damage, and/or they may mediate toxicity by triggering neuroinflammation (Benilova et al., 2012).

However, the prominent position given to Aβ in the cascade hypothesis may only partly be right for the early-onset AD and not late-onset AD, which seems to arise due to a combination of environmental, genetic and idiopathic factors, dissociated from APP. The strongest risk factor for the late-onset AD is the ApoE-4 allele (Liu et al.,

2013). Recent genome-wide associated studies (GWAS) have identified more genes that pose low risk to AD (Lambert et al., 2013), and importantly, a variant of the triggering receptor expressed on myeloid cells 2 gene *(*TREM2*)* has been identified as a major risk factor for the late-onset AD (Guerreiro et al., 2013, Jonsson et al., 2013). The GWAS studies and other studies on AD employing transcriptional profiling, clearly reveal the complexity of the disease, beyond Aβ (Morris et al., 2014). Coupled with the recent clinical failures of amyloid cascade hypothesis targetted treatment (Morris et al., 2014), this has partly led to increased interest in the involvement of tau protein, which constitutes the other hallmark of AD. Tau is now considered by many in the field to be important in the causation of neurodegeneration in AD pathology; as such, interests are rising on the potentials of targeting it as a drug target (Brier et al., 2016, Götz et al., 2012). Therefore, we next review tau protein and evidence implicating it in AD.

1.2 Tau protein gene, transcripts, isoforms, and localisation

Tau (tubulin-associated unit) is a small molecular weight protein, first identified by Weingarten *et al.* with a capacity to promote microtubule assembly *in vitro* (Weingarten et al., 1975). It is expressed in higher eukaryotes and found in both neuronal and non-neuronal cells, but predominantly in neurons (Rossi et al., 2008, Loomis et al., 1990, Stoothoff and Johnson, 2005, Martin et al., 2011). Besides its widely known role in microtubule assembly and stability, tau has become known for many other functions, such as the maintenance of axonal transport and providing linkage for signal transduction (Buee et al., 2000, Martin et al., 2011, Dixit et al., 2008, Ittner et al., 2010). Tau is a product of the microtubule-associated protein (MAPT) gene, located on chromosome 17q21.1 (Neve et al., 1986, Andreadis et al., 1992, Andreadis, 2005). A complex post-transcriptional processing of the tau transcript

yields predominantly three transcripts: a less abundant 2kb tau transcript which encodes for a tau mainly targeted to the nucleus (Wang et al., 1993); 6kb transcript which encodes for tau predominantly directed to the soma/axons in the CNS (Andreadis, 2005, Liu and Götz, 2013); and 8/9 kb transcript producing a tau preferentially expressed in the retina and PNS and with apparent molecular weight of about 110–120 kDa, often called high molecular weight tau (Georgieff et al., 1993, Nunez and Fischer, 1997). The 8/9 kb transcript arises from the inclusion of exon 4A from the tau gene during tau pre-mRNA processing. The 2 kb and 6kb transcripts result from the same pre-mRNA polyadenylated on different sites, with the 2 kb transcript having poly-A tail addition about 3.5 kb before that of the 6 kb transcript. This may be responsible for the different preferential localisation of their products and may impact on their function and stability (Sadot et al., 1994, Behar et al., 1995, Gupta et al., 2014, Nunez and Fischer, 1997).

The tau gene has 16 exons (Fig. 1.5), of which, exon 2, 3, 4A, 6, 8, 10 and 14 are alternatively spliced. Theoretically, splicing of this gene could yield up to 30 different variants of tau protein, thus creating an additional layer of complexity to the distribution of tau in different tissues (Andreadis, 2005, Andreadis et al., 1992, Georgieff et al., 1993, Luo et al., 2004, Shea and Cressman, 1998). The alternate splicing of exon 2, 3 and 10 generates the six widely known isoforms of tau in the CNS, ranging from 352–441 amino acids in length and 48–67 kDa on SDS-PAGE (Fig. 1.5) (Buee et al., 2000, Martin et al., 2011). The smallest isoform is found in the foetal brain, expressing three microtubule-binding repeats on its C-terminal (3R) and zero N-terminal inserts, and is called foetal tau. The other five isoforms are larger and predominantly found in the adult brain, having either three or four (3R/4R)

microtubule binding repeats and the presence or absence of one or two (1/2) N-terminal inserts (Buee et al., 2000).

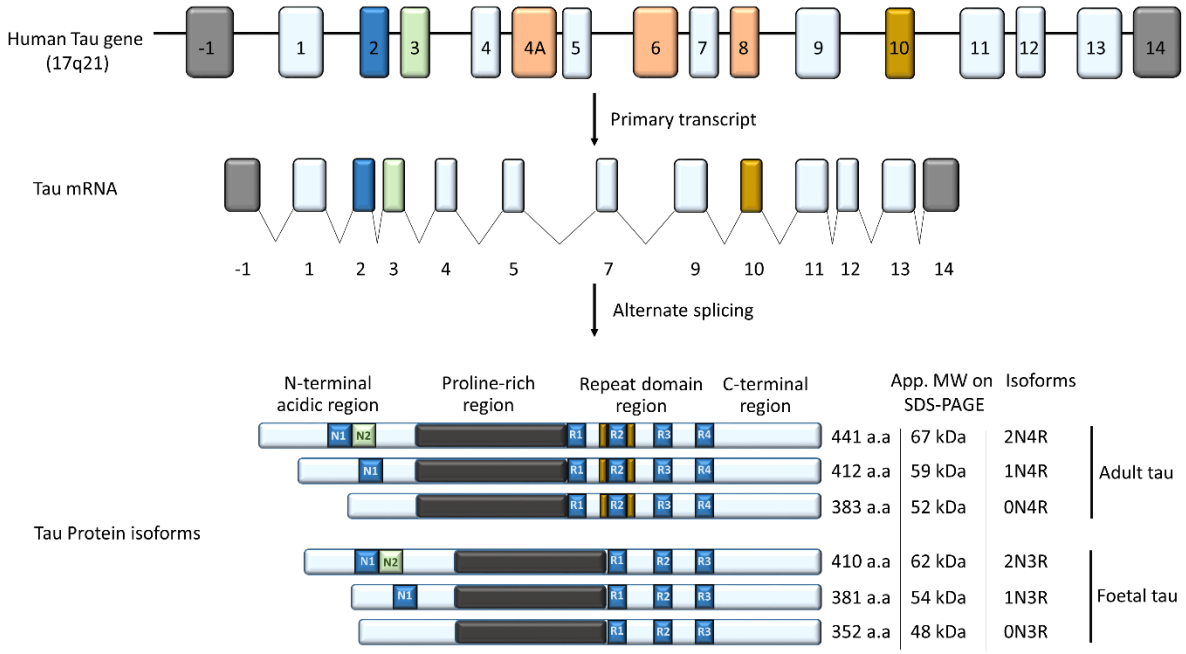

Figure 5.5 Tau gene, primary transcript, protein isoforms, and structure.
The tau gene has 16 exons; exon 1, 4, 5, 7, 9, 11, 12 and 13 (light blue) are constitutively transcribed in the CNS (Martin et al., 2011). Exon 4A, 6 and 8 (orange) are rarely expressed in the brain but included in mRNA of most peripheral tissues, while exon 14 forms part of the 3′ untranslated region of the tau mRNA (Andreadis, 2005, Connell et al., 2005). Alternate splicing of exon 2 (blue), 3 (Green) and 10 (Yellow) in the CNS generates the widely known six isoforms of tau; 352–441 amino acids in length and 48–67 kDa on SDS-PAGE (Martin et al., 2011). Depending on the inclusion and/or exclusion of exon 2, 3 and 10, tau have zero, one or two (0/1/2) N-terminal inserts and three or four (3R/4R) microtubule binding repeats, leading to the six isoforms of tau in the CNS. Structurally, the tau molecule is subdivided into four regions; an N-terminal acidic region; Proline-rich region/domain (PRD), repeat domain region and a C-terminal region.

Structurally, tau is subdivided into four regions; an N-terminal acidic region; a proline-rich domain (PRD), microtubule-binding repeat domain region (MBD) and a C-terminal region, and the epitopes across these areas vary depending on the tau isoform (Buee et al., 2000, Martin et al., 2011). Isoform localisation preference also exists between developmental stages, tissues, cell lines, brain regions and intracellular compartments (Luo et al., 2004, Shea and Cressman, 1998, Wang et al., 1993, Nunez and Fischer, 1997, Cross et al., 2000, Liu and Götz, 2013). For instance, in the murine

brain, 1N tau isoforms are overexpressed in the pituitary gland, compared to the cortex or hippocampus and underexpressed in the olfactory bulb. The 2N isoforms are more enriched in the cerebellum compared to any other brain region, but underexpressed in the olfactory bulb while the 0N isoform showed the highest expression in the olfactory bulb followed by the cortex (Liu and Götz, 2013). Intracellularly, in the murine brain, the 1N isoforms predominate in the nuclear fraction, the 0N isoforms predominate in the cell body/axons (Liu and Götz, 2013); while in human cells, like SHSY5Y neuroblastoma cells, high and low molecular weight tau both exist, and tau may predominantly localise to the nucleus or cytoplasm depending on whether the cells are differentiated or not (Uberti et al., 1997).

1.2.1 Tau protein in Alzheimer's disease

It is clear that the complex post-transcriptional processing of the tau message yields many isoforms with different localisation. However, tau researchers have placed more focus on the tau localised to the axons probably due to its well-known role in microtubule stability and dynamics, axonal transport and involvement in tauopathies. In AD, tau misfolds to form paired helical filaments (PHF) which are deposited in tangles (Martin et al., 2011), which together with plaques, are the principal hallmarks of the disease as described by Alois Alzheimer (Alzheimer et al., 1995). Electron microscopy reveals that PHFs are made up of a twisted ribbon-like structure, whereby two filaments twist around one another (Crowther and Wischik, 1985). Structurally, both tau filaments from human brain and from *in vitro* assembly of recombinant tau protein have cross-β structure, comprised of packed β-sheets, with the β-strands perpendicular to the fibre axis (Berriman et al., 2003, Giannetti et al., 2000). However, the exact mechanism and trigger for tau assembly into PHFs are still not well

understood. Many post-translational modifications have been proposed as key molecular events in the abnormal tau aggregation leading to the formation of PHFs.

Although, tau is known to be post-translationally modified in a variety of ways; glycosylation, glycation, truncation, prolyl-isomerisation, polyamination, nitration, oxidation, ubiquitination, sumoylation, and phosphorylation; the most studied thus far is tau phosphorylation (Martin et al., 2011, Guo et al., 2017). Eighty phosphorylation sites have been described on the longest isoform of tau driven by proline-directed protein kinases (PDPK) (e.g., GSK3), cyclin-dependent kinases (e.g. cdk5), non-proline directed protein kinases (e.g. PKA, CaMK II and CK II) and tyrosine-specific protein kinases (e.g. Src family of kinases (e.g. Src and Fyn). However, protein phosphatases (PP) are also important regulators of the tau molecule. Indeed, it has been proposed that a balance between kinases and phosphatases help in regulating the activity of tau and aberration in this equilibrium through either downregulation or inactivation of phosphatases relative to the kinases and vice versa could exacerbate AD by reducing tau dephosphorylation and increasing its phosphorylation (Trojanowski and Lee, 1995, Sontag et al., 1999). Indeed, it was found that GSK3β is up-regulated while PP2A is downregulated in the TgCRND mouse model of AD (Nicolia et al., 2010).

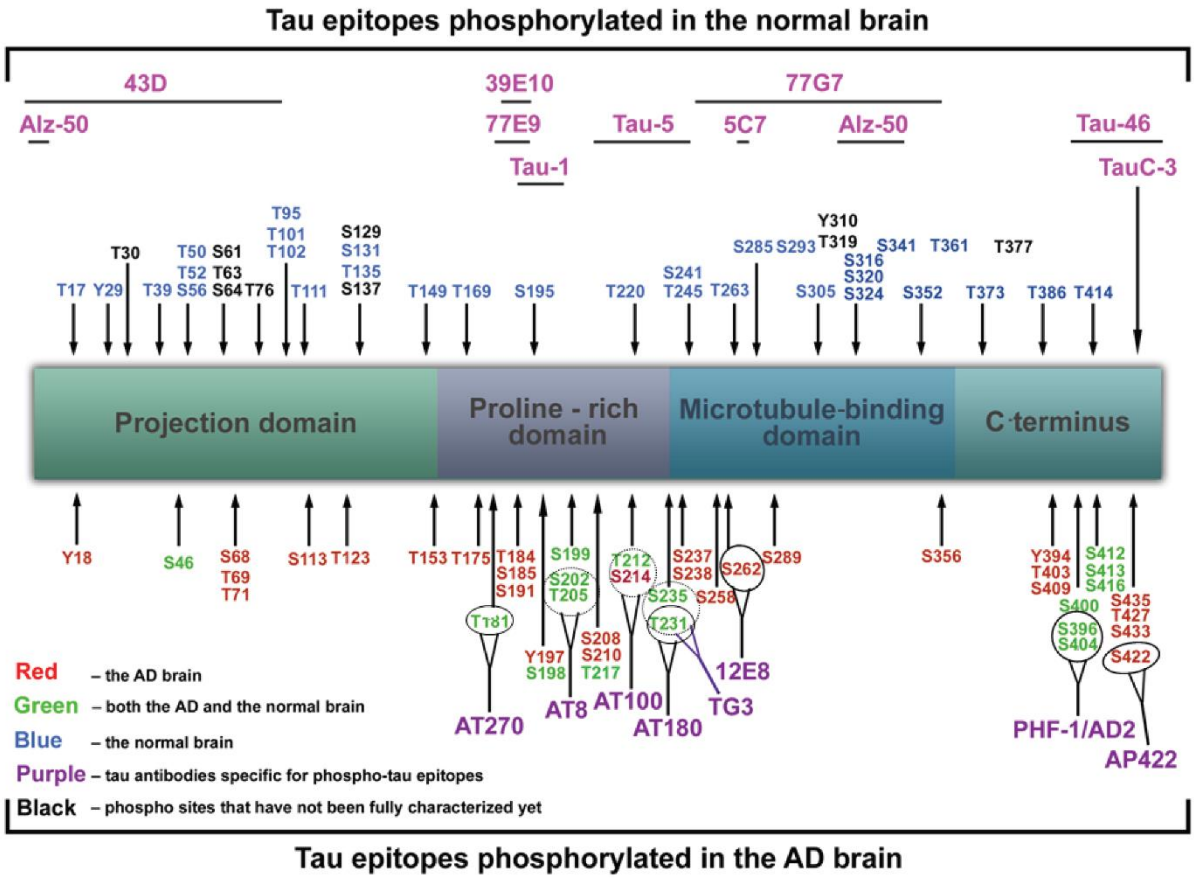

Figure 6.6 Putative phosphorylation sites on human tau protein.
Taken from Simic et al. (Simic et al., 2016), showing tau phosphorylation sites based on the longest isoform of human tau, with phosphorylation sites only found in AD (red), in normal brain (blue), in AD and non-AD brain (green) and those that have not been proven to be phosphorylated both in vitro and in vivo (black). Purple denotes tau antibodies specific for phospho-tau epitopes.

Many residues on the tau molecule (e.g., Ser 202, Ser 396, Ser 404, Thr 181, Thr231, Ser 235, and Ser 262) are abnormally phosphorylated in PHF (Fig. 1.6). The abnormal phosphorylation of tau on such residues, especially, S262, S293, S324 and S356 that are located on the KXGS motif of R1, R2, R3 and R4, reduces its affinity for binding to the microtubules and therefore reduces the number of microtubule-bound tau molecules (Martin et al., 2011). The destabilisation of the microtubules may lead to its depolymerisation, alter axonal transport and contribute to neurodegeneration – this has been one of the main arguments for the tau hypothesis of AD (Dixit et al., 2008, Trojanowski and Lee, 2005). The detachment of tau from the microtubules could also

lead to an increase in free soluble tau species that could aggregate to form dimers, then oligomers, which constitute subunits of filaments called protomers, that form PHFs and tangles (Martin et al., 2011, Buee et al., 2000). The PHFs could also promote the neuronal degeneration by sequestering normal tau and blocking anterograde and retrograde axonal transport to the synapse (Trojanowski and Lee, 1995).

While much attention has focused on hyperphosphorylation as a mechanism to induce the self-assembly of tau, an alternative hypothesis argues that tau truncation is the trigger of tau self-assembly (Spillantini and Goedert, 2013, Alonso et al., 2001, Novak et al., 2012, Kovacech and Novak, 2010). Many studies have proposed that tau phosphorylation is associated with tau assembly into PHFs (Alonso et al., 2001, Spillantini and Goedert, 2013). However, these studies have been based upon *in vitro* experiments and animal models, and so it has been argued that further supporting evidence is required using human AD samples. It has been shown that the core of PHFs contains truncated forms of tau protein (Wischik et al., 1988). Novak *et al.* demonstrated that truncation is mediated by specific cleavage events *in vivo* (Zilka et al., 2012, Novak et al., 2012). Furthermore, it has been suggested that truncated tau can serve as a nucleus for the assembly of endogenous tau into neurofibrillary tangles (Zilka et al., 2006). To understand and gain more insight into the relationship between phosphorylation and truncation, a more recent immunohistochemical study of AD brain tissue revealed that truncated tau represents an early neurotoxic form and proposed that phosphorylation may play a neuroprotective role by inhibition of tau aggregation (Flores-Rodriguez et al., 2015) or Aβ toxicity in AD (Ittner et al., 2016). Truncation of tau by protease (e.g. calpain-2) could also enhance its aggregation and might lead to further modifications such as glycation (Martin et al., 2011), which could induce oxidative stress via the production of reactive oxygen species (ROS) via the

formation of AGEs (Yan et al., 1994). Glycation can also enhance the aggregation of tau and therefore promote the transition of tau aggregates to tangles (Ledesma et al., 1996). It appears that the tau molecule is vulnerable to various modifications that could promote neurotoxicity and neurodegeneration in AD (Martin et al., 2011).

1.2.2 Tau as a disease driver of neurodegeneration in tauopathies.

Although in AD, the aberration of tau could not be the trigger of the disease, tau alone can cause neurodegenerative diseases. In 1998 three groups reported the discovery of tau pathology arising from tau mutations causing frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Hutton et al., 1998, Poorkaj et al., 1998, Spillantini et al., 1998). FTDP-17 is a neurodegenerative disease that often affects personality, behaviour, language, movement, and cognition. It belongs to a group of disorders involving tau which are called frontotemporal dementia (FTD) also including pick disease, corticobasal degeneration, progressive supranuclear palsy, sporadic multiple system tauopathy with dementia, argyrophilic grain disease, and neurofibrillary tangle dementia. FTD is characterised by progressive neuronal loss predominantly involving the frontal and temporal lobes and mostly affects people below 65 years of age (Cardarelli et al., 2010).

Other proteins, such as TAR DNA-Binding protein 43 (TDP-43) and Fused in Sarcoma (FUS) are also involved in some cases of FTD (Cairns et al., 2007, Mackenzie et al., 2006). However, the finding of tau mutations causing FTDP-17 provided strong evidence that tau could exclusively cause neurodegenerative diseases and that it could contribute to neurodegeneration in AD. Over 50 different tau mutations have now been identified, some showing no pathogenicity, a full list of these mutations can be accessed on the AD&FTLD and PD mutation databases (http://www.molgen.vib-

ua.be/FTDMutations/) (Cruts et al., 2012). The pathogenic tau mutations show a different degree of effect on tau at the protein or RNA level, with varying effect on its microtubule-binding ability and aggregation (Table 1) (Goedert and Jakes, 2005, Wolfe, 2009). Three mutations constitute about 60% of known cases of tau mutation; the P301L and N279K mutations and a splice site mutation (exon 10 +16) (Wszolek et al., 2006), all exhibiting mainly constituting of 4R tau (See Table 1). Some mutations, such as G272V and V337M produce tau molecules that are more favourable substrates for phosphorylation by kinases compared to wild-type tau (Alonso et al., 2004) and this changes can drive the formation of tau aggregates (Alonso et al., 1996). *In vitro* evidence using heparin or arachidonic acid to induce tau aggregation revealed that some mutations increase tau's propensity to assemble. This is particularly marked for P301L and P301S mutations (Goedert and Jakes 2005). Paradoxically, some mutations, such as Q336R, slightly increase tau's capability to promote microtubule assembly (Table 1). All these indicate that most of the pathogenic tau mutations promote neurodegeneration by altering the normal function of tau, such as its microtubule-binding function, increasing pathologic tau species and enhancing tau phosphorylation and aggregation.

Table 1 Tau mutations, isoforms affected and effect on the microtubules (MT). Taken from (Liu and Gong, 2008)

Mutation	Location	E10 inclusion	MT-binding	Insoluble tau	Phenotype
R5L	Exon 1			Mainly 4R	PSP-like
R5H R	Exon 1			4R+1N3	AD-like
K257T	Exon 9		↓	3R > 4R	PiD-like
I260V	Exon 9			Mainly 4R	
L266V	Exon 9	↓	↓	Mainly 3R	PiD-like
G272V	Exon 9	→	↓	Mainly 3R	PiD-like
E9+33	Intron 9	↓			
N279K	Exon 10	↑	Variable	Mainly 4R	PSP-like
Δ280K	Exon 10	↓	↓	3R>>4R	FTDP-17
L284L	Exon 10	↑	→	4R?	AD-like
N296N	Exon 10	↑	→	Mainly 4R	CBD-like
N296H	Exon 10	↑		Mainly 4R	FTDP-17
Δ296N	Exon 10		↓		PSP-like
P301L	Exon 10	→	↓	Mainly 4R	FTDP-17
P301S	Exon 10	↑		Mainly 4R	FTDP-17, CBD-like
G303V	Exon 10	↑		Mainly 4R	PSP-like
S305N	Exon 10	↑	→	Mainly 4R	CBD-like
S305S	Exon 10	↑		Mainly 4R	PSP-like
S305I	Exon 10	↑		Mainly 4R	AGD
E10+3	Intron 10	↑	→		FTDP-17
E10+11	Intron 10	↑	→		FTDP-17
E10+12	Intron 10	↑	→	Mainly 4R	FTDP-17
E10+13	Intron 10	↑	→		FTDP-17
E10+14	Intron 10	↑	→	Mainly 4R	FTDP-17, PSP-like
E10+16	Intron 10	↑	→	Mainly 4R	PSP/CBD-like
E10+19	Intron 10	↓	→		
E10+29	Intron 10	↓	→		
L315 R	Exon 11	→	↓		PiD-like
L315L	Exon 11		→		
S320F	Exon 11	→	↓		PiD-like
S320Y	Exon 11				PiD-like
Q336R	Exon 12	→	↑		PiD-like
V337M	Exon 12	→	↓		FTDP-17
E342V	Exon 12	↑		Mainly 4R	FTDP-17, PiD-like
S352V	Exon 12				
K369I	Exon 12			3R + 4R	PiD-like
G389R	Exon 13	→	↓	4R > 3R	PiD-like
R406W	Exon 13	→		3R + 4R	PSP-like

↑ increased; ↓ decreased; → unchanged.

Apart from tau mutations, tau gene polymorphism has been linked with the development of tauopathies. The H1 haplotype of the tau gene which is comprised of single nucleotide polymorphism shows increased risk of progressive supranuclear palsy (Pittman et al., 2004), Parkinson disease (Kwok et al., 2004, Zabetian et al., 2007) and AD (Myers et al., 2005). Interestingly, an aberration in tau protein has also been reported in other diseases, such as postencephalitic parkinsonism and amyotrophic lateral sclerosis/Parkinson–dementia complex (Ludolph et al., 2009), temporal lobe epilepsy and chronic traumatic encephalopathy (Puvenna et al., 2016). All these evidence support a role for tau in driving neurodegeneration in many tauopathies and strongly supports a causal role for tau pathology in AD progression.

1.3 Beyond Amyloid beta and Tau: Nuclear factories altered in Alzheimer's disease

Although tau mutations cause some forms of tauopathies (Table 1), not AD, and the amyloid cascade hypothesis placed Aβ upstream of tau, the relationship between the two giants can best be described as cooperative (Rhein et al., 2009). Several cell culture and animal studies have shown the capacity of Aβ to induce changes in tau protein. For instance, Aβ induces tau dissociation from the microtubule (King et al., 2006), induces tau phosphorylation in cultured neurons, animal models with mouse or wild-type human tau (Zheng et al., 2002, Zhang et al., 2014, Guo et al., 2013). However, Aβ cannot achieve its mischief without tau. Tau-depleted neurons are protected from Aβ-induced neurotoxicity (Rapoport et al., 2002), the reduction of endogenous tau in an AD mouse model improved their cognitive deficit induced by Aβ (Roberson et al., 2007), tau gene knockout prevented Aβ-induced LTP impairment (Shipton et al., 2011), tau phosphorylation is critical for Aβ-induced neurotoxicity in a transgenic *Drosophila* model of AD (Iijima et al., 2010) and tau is required for Aβ-induced

excitotoxicity in APP23 mouse model (Ittner et al., 2010). Both seem to be important and apparently cooperate to cause insults to neurons in AD. Based on what has been reviewed thus far, it is also evident that in AD, both Aβ toxicity and tau aberration can promote neurodegeneration. In the AD field, much focus has been on synaptic impairment, oxidative stress, and neuroinflammation as the key pathological signatures of the disease. It appears that some pathways that are potentially important for understanding the disease are neglected or yet to be explored. Finding these molecular factories affected early in the disease, induced by Aβ toxicity, non-Aβ AD factors or tau dysfunction would provide alternate avenues that would enhance our understanding of the disease and provide a possible pathway to drug discovery. This is the case for protein synthesis restoration, which has been shown to alleviate neurodegeneration in a mouse model of tauopathy and prion disease (Halliday and Mallucci, 2015, Halliday et al., 2017). Several studies have implicated same protein synthesis pathway in AD (Smith and Mallucci, 2016, Hoozemans et al., 2009, Hoozemans et al., 2005). However, protein synthesis lies downstream of nucleolar ribosomal DNA (rDNA) transcription and depends on the availability of RNA, which in turn depends on chromatin's transcriptional output. Interestingly, nucleolar stress and chromatin alteration have both been implicated in AD. Some forms of tau protein have been localised in the nucleus and nucleolus and may serve as a link between the nucleolus and the heterochromatin (Bukar Maina et al., 2016). In light of this, it is important to assess the involvement of the nucleolus and heterochromatin in AD and explore the link between Aβ toxicity and tau in the context of the nucleolus and chromatin.

1.3.1 The Nucleolus

The nucleolus is a molecular factory, easily recognisable under both light and electron microscopes, which lies within the nucleus of the eukaryotic cell, not surrounded by a membrane and serving as the most active site of transcription within the nucleus (Németh and Längst, 2011). First reports describing the nucleolus were published in the 1800s. Specifically, 1835, 1836 and 1839 and it took nearly a century when it was found that the nucleolus forms around the nucleolar organiser regions (NORs) (McClintock, 1934, Pederson, 2011). Subsequently, it was discovered that the nucleolus forms around the short arm of the acrocentric chromosomes; 13, 14, 15, 21, and 22. This remarkable structure is a host for many proteins, RNAs, but mainly comprised of tandemly repeated DNA separated by non-transcribed spacer DNA (Fig. 1.7). This DNA was shown in the 1960s to hybridise with the ribosomal RNA (rRNA), leading into a new age for research on the nucleolus (Pederson, 2011). Indeed, the acrocentric chromosomes have multiple copies of the 18S, 5.8S and 28S rRNA genes.

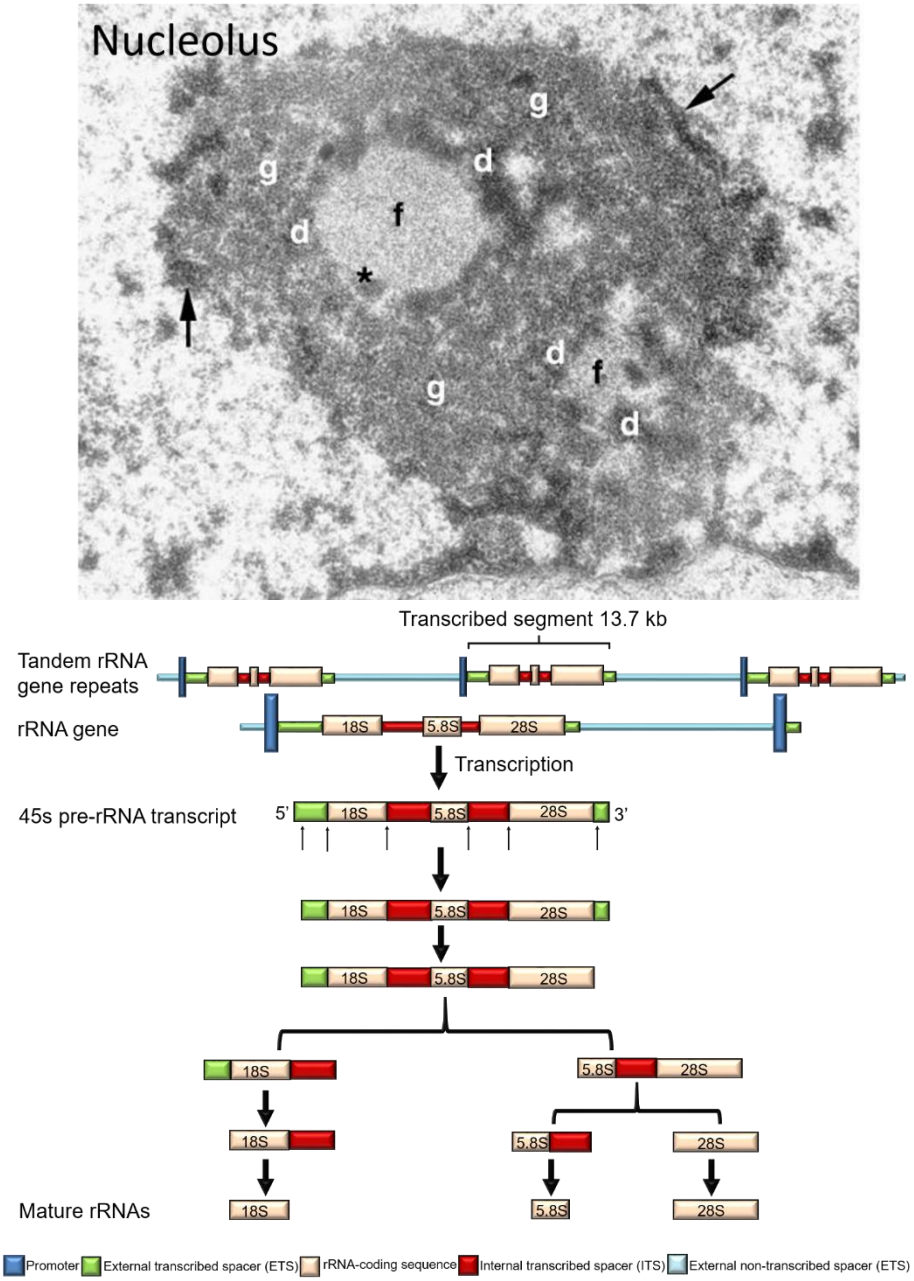

Figure 7.7 The organisation of the nucleolus and rRNA synthesis and processing.
(Top) Adapted from (Raška, 2003) showing a transmission electron micrograph of a mouse fibroblast, black arrow indicating nucleolus-associated heterochromatin; f, d and g show FC (fibrillar centre), DFC (dense fibrillar component), GCs (granular components), respectively. Asterisk (*) indicates the presence of DFC within the FC. (Bottom) Schematic to describe the tandemly repeated rRNA genes, which upon transcription yield 45S pre-rRNA, containing 18S, 5.8S, and 28S rRNAs as well as transcribed spacer regions. Processing of the pre-rRNA begins during the transcription as a cleavage within the ETS near the 5′ end of the pre-rRNA. Upon the completion of transcription, the ETS at the 3′ end of the pre-rRNA is removed, followed by a cleavage at the 5′ end of the 5.8S region, yielding separate precursors for the 18S and 5.8S + 28S rRNAs. Subsequent cleavages result in the formation of the mature rRNAs.

Morphologically, the nucleolus is compartmentalised into the fibrillar centre (FC), dense fibrillar component (DFC) and granular component (GC) (Fig. 1.7). The rRNA genes are located in the FC, and one of the primary functions of the nucleolus is rRNA synthesis which occurs at the FC or the border of the FC and DFC, driven by RNA polymerase 1 (Cooper, 2000). Some rDNA are maintained in a repressive configuration, associated with repressive marks, such as DNA methylation, and this is facilitated by the nucleolar remodelling complex (NoRC) comprised of TIP5 (Transcription Termination Factor I-Interacting Protein 5) and Snf2H, the mammalian homolog of the ATPase ISWI (Santoro et al., 2002). The silencing activity of the NoRC helps in promoting stability of the rDNA. However, the rDNA that is transcriptionally permissive is associated with the RNA polymerase I transcription factor upstream binding factor (UBF) (Németh and Längst, 2011). The transcription of the rDNA leads to the production of a large precursor - 45s pre-rRNA, which becomes processed to 18S, 5.8S and 28S rRNAs (Fig. 1.7). The pre-rRNA processing is initiated at the DFC and continues in the GC, during which ribosomal proteins are recruited to the newly transcribed rRNA, leading to the formation of the 40S ribosomal subunit comprised of 18S rRNA and approximately 32 ribosomal proteins. A 60S subunit is also formed, consisting of 5S and 28S rRNAs, a 5.8S rRNA synthesised in the nucleoplasm, and approximately 47 ribosomal proteins. Both subunits subsequently become exported to the cytoplasm, where functional ribosome is formed. Interestingly, cells actively engaged in protein synthesis usually have large nucleoli, indicating that its size is regulated by the metabolic activity of cells (Cooper, 2000). Recent evidence has implicated it as a multifunctional hub involved in other functions, such stress response (Boisvert et al., 2007).

1.3.2 The Nucleolus in Alzheimer's disease

Since the early 1970s, nucleolar volume/size has been used as a readout of the metabolic activity or protein synthesis of cells. To investigate if this function is altered in AD, Dayan and Ball (Dayan and Ball, 1973) showed that in the cortices of the AD brain, tangle-bearing neurons had reduced nucleoli diameter compared to adjacent non-tangle bearing cells. A similar decrease in nucleolar volume was subsequently reported in the temporal cortex, cerebellum, pons and medulla oblongata of the AD brain (Mann and Sinclair, 1978, Mann et al., 1977, Mann et al., 1980, Mann et al., 1981b). Interestingly, this reduction in nucleolar volume was observed not just in tangle-bearing neurons, but even in non-tangle bearing neurons, indicating that the nucleolus was affected before the accumulation of tangles (Mann et al., 1981b). David Mann and colleagues subsequently published many articles in the 1980s, establishing that the nucleolus is indeed affected in AD (Mann et al., 1984a, Mann et al., 1984b). Reasoning that the nucleolus form around the acrocentric chromosomes, one of which is the APP gene carrying chromosome 21, Payao et al. subsequently investigated whether the NORs in AD were affected. Working with peripheral lymphocyte cultures from AD and control individuals, they found that the NOR around chromosome 21 is significantly reduced in AD (Payao et al., 1994), consistent with other studies that established a reduction in nucleolar volume in the disease. The decrease in NORs in AD was further shown in hippocampal neurons of the AD brain (Lu et al., 1998). All these indicate a decline in rRNA production, which would impact on ribosome production and protein synthesis. Indeed, it was subsequently demonstrated in peripheral blood cells from AD patients with moderate dementia that the disease leads to the reduction in the 28S/18S rRNA ratio (da Silva et al., 2000, Payao et al., 1998).

These early studies established that nucleolar dysfunction is a signature of AD occurring earlier before tangle formation. Indeed, the nucleolus is now known as a stress detector, and a prominent feature of "nucleolar stress" is the translocation of nucleolar proteins like nucleophosmin and fibrillarin and reduction of rDNA transcription. This stress mechanism is thought to be an early event to cellular dyshomeostasis, preceding apoptosis and such a stress mechanism occurs in neurodegeneration (Avitabile et al., 2011, Yang et al., 2016, Erickson and Bazan, 2013, Tsoi et al., 2012). Interestingly, Ding and colleagues subsequently found that ribosomal dysfunction and decreased levels of rRNA and transfer RNA occurs in the brains of people with mild cognitive impairment (MCI), indicating that changes in protein synthesis machinery in the disease precede neuronal loss (Ding et al., 2005). Redistribution and a decrease of many nucleolar proteins and their mRNA have been recently reported to occur progressively in AD (Hernandez-Ortega et al., 2015). Since early stage of the disease is associated with less neuronal loss (Derenzini et al., 2009, Greco, 2009), this seems to suggest that the rRNA production and processing become altered before the onset of full AD, progressing with the disease. One possible mechanism of such aberration could be due to oxidative stress, which is known to be a feature of AD. Accordingly, markers of rRNA oxidation have been found in neurons from the hippocampus, subiculum, and entorhinal cortex as well as frontal and occipital neocortex (Nunomura et al., 1999, Honda et al., 2005). Interestingly, rRNA oxidation was also observed in the ribosome complex from MCI and AD subjects (Ding et al., 2006), indicating that the oxidative damage to the rRNA precedes the neurodegeneration in AD. One possible reason for the reduction in rRNAs in the disease could be due to epigenetic silencing of the rDNA. Indeed, the rRNA genes are kept in a balance of transcriptionally active and silent configuration (Grummt, 2010).

Although no significant changes in rDNA methylation were observed in peripheral blood cells from AD patients (Sperança et al., 2008), a recent re-examination in a relevant tissue - parietal and prefrontal cortices, from both MCI and AD revealed a significant hypermethylation of the rDNA (Pietrzak et al., 2011). This again indicates that the hypermethylation occurs before overt neurodegeneration in AD. The methylation of gene promoter prevents the recruitment of transcriptional machinery or recruits proteins that inhibit gene expression (Moore et al., 2013). Thus, the hypermethylation of the rDNA promoter could be an additional mechanism leading to rRNA deficit observed in the MCI and AD brain (Ding et al., 2005). All these can result in the reduction of protein synthesis.

It is clear from the evidence reviewed above that nucleolar dysfunction is an early event in AD since the changes in the nucleolus begin in MCI. Moreover, these changes were not restricted to dying cells represented by tangles. Instead, even non-tangle bearing neurons show changes in nucleolar function (Mann et al., 1981b). So far, there is no direct evidence of the cause of these changes. They could arise due to Aβ toxicity that occurs during the disease, inflammation, tau toxicity, oxidative stress, or an entirely different mechanism. One of the key objectives of this thesis is to identify the culprit behind the nucleolar stress in AD. However, this goal would be deficient without an understanding of the heterochromatin. In fact, a discussion about the nucleolus would be incomplete without a similar discussion on the heterochromatin. This is due to the tight relationship between the two molecular factories (Németh and Längst, 2011). Proteins like TIP5, HP1α, H3K9me2, and H3K9me3 are constituents of both nuclear and nucleolar heterochromatin (Bártová et al., 2010, Harničarová Horáková et al., 2010, Saksouk et al., 2015). A layer of heterochromatin tightly wraps the nucleolus (Fig. 1.7), called the nucleolar-associated chromatin domains (NADs)

which were found to be comprised of different DNA sequences, such as alpha and beta-satellite repeats, transfer RNA genes and transcriptionally active 5S rRNA (Németh and Längst, 2011, Németh et al., 2010). Tau protein has been found to associate with some of these satellite repeats and the NADs (Sjoberg et al., 2006). The satellite sequences of the NADs are thought to contribute to the heterochromatin formation around the nucleolus (Németh and Längst, 2011), and heterochromatin is essential for the stability and function of the rDNA (Larson et al., 2012, Peng and Karpen, 2007). Therefore, we will next discuss the heterochromatin and its involvement in AD.

1.3.3 The Heterochromatin

In his seminal work of 1928, Emil Heitz published his observation that some areas of the nucleus do not change their degree of condensation during the cell cycle, compared to some other areas. Heitz termed those tightly condensed regions as the heterochromatin and those regions that become visible at late telophase as euchromatin (Passarge, 1979). Before his time, the chromatin has been described by Walther Flemming as the component within the cell that reacts with basic dyes (Trojer and Reinberg, 2007). Decades of research now show that the chromatin is a complex structure comprised of DNA, histone proteins and non-histone protein components. The heterochromatin is mostly a transcriptionally inactive region due to its low permissivity to transcription factors and RNA polymerases and usually localised around the nucleolus or nuclear lamina. In contrast, euchromatin constitutes an area with an open chromatin, accessible to transcription factors, hence allowing for transcription of genes. Recent evidence has further categorised the heterochromatin into facultative and constitutive domains. Based on the Latin word for opportunity - “facultas”, facultative heterochromatin is thought to be a dynamic heterochromatin domain that can adopt both open and closed conformation depending on molecular

cues, such as developmental cues (Trojer and Reinberg, 2007). The constitutive heterochromatin instead is considered more stably condensed and comprised of mostly long repetitive sequences, such as satellite DNA, which are rich in the centromere and telomeres forming centromeric and telomeric heterochromatin (Hughes and Hawley, 2009). Recent evidence in epigenetics revealed that the heterochromatin is marked by specific pattern of hypoacetylation and global methylation which lead to chromatin compaction and this can be identified with histone marks, such lysine methylation at different amino acids of the histones e.g., H4K20me1, H3K9me2, H3K9me3, and H4K20me3, driven by the lysine methyltransferases often called 'writers' (Saksouk et al., 2015). However, some of these markers further show preference to some chromatin domains. For instance, H3K9me2 is more enriched in the facultative heterochromatin (Trojer and Reinberg, 2007), while the heterochromatin proteins - HP1α and HP1β, H4K20me1 and H3K9me3 show more preference to the constitutive heterochromatin (Trojer and Reinberg, 2007, Saksouk et al., 2015). Of these, HP1, first discovered in *Drosophila*, is integral to heterochromatin formation. It is important for the establishment and propagation of the heterochromatin state through its interaction with different epigenetic marks, especially H3K9me3 (Fig. 1.8) (Saksouk et al., 2015, Lomberk et al., 2006). It is also required for the recruitment of SUV420H and other DNMTs, leading to H4K20me3 and DNA methylation (Saksouk et al., 2015). The bulk of constitutive heterochromatin is formed at pericentromeric regions (Saksouk et al., 2015), which are closely decorated with many centromere proteins, mainly CENP-A, which is an integral part of the centromere nucleosome that associates with other centromere proteins e.g. CENP-B and CENP-C (Fig. 1.8) (Dunleavy et al., Pidoux and Allshire, 2005, Ohzeki et al., 2016, Gonzalez-Barrios et al., 2014, Earnshaw, 2015, McKinley and Cheeseman,

2016). Interestingly, TIP5, a non-histone protein recognised for its role in silencing rDNA (Santoro et al., 2002), has emerged as an important player that associates, facilitates and regulates the formation and stability of the constitutive heterochromatin at centromeres and telomeres (Postepska-Igielska et al., 2013). These distinct proteins now serve as powerful markers used with many techniques (e.g. Chromatin immunoprecipitation, Fluorescence and Electron Microscopy) to identify these chromatin domains and their interacting partners.

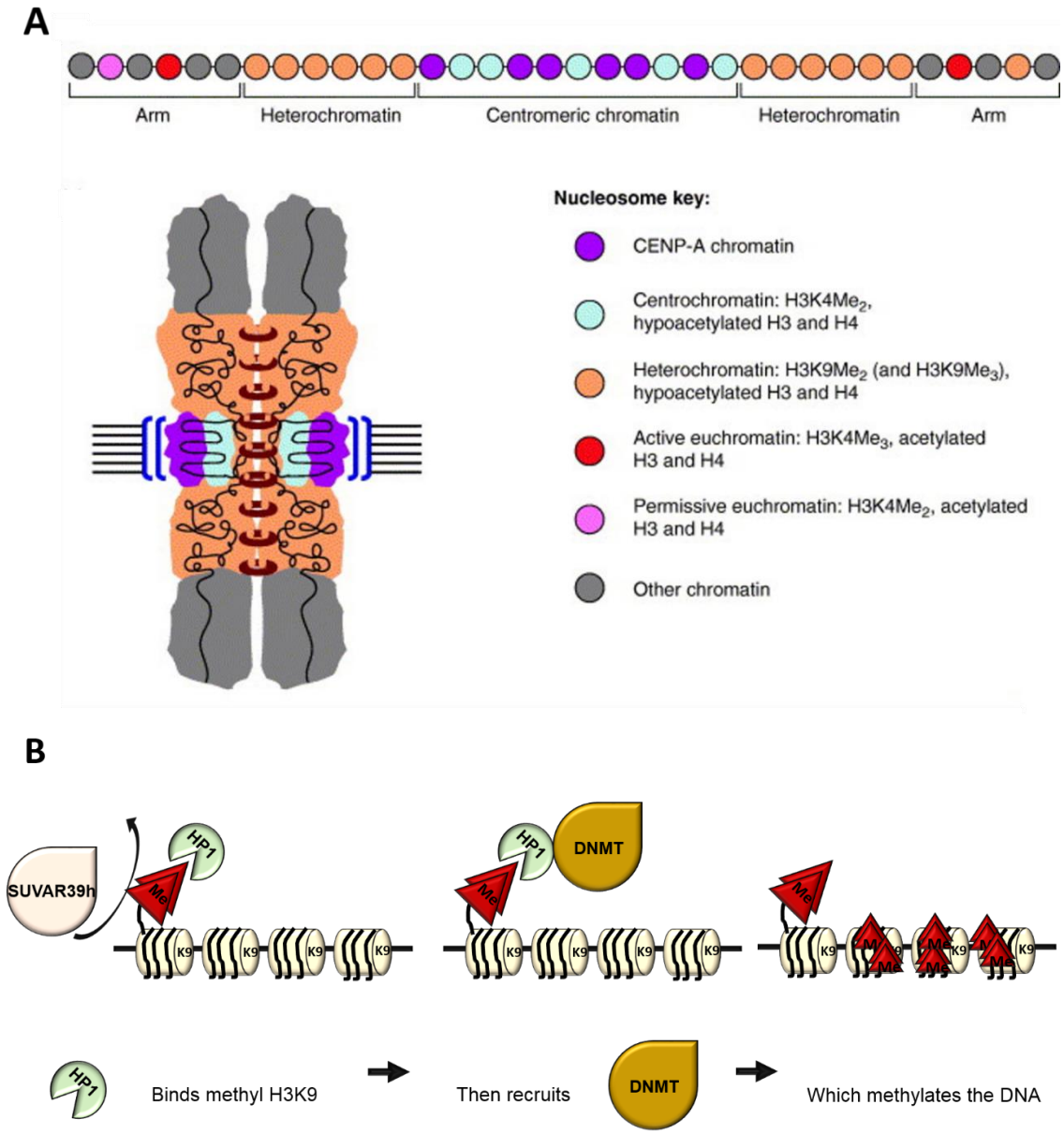

Figure 8.8. The organisation and formation of the heterochromatin.
(A) Taken from (Dunleavy et al.). CENP-A marks the centromeric chromatin domain (purple). Associated to the CENP-A chromatin are H3-containing nucleosomes, containing H3K4Me2 and hypoacetylated H3 and H4 N-terminal tails, forming the 'centrochromatin' (light blue). The centromeric heterochromatin marked by hypoacetylation of H3 and H4, and H3K9Me2 and H3K9Me3 flanks the centromeric domain (orange). The chromosome arms contain a mixture of heterochromatin, active euchromatin (red), permissive euchromatin (pink) and other chromatin (grey). (B) A simplified model of heterochromatin formation and spreading. The methyltransferase SUVAR39H methylates Lysine 9 of histone H3, which serves as a binding site for HP1, which in turn can recruit DNA methyltransferases to methylate the DNA and reinforce chromatin compaction and silencing.

1.3.4 The Heterochromatin and Alzheimer's disease

It is hard to trace the first report about a global aberration of the heterochromatin in AD, but a PUBMED search of published articles since 1906, date of the first description

of AD, revealed that Crapper and colleagues did the first comprehensive documentation of chromatin changes in AD (Crapper et al., 1979). Working on the reports that implicated reduced RNA level in AD and knowing that the chromatin is the home for RNA transcription, Crapper et al. quantified the changes in the chromatin from the nuclei isolated from the cerebral cortices of control and AD patients. Interestingly, this revealed a reduction in the quantity of euchromatin in the AD brain compared to control cortices. However, the decrease in the euchromatin from the same AD cortex showed variation between different regions, while those of the control brain was more consistent. They categorised the euchromatin into an intermediate euchromatin and light euchromatin, confirming the decrease in the euchromatin in the AD brain compared to the control brain. Crapper and colleagues further found that the AD-associated heterochromatinisation occurred in both neuron- and glia-enriched fractions and these changes were further associated with a decrease in neuronal cell size. Given the previously documented reduction in RNA content observed in the AD brain (Mann and Sinclair, 1978, Mann et al., 1981a), it was argued that this could be related to the increased heterochromatin formation in the AD brain, providing low accessibility of RNA polymerases to initiate transcription (Lewis et al., 1981).

Lewis and co-workers subsequently found that the chromatin from neurons and glia from the AD brain cortex provides less accessibility for micrococcal nuclease, an enzyme that cleaves between linker regions on the nucleosomes, further indicating enhanced heterochromatin formation in the AD brain (Lewis et al., 1981). Interestingly, the nuclei from the brains with fewer plaques and tangles showed moderate heterochromatinisation, compared to the brain with a higher number of these deposits (Lewis et al., 1981). Lewis et al. further found a significant increase in three linker histones in the AD brain (Lewis et al., 1981). In a subsequent study by McLachlan and

colleagues (McLachlan et al., 1984), micrococcal digestion of the chromatin from the AD brain was found to result in the liberation of dinucleosomes, particularly abundant in the methionine-containing histones H1.0 and non-methionine containing H1oo, thus confirming the previous observation that found an increase in three linker histones in the AD brain (Lewis et al., 1981). The increase in the linker histones was peculiar to the brain, as no such increase was found in the liver (McLachlan et al., 1984). H1.0 containing nuclear fraction has been found to associate with repressed genes (Lukiw and Crapper McLachlan, 1990, Roche et al., 1985). H1.0 is enriched in heterochromatin-associated regions like the repetitive sequences of rDNA (Hergeth and Schneider, 2015). Indeed, McLachlan et al. (McLachlan et al., 1984) showed that the histones from the AD brain were unusually resistant to salt-induced chromatin release compared to controls. Interestingly, these changes were found to be specific to the AD brain, as other forms of dementia such as Lewy body dementia and multi-infarct dementia show no such changes (McLachlan et al., 1984). In a DNA binding assay, the increased linker histones isolated from the AD brain were found to possess increased affinity to the DNA compared to the histones isolated from scrapie-affected brains (McLachlan et al., 1986). This indicates an enhanced preference for DNA binding and condensation in the AD brain.

Subsequently, the increased chromatin compaction was found around the neurofilament light chain gene, with an associated decrease in its mRNA (Lukiw and Crapper McLachlan, 1990). All these studies indicate a shift in chromatin arrangement in AD (McLachlan et al., 1991), which is associated with heterochromatinisation of the chromatin. These early studies provided a strong link between the RNA and protein deficits with the globally increased tightness of the DNA and nucleolar dysfunction in AD.

Although looking at changes at the individual gene level and brain regions, the pattern of changes for gene repression is highly variable and difficult to use as indices for mapping global chromatin changes associated with AD. In AD, some genes show more transcriptional activity, while others show reduced activity. Surprisingly, two recent studies on AD hippocampi using the heterochromatin epigenetic markers revealed chromatin relaxation in AD. Frost et al. showed that the pyramidal neurons from AD hippocampi undergo a significant reduction in HP1α and H3K9me2 with an associated increase in the transcription of the heterochromatic genes (Frost et al., 2014). Similarly, Hernandez-Ortega and co-workers demonstrated a gradual decrease in hippocampal H3K9me2 with AD progression (Hernandez-Ortega et al., 2015). Results from the 3xTg AD mouse model showed an increase in H3K9 methylation with ageing (Walker et al., 2013). While the levels of H3K9me3 in the p25/cdk5 AD mouse model revealed no substantial difference in the heterochromatin (Gjoneska et al., 2015). Based on the epigenetic markers used, these studies show conflicting results on the heterochromatin status of the AD brain, which needs to be addressed by future studies (Sanchez-Mut and Gräff, 2015). The majority of the early studies showed the enhancement of chromatin compaction in AD, in contrast to the recent studies showing heterochromatin loss. These differences could indicate region-specific changes in the heterochromatin associated with the AD pathology, for the following reasons. 1) Findings of heterochromatin loss by Frost et al. (Frost et al., 2014) and Hernandez-Ortega et al. (Hernandez-Ortega et al., 2015) were based on samples from the hippocampus. 2) The increased heterochromatin found in the 3xTg mouse model which support earlier studies was based on data collected from hippocampal and cortical mixed neuronal cultures (Walker et al., 2013). 3) All the pioneering studies showing increased heterochromatin formation in the AD brain were based on samples

taken from brain cortices (Crapper et al., 1979, Lewis et al., 1981, McLachlan et al., 1984, McLachlan et al., 1986, Lukiw and Crapper McLachlan, 1990, McLachlan et al., 1991). Therefore, this may indicate that the increase heterochromatin formation in the AD brain is specific to cortical neurons. Regardless, all these studies suggest an aberration of the heterochromatin that occurs in AD, similar to changes in the nucleolus that occurs with the disease. Considering the close relationship between the nucleolus and the heterochromatin, future studies are required to address whether in AD, changes in one of these molecular factories leads to the aberration of the other or whether both become affected at the same time.

Another dimension to the nucleolus-heterochromatin axis is the finding that tau protein localises to the both compartments. Indeed, many studies revealed that tau localises to multiple cellular compartments (Bukar Maina et al., 2016). This has led to the suggestion that tau is a multifunctional protein. Next, we discuss the unconventional localisation and function of tau, especially, as it relates to the nucleus, and how it may play a role in neurodegenerative diseases like AD.

1.4 Nuclear Tau and Alzheimer's disease

1.4.1 Cellular localisation of Tau

Tau is a cytosol-enriched protein, distributed within the somatodendritic compartment, but predominating in the axons (Papasozomenos and Binder, 1987, Binder et al., 1985), that regulate microtubule assembly and stability, and axonal transport of vesicles and organelles (Trinczek et al., 1999, Dixit et al., 2008). However, numerous studies have identified it in different subcellular compartments (Fig. 1.9). Its localisation to the microtubules of growth cones (DiTella et al., 1994, Black et al., 1996) and mitotic spindle (Preuss et al., 1995) has raised questions as to whether it has

a non-microtubule polymerising function since the microtubules in these two locations are more dynamic than axonal microtubules (Lee, 2005). Tau, mostly in a dephosphorylated state, has been localised in the plasma membrane of different cell lines (Arrasate et al., 2000). Its N-terminal domain mediates the interaction with the plasma membrane (Buee et al., 2000). In rat cortical neurons, a significant quantity of tau is localised to the membrane, and this tau species is dephosphorylated at Tau-1, AT8, and PHF-1 epitopes (See Fig. 1.6) (Pooler et al., 2012). This tau-membrane interaction is highly dynamic and depends on phosphorylation, such that inhibiting CK1 or GSK3β significantly increased tau translocation to the membrane and mimicking tau N-terminal phosphorylation prevented the tau-membrane localisation (Pooler et al., 2012). Tau has also been identified in the lipid rafts of the human AD brain, as well as brains from the Tg2576 AD mouse which harbours the Swedish mutation (Kawarabayashi et al., 2004). Similarly, it has been found in the lipid rafts of primary neurons, where it is regulated by Aβ oligomers (Williamson et al., 2008). In neurons, it localises in a good quantity within the synapses (Sahara et al., 2014). This different localisation of tau provides evidence for its role in non-microtubule-associated functions, such as signal transduction (Buee et al., 2000, Lee, 2005). In support of this, Ittner *et al.* (Ittner et al., 2010) showed that tau localised to the dendrites mediates Aβ toxicity by targeting FYN kinase to post-synaptic NMDAR in the mouse brain. Hyperphosphorylation has also been shown to mislocalise tau to the dendrites, where it alters synaptic function by affecting glutamate receptor trafficking (Hoover et al., 2010). These studies collectively suggest that tau-associated factors that promote neurodegeneration, such as tau phosphorylation, could change the localisation of tau and its functions, and trigger its somatodendritic accumulation, axonal microtubule

disassembly, PHF formation and downstream neurodegeneration (Pooler and Hanger, 2010).

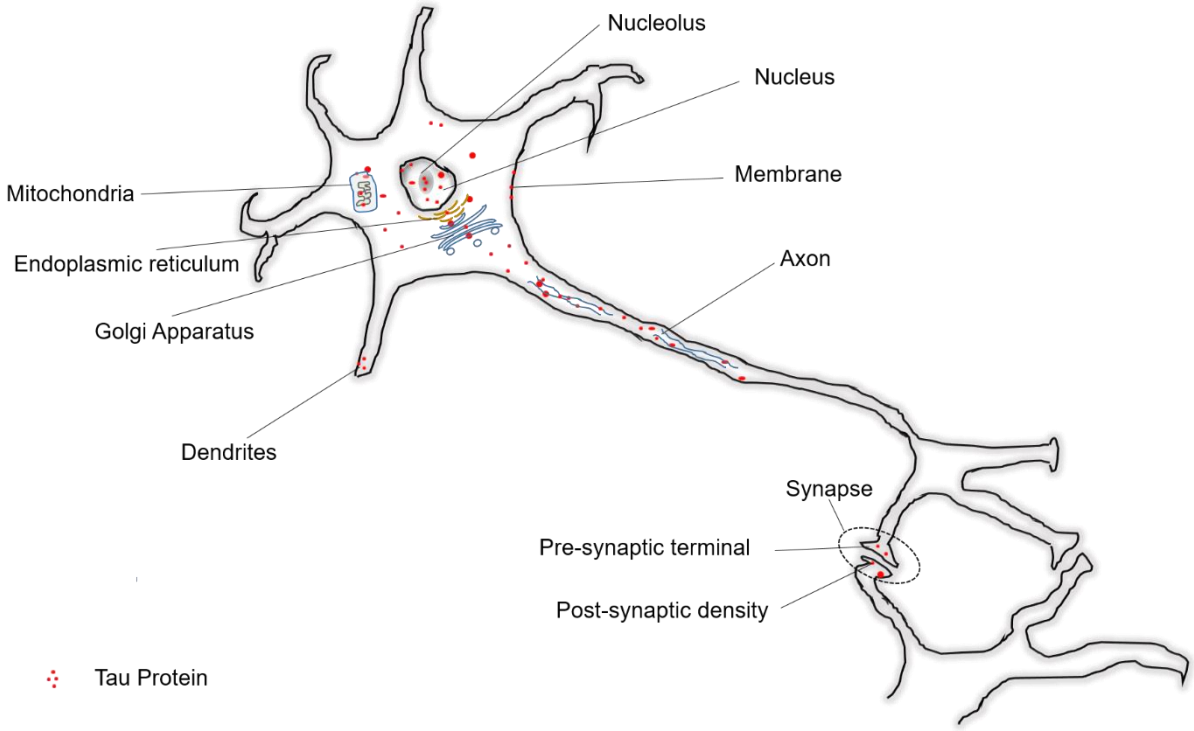

Figure 9.9 Cellular localisations of tau protein.
Evidence from the past two decades revealed that tau protein localises to the nucleolus, nucleoplasm, cytoplasm associated with the mitochondria, ribosomes, rough endoplasmic reticulum and Golgi apparatus. It also localises to the dendrites, pre- and postsynaptic sites and axons. See text for references.

Apart from the compartments above, tau has been localised to the ribosomes of both neurons and astrocytes in the AD brain (Papasozomenos and Binder, 1987, Papasozomenos, 1989). Papasozomenos and Su also found abnormally phosphorylated tau associated with purified ribosomes from AD brains but not from control brains (Brady et al., 1995). Its association with the ribosomes raised interesting questions on whether it plays any function related to protein synthesis. To date, the functional relevance of this association is still elusive. In the human brain, an antibody against total but not hyperphosphorylated tau partially colocalises with markers of endoplasmic reticulum and Golgi apparatus. Both the total tau and

hyperphosphorylated tau show increased colocalisation with these markers and the mitochondria in the AD brain indicating a disease associated changes in tau in these compartments (Tang et al., 2015). Aside from these extra-nuclear locations of tau, it has been localised within the nucleus of the mouse brain neurons (Sultan et al., 2011, Violet et al., 2014), within the nucleus and nucleolus of different undifferentiated primate cell lines (Loomis et al., 1990), and the nucleus of the AD and control brain (Brady et al., 1995). The evidence reviewed so far demonstrate that tau is a ubiquitous protein, highly dynamic, with a broad range of potential functions and whose functions and localisation are altered during neurodegeneration. Considering the importance of the nucleus in cellular homeostasis, out of all the different localisations of tau, nuclear tau is intriguing, as this raises many questions regarding its likely role in the nucleus, and how this is affected in tauopathies. Hence, we next review the evidence of nuclear tau and its potential role.

1.4.2 Nuclear Tau: three decades of discovery

In 1988, Metuzals and co-workers published a paper demonstrating the presence of PHF profiles within the nucleus of AD brain biopsies (Metuzals et al., 1988). Around the same time, using immunofluorescence microscopy with a Tau-1 antibody which detects nonphosphorylated tau on serine 195, 198, 199 and 202, tau was localised within the nucleus of CG human neuroblastoma cells, specifically at the NORs and the fibrillar region of the nucleolus in the interphase cells (Loomis et al., 1990). Similar tau staining patterns were found in other primate cell lines, but no tau immunoreactivity was observed in the non-primate cell lines used in their study. This finding was further confirmed by the same group with immunoblotting (Wang et al., 1993), and sense and antisense transfection strategies (Thurston et al., 1997) in neuroblastoma cells. Similarly, the neuroblastoma LAN-5 cell line also showed

nucleolar tau localisation (Greenwood and Johnson, 1995), and Thurston *et al.* (Thurston et al., 1996) revealed that other human non-neuronal cells like fibroblasts and lymphocytes contain nucleolar tau. This discovery of nuclear tau was very significant, as it provided substantial evidence for a potential non-microtubule associated function for tau at that time. Accordingly, Lu and Wood (Lu and Wood, 1993) showed that the microinjection of fluorescently-tagged bovine tau to cultured Chinese hamster ovary (CHO) cells (which do not normally express tau protein) showed the accumulation of tau within both the nucleolus and the centrosome. Cross *et al.* (Cross et al., 1996) localised tau with the centrosome of interphase cells of the Huh-7 cells, SW-13 cells, and normal human fibroblasts. Using *in vitro* assays, they also showed that the centrosomal tau promotes microtubule assembly at the centrosomes (Cross et al., 1996).

Subsequently, nuclear tau was localised in non-primate cell lines; such as the rat brain cell line B103 (Lambert et al., 1995), cultured mouse cortical neurons (Sultan et al., 2011), and the mouse brain (Liu and Götz, 2013, Violet et al., 2014). All this evidence makes a strong case that tau is a bonafide nuclear protein. It also suggests that although nuclear tau could be a shared phenomenon between different cell types of different species, some cells, especially primate cells, show prominent nucleolar tau localisation (Loomis et al., 1990, Sjoberg et al., 2006). Considering that nucleolar tau was found predominantly in dividing cells, it was assumed that it functions only in dividing cells, such that its expression ceases after differentiation (Wang et al., 1993). However, the same group later identified nuclear tau in the normal and AD brain with occasional scant nucleolar staining (Brady et al., 1995). The tau staining from their study is mostly extranucleolar, prompting the authors to suggest that tau may have a

nuclear function in postmitotic neurons, and the absence of nucleolar tau may indicate its function is no longer necessary in terminally differentiated cells.

We are still far from understanding the complete function of tau, especially as it relates to the nucleus. This is partly complicated by the presence of many isoforms of tau in primate and non-primate cells and the human brain. Indeed, the identity of nuclear tau, such as its post-translational modifications and the isoforms that exist within the nucleus is largely elusive. Post-translational modifications of tau are important disease modifiers in tauopathies (Martin et al., 2011), and the ratio of the different isoforms of tau also changes in some tauopathies (Connell et al., 2005). To appreciate the role of nuclear tau in physiology and pathology, there is the need to understand the real identity of the nuclear tau.

1.4.3 The identity of nuclear Tau

Alternate polyadenylation and alternative splicing could generate over 30 variants of tau. Most of our knowledge has focused mainly on the 6 kb transcript of tau and the six widely known isoforms in the CNS (see Fig. 1.5). Whether nuclear tau is generated from a distinct transcript and whether specific nuclear isoforms exist is not clear. It is possible that the tau that localises to the microtubules is different from the one that localises to the nucleus. Otherwise, localisation of tau with a microtubule-binding function would render the microtubule undersupported and vulnerable. To begin to tackle this question, Liu *et al.* observed that 1N4R tau isoform in the murine brain localises mainly to the nucleus, with some quantity in the soma, and dendrites, but not the axons (Liu and Götz, 2013). Interestingly, Wang *et al.* previously showed that the 2 kb tau transcript produces mainly nuclear tau, with a small amount of cytosolic tau showing non-microtubule binding function (Wang et al., 1993). Whether the murine

brain also contains a 2 kb tau transcript is not known, but it would be interesting to investigate if there is a specific targeting at the transcript level that specifies the distribution of the 1N4R message to the nucleus. Other isoforms (e.g., 2N4R) and products of the 6 kb tau transcript can also localise in small quantities within the nucleus (Liu and Götz, 2013, Wang et al., 1993). Decoding the transcripts targeting and isoform localisation of nuclear tau would enable a detailed understanding of tauopathies, some of which arise from altered splicing and balance of tau isoforms (Connell et al., 2005).

Apart from the post-transcriptional processing that yields different tau transcripts and isoforms, tau undergoes many post-translational modifications, such as phosphorylation (Martin et al., 2011). Tau phosphorylation has been suggested to be a major modifier of its function and the induction of toxicity in neurons (Frost et al., 2014, Pooler et al., 2012). It is therefore important to investigate whether phosphorylated tau can also localise to the nucleus and the functional relevance of this localisation. Indeed, Greenwood and Johnson (Greenwood and Johnson, 1995) found that nuclear tau in LAN-5 neuroblastoma cells may exist in both phosphorylated and native state, to a similar extent to the tau pool in the cytoplasm. *In vitro* phosphorylation of an intact isolated nucleus incubated with ATP Gamma ^{32}P revealed that the nuclear tau is likely not to be phosphorylated in the nucleus, but rather in the cytoplasm before translocation into the nucleus. Other studies confirmed that a quantity of nuclear tau could exist in a phosphorylated state in normal cell lines, mouse brain and the human brain (Shea and Cressman, 1998, Lambert et al., 1995, Lu et al., 2013a, Brady et al., 1995). However, some studies showed that tau, especially nucleolar tau, can only be detected with Tau 1 antibody (see Fig. 1.6) (Loomis et al., 1990, Wang et al., 1993, Thurston et al., 1996). Tau in the mouse brain also exists mainly in a non-

phosphorylated state (Lu et al., 2014), especially under stress conditions (Sultan et al., 2011). Nuclear tau in the human brain can also exist in a non-phosphorylated state (Brady et al., 1995). Generally, these findings appear to suggest the existence of both phosphorylated and non-phosphorylated nuclear tau, which may vary depending on cell type and intranuclear localisation, with the nucleolus restricted to mostly non-phosphorylated tau. So far, it is not entirely clear what functional role is played by the nuclear phosphorylated and non-phosphorylated tau. The nuclear compartment could also harbour an abnormal tau or tau on the path of transformation to a pathological state. Using both Pr5 mice that express the P301L tau mutation, and ΔTau74 mice, which express a truncated form of wild-type human tau with no microtubule-binding domain, Lu *et al.* showed that tau accumulates in the nucleus (Lu et al., 2014). Further, the first paper that hinted the localisation of tau to the nucleus identified PHF strands within the AD brain nucleus (Metuzals et al., 1988). Tau rod-like deposits with ordered filamentous ultrastructure were also localised within the brain, in nuclei of subjects with AD and Huntington disease (Fernandez-Nogales et al., 2014). These findings collectively suggest a role for tau within the nucleus in normal and disease conditions. We generally have a modest understanding of the functional role of tau isoforms, but the work of Wang *et al.* (Wang et al., 1993) and Liu *et al.* (Liu and Götz, 2013) strongly suggests that the bulk of nuclear tau may arise from a distinct transcript and comprised of a dominant isoform. It is crucial to decipher whether this nuclear tau typically interacts with and influences the DNA, and if/how this is important in pathology.

1.4.4 Capacity of Tau to interact with the DNA

As far back as 1975, Bryan *et al.* showed that RNA could inhibit microtubule assembly *in vitro*, through the reduction of the activity of a protein essential for tubulin assembly (Bryan et al., 1975). They showed that tau protein could serve as a protein whose

activity is reduced by the RNA. This provided preliminary evidence for interaction between tau and RNA. Indeed, two decades later, it was further shown that RNA could induce the aggregation of tau into AD-like PHFs (Kampers et al., 1996). Considering the relationship between the DNA and RNA, this finding also suggested a possible interaction between tau and the DNA. Indeed, Corces *et al.* showed that brain depolymerised microtubule-associated proteins bind to DNA with high affinity (Corces et al., 1978). Using an *in vitro* assay, Corces *et al.* further demonstrated that DNA inhibits microtubule assembly in a concentration-dependent manner, indicating that microtubule-associated proteins have more affinity to the DNA than to the microtubules (Corces et al., 1980). In the study, they explicitly showed that tau protein-containing microtubule fractions bind to DNA. Hua and He later found that addition of native tau to a solution of Calf Thymus DNA increased the melting temperature (T_m) of the DNA from 67 °C to 81 °C in a concentration-dependent manner (Hua and He, 2000). Similarly, tau protected pBluescript-II SK DNA from denaturation by raising its T_m from 75 °C to 85 °C. Kinetics study of tau and DNA further showed that tau could stabilise double-stranded DNA (Hua and He, 2000). This study made a strong case for tau as a DNA binding protein *in vitro*. However, it was unclear whether the DNA interacts only with native tau or whether it can interact with tau modified by post-translational modifications, such as phosphorylation. Hence, the same group looked at the interaction of phosphorylated or aggregated tau with the DNA. Using an *in vitro* approach, they showed that tau phosphorylated by a neuronal cdc2-like kinase (NCLK) retains its ability to bind the DNA and also increases the Calf Thymus DNA melting T_m (Hua and He, 2000). Interestingly, Hua and He observed that when the conformation of tau is changed by aggregation using formaldehyde, electrophoretic mobility shift assay and agarose gel retardation assay both showed that

phosphorylated and native tau lose the ability to bind the DNA (Hua and He, 2000). However, a recent study to further characterise the nature of this interaction revealed strongly reduced or loss of capability of tau phosphorylated by GSK-3β for binding and protecting the DNA against thermal denaturation (Lu et al., 2013b). Although both NCLK and GSK-3β phosphorylate tau on multiple epitopes, including the PHF epitopes, GSK-3β can phosphorylate tau on additional epitopes that are not phosphorylated by NCLK and has been suggested to play a dominant role in tau phosphorylation (Tenreiro et al., 2014). Hence, this could be the reason for the discrepancy between the two studies on phosphorylated tau–DNA interactions (Hua and He, 2002). Using other approaches, other studies also showed a similar reduction of the phosphorylated tau-DNA interactions (Camero et al., 2014b, Qi et al., 2015). Based on these findings, it was postulated that aberrant phosphorylation of tau, such as in AD, might lead to its aggregation, thereby affecting its ability to both stabilise the microtubule and protect the DNA (Hua et al., 2003). To further characterise the nature of the Tau–DNA interaction, Hua *et al.* investigated the binding of tau to double-stranded (dsDNA) or single-stranded (ssDNA) DNA and the nature and flexibility of this binding. Their results revealed that tau binds to dsDNA, but not ssDNA and that this binding is rapid, dynamic and reversible and occurs in a cooperative, rather than sequence-specific fashion (Hua et al., 2003). It was suggested that the binding probably occurs via a charge effect since incubation of tau–DNA solution in an increasing concentration of a high ionic strength buffer (NaCl) led to a NaCl concentration-dependent binding of tau to the DNA. Transmission electron microscopy showed that tau clustered around the DNA like a necklace. In contradiction to Hua *et al.* (Hua et al., 2003), a study by Krylova *et al.* (Krylova et al., 2005), using kinetic capillary electrophoresis found that tau could not only bind dsDNA, but it can also bind a ssDNA in sequence-specific

fashion, and that it induces the denaturing of dsDNA by binding to one of its strands, sequence specifically. Hence, in an effort to clearly elucidate the interaction of tau with the DNA and therefore understand the functional significance of this interaction, Qu *et al.* using atomic force microscopy, showed that monomeric tau molecules bind the DNA at a molar ratio of about 1:10 (Tau/DNA), the equivalent to about 1 tau molecule to 700 bp of dsDNA (Qu et al., 2004). Further studies revealed that tau binds and bends the DNA through AT-rich minor groove of the DNA, likely through an electrostatic interaction with the ε-amino group of its lysine residues on its PRD and MBD; and that tau preferentially binds DNA sequences of about 13 bp or longer (Wei et al., 2008). However, a recent study using nuclear magnetic resonance spectroscopy (NMR) further revealed that this interaction might be through the second half of the PRD of tau (R209 to A246), and a second interaction site on its MBD in the R2 repeat region (K267 to S289) (Qi et al., 2015). The authors also showed that tau interacts with not only AT-rich regions but GC-rich oligonucleotides, indicating generic binding with the DNA backbone, independent of the bases. Hydrophobicity has also been suggested to be essential in the tau–DNA interaction (Camero et al., 2014b). Therefore, there is sufficient evidence to indicate the capacity of tau to interact, stabilise and bend the DNA. However, most of these experiments showing tau–DNA interaction were conducted outside a cellular environment, mostly using recombinant proteins.

Greenwood and Johnson earlier discovered that of the total tau in LAN-5 neuroblastoma cells, 14% are localised within chromatin fraction containing DNA, chromatin, and associated proteins (Greenwood and Johnson, 1995). This confirmed that tau could form a complex with the DNA within a cellular environment. Moreover, they further showed that the tau in the chromatin fraction could exist in a phosphorylated state, supporting the work of Hua and He which showed the capacity

of phosphorylated tau to interact with the DNA (Hua and He, 2002). Using immunofluorescence microscopy, Sjoberg *et al.* provided evidence *in situ* of tau-DNA interaction (Sjoberg et al., 2006). They showed that tau colocalised with H3K9me2 and centromeric α satellite DNA in human fibroblasts. They further showed that it also binds to human α satellite DNA sequences and murine γ-satellite DNA sequences. Rossi *et al.* also showed that tau localises to the spindle poles and the mid-body in dividing cells (Rossi et al., 2008). Sultan *et al.* using immunoprecipitation in primary neurons, further showed that tau interacts *in situ* with the DNA (Sultan et al., 2011). Using netropsin—a polyamide that binds the minor groove of dsDNA through AT-rich sequences; and methyl green—a major groove binding drug—Sultan *et al.* confirmed that tau binds the minor groove of the DNA. All these studies indicate that tau is a DNA-binding protein, so the question now is – what is the functional role of nuclear tau?

1.4.5 Functional role of nuclear Tau

Tau's localisation within the nucleus is particularly interesting, considering the importance of the nucleus in cellular processes. Looking at cell lines and the human brain, nuclear tau exists in different isoforms, with some cells showing diffuse nuclear staining of tau and some showing major nucleolar tau signal (Loomis et al., 1990, Wang et al., 1993, Liu and Götz, 2013, Lu et al., 2014, Cross et al., 1996, Brady et al., 1995). While discussing the role of nuclear tau, it may be important therefore to make a distinction between the nuclear tau ubiquitously distributed within the nucleus and tau predominantly localised to the nucleolus. The difference in their nuclear distribution could assign different roles for them in neuronal physiology and pathology. The localisation of tau to the nucleolus in interphase cells or the NORs of dividing cells is very intriguing considering the role of the nucleolus in the cell in rRNA production and

downstream protein synthesis. NORs contains rRNA genes and is the source for the formation of the nucleolus (Olson and Dundr, 2001). The nucleoli play numerous cellular functions, the prominent of which is rRNA synthesis. Tau localises to the DFC (Sjoberg et al., 2006) and the ribosomes (Papasozomenos and Binder, 1987, Papasozomenos, 1989). Tau may not play a role in nucleolar assembly or formation (Thurston et al., 1997), but together, these studies suggest it could be involved in rDNA transcription and/or rRNA processing (Fig. 1.10). The synthesis of ribosomes begins within the nucleolus, with final maturation occurring in the cytoplasm. Hence, the localisation of tau both within the DFC of the nucleolus and to the ribosomes may suggest that it plays a role in the processing of ribosomes from nascent pre-rRNA to maturation in the cytoplasm. Alternatively, nucleolar tau could play a part in the heterochromatinisation of rDNA (Sjoberg et al., 2006). The majority of rDNA are kept in a transcriptionally inactive state through epigenetic mechanisms. The silenced rRNA genes are packaged to form nucleolar heterochromatin localised to a region adjacent to the perinucleolar heterochromatin (Carmo-Fonseca et al., 2000). Sjoberg *et al.* showed that tau interacts with the perinucleolar heterochromatin and α-satellite of pericentromeric DNA (Sjoberg et al., 2006). They proposed that tau could serve as a link between rDNA repeats and pericentromeric heterochromatin, through which it could play a role in rRNA gene silencing. Tau's interaction with the perinucleolar heterochromatin makes it a potential regulator of rDNA stability, especially against illicit recombination (Carmo-Fonseca et al., 2000). In dividing cells, its association with the NORs of acrocentric chromosomes (Loomis et al., 1990) also suggests a potential role for it in chromosomal stability (Rossi et al., 2008). It's role in chromosomal stability is supported by the observation that cells with tau mutations showed a high degree of structural, numerical and stable chromosomal defects and splenocytes from tau

knockout (KO) mouse accumulate chromosomal abnormalities including aneuploidy (Rossi et al., 2013, Rossi et al., 2008, Granic et al., 2010).

The capacity of tau to interact with nucleic acids, such as the RNA and DNA (Bryan et al., 1975, Kampers et al., 1996, Corces et al., 1978, Corces et al., 1980), and raise the melting temperature of the DNA suggests it could also play a role in DNA protection (Hua and He, 2000). *In vitro* evidence showed that tau could protect dsDNA from thermal denaturation and enhance its renaturation (Hua et al., 2003). Hydroxyl free radicals (•OH) are known to induce dsDNA breakage. *In vitro* evidence implicates tau in DNA protection from the –OH radical-induced DNA damage in a concentration-dependent manner (Hua et al., 2003, Wei et al., 2008, Lu et al., 2013b, Camero et al., 2014a). How this DNA protection occurs is not clear *in vivo*, especially because tau is mostly a cytosolic protein although a permanent nuclear pool appears to exist. In addition, it's dynamic and reversible nature of interaction with the DNA (Hua et al., 2003) suggests that it may shuttle between the cytosol and the nucleus, similar to heat-shock proteins which shuttle between the cytoplasm and the nucleus following heat shock (Welch and Feramisco, 1984, Chu et al., 2001, Welch and Mizzen, 1988). Like tau, some of these heat-shock proteins are enriched in both cytoplasmic and nuclear compartments (Mandell and Feldherr, 1990). Heat-shock was previously shown to impact on tau protein phosphorylation in male and female rats (Papasozomenos and Su, 1991). Papasozomenos also found widely distributed tau immunoreactivity in the nucleus of an autopsy specimen of a subject with presenile dementia with motor neuron disease, cited in (Thurston et al., 1996). These findings led Papasozomenos to postulate that tau may play a role in stress response acting like heat-shock proteins, which upon heat shock translocate into the nucleus and to the nucleolus to maintain the integrity of the nuclear and nucleolar DNA and then subsequently exit to the cytoplasm (Welch and

Feramisco, 1984, Welch and Mizzen, 1988, Kotoglou et al., 2009). Hsp70, which is also known to translocate to the nucleus upon heat shock, has been shown to protect the DNA (Niu et al., 2006). In response to oxidative stress, Hsp72 was also shown to translocate to the nucleus, where it interacts with many nuclear proteins, including HMGB1, thus conferring cytoprotection by preventing the cytoplasmic translocation and release of HMGB1 from the injured cells (Tang et al., 2007). In a somewhat similar scenario, tau has been shown to translocate to the nucleus following cellular distress to bind and protect the DNA in mouse primary neurons (Sultan et al., 2011). The immunogold electron microscopy image from Sultan *et al.*'s study (Sultan et al., 2011) also showed dense nucleolar tau staining following the heat-stress, reminiscent of Hsp70 that translocates to the nucleus and the nucleolus to protect nucleoplasmic and ribosomal DNA from DNA breaks (Kotoglou et al., 2009). Similar to heat-shock proteins after the distress, Sultan *et al.* (Sultan et al., 2011) showed that tau translocates back to the cytoplasm. Protein dephosphorylation has been previously shown to be essential for the translocation of Hsp70 into the nucleus in HeLa cells (Chu et al., 2001). Interestingly, Sultan *et al.* showed that when cells are under heat stress, nuclear tau is mainly non-phosphorylated (Sultan et al., 2011). Although tau KO mice are viable and show no apparent neuronal aberration (Ke et al., 2012), evidence from tau KO background also supports the DNA protective role of tau. Sultan *et al.* (Sultan et al., 2011) showed that tau KO cortical neurons are vulnerable to heat stress-induced DNA damage while overexpression of hTau44 restored the DNA protective function of tau in these cells. Using wild-type and tau KO mice, the same group further showed a role for tau in RNA quality control and its reversible nuclear accumulation following cellular distress to protect the DNA (Violet et al., 2014). Recently, the same group provided evidence to support a role for tau in chromatin

stability (Mansuroglu et al., 2016). Collectively, these results suggest a role for tau in DNA protection and chromatin stability. They also seem to suggest that the tau that shuttles between the nucleus and the cytoplasm is a different isoform from the tau mainly localised to the nucleolus in neuronal and non-neuronal cell lines (Loomis et al., 1990, Sjoberg et al., 2006).

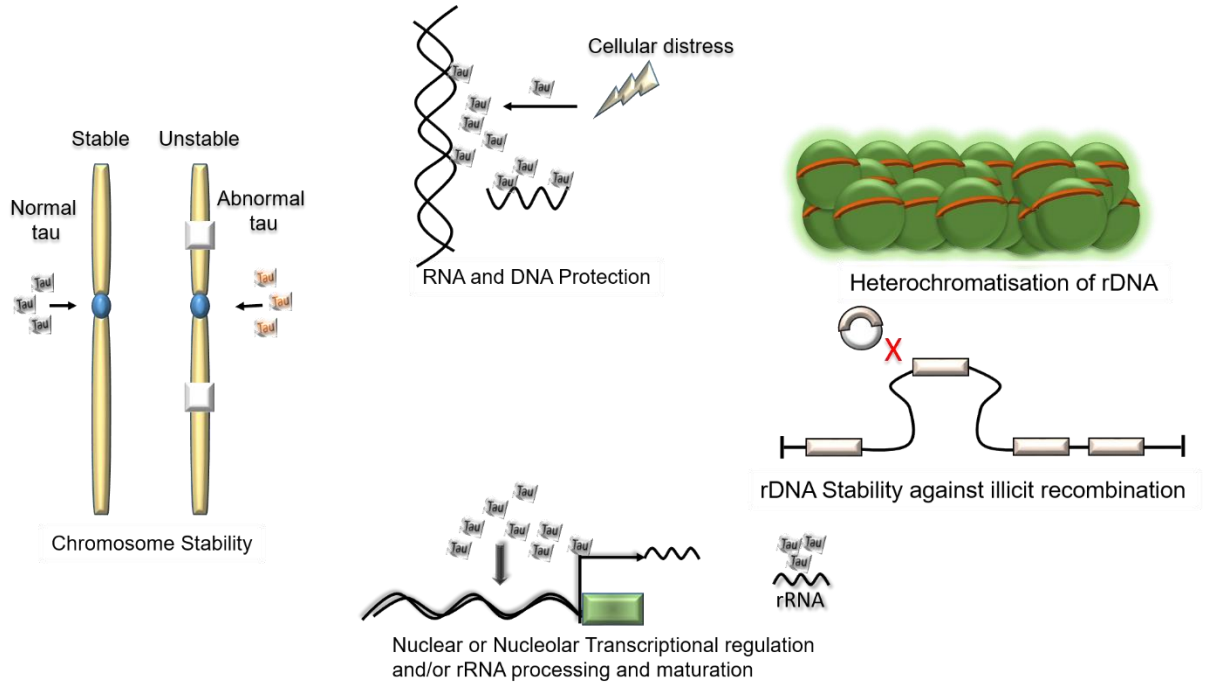

Figure 10.10 Potential functions of nuclear tau.
Tau has been shown to protect the DNA and RNA from cellular distress (Sultan et al., 2011, Violet et al., 2014). It has been localised within the nucleolus, at the vicinity of the rDNA and is associated with a marker of the heterochromatin, within the nucleolus. It specifically localises to the DFC —a region involved in rDNA transcription and processing of nascent pre-rRNA; collectively, this suggests a potential role for tau in either rDNA heterochromatinisation and stability, rDNA transcription and/or rRNA processing and maturation (Sjoberg et al., 2006, Loomis et al., 1990, Thurston et al., 1996). Data from cell culture and human cells with varying tau mutations also provides strong evidence for tau in the maintenance of chromosomal stability (Rossi et al., 2008, Rossi et al., 2013, Malmanche et al., 2017). The nature of the interaction of tau–DNA interaction also suggests it could be involved in nuclear transcriptional regulation (see below for more detailed discussion). All these functions need further research to be completely validated.

Tau has also been suggested to possess the quality of transcriptional regulator due to its ability to induce the separation of dsDNA to ssDNA (Krylova et al., 2005). *In vitro* evidence by Padmaraju *et al.* also showed that tau could alter gene expression by causing a change in DNA conformation from the standard B conformation to A–C

conformations (Padmaraju et al., 2010). Qi *et al.* showed that tau could bind AT and GC-rich oligonucleotides (Qi et al., 2015). CpG islands are found in GC-rich DNA regions and are essential regulators of transcription (Deaton and Bird, 2011, Kolell and Crawford, 2002). Some proteins with cytoplasmic localisation, such as stress-responsive transcriptional activator MSN2, have also been shown to undergo dephosphorylation following cellular distress (e.g., Heat-shock) and translocate to the nucleus, where it functions as a transcription factor (Gorner et al., 1998, Hao et al., 2013). Evidence from Tau KO mice showed that tau could regulate the *smarce1* gene (Gómez de Barreda et al., 2010), and regulate pericentromeric heterochromatin transcription (Mansuroglu et al., 2016). In an analysis of ~11,000 mRNAs using microarray screening from wild-type and tau KO mice, about 74 mRNAs were found to be significantly altered in the brain of 8-week-old KO mice. Further analysis using qPCR, showed a significant rise in 13 mRNA in the KO mice brain (Oyama et al., 2004). These findings support a role for tau in gene regulation (Ke et al., 2012). Therefore, a role for tau in transcriptional regulation is something worth investigating.

1.4.5 Potential role of nuclear Tau in neurodegeneration

Rossi *et al.* demonstrated that tau in non-neuronal cells carrying the P301L tau mutation consistently present with a higher degree of structural, stable and numerical chromosome lesions, chromatin bridges and decondensed chromosomes (Rossi et al., 2008). This finding was further confirmed in other non-neuronal cells with varying tau mutations, making them more susceptible to genotoxic agents (Rossi et al., 2013). Accordingly, a recent study revealed that human tau overexpression in *Drosophila melanogaster* leads to mitotic aberrations (Malmanche et al., 2017). Considering the localisation of tau to chromosomes (Loomis et al., 1990, Rossi et al., 2008, Wang et al., 1993), its capacity to bind and protect the DNA *in vitro* and *in vivo* (Wei et al., 2008,

Sjoberg et al., 2006, Sultan et al., 2011), tau was suggested to be essential for the maintenance of chromatin stability (Rossi et al., 2013). This suggests that the absence of normally functioning tau due to mutation can affect tau's role in genome protection and render cells susceptible to chromosomal instability. Although tau's potential role in chromosomal stability was demonstrated in non-neuronal cells (Rossi et al., 2013, Rossi et al., 2008), it does not preclude the potential of tau in stabilising the chromosome of neurons. Also, tau mutations do not cause AD, but relationship exists between chromosomal instability and AD. For instance, an aberration in the PS-1 gene has been shown to cause nondisjunction (Boeras et al., 2008). It was further demonstrated that fAD mutant APP (V717F) transgenic mouse and fAD-APP transfected cultured human cells, both produced chromosome missegregation, and aneuploidy in both brains and peripheral cells (Granic et al., 2010). These authors also showed that Aβ could also cause aneuploidy, including trisomy 21, in cultured cells. Interestingly, using splenocytes from tau KO mice, they demonstrated that tau KO cells also harbour the aneuploidy, which was only enhanced by Aβ treatment, indicating that tau is required for the chromosome stability (Granic et al., 2010). Increased level of aneuploidy specific to chromosome 21 has also been observed in the cerebral cortex of the AD brain (Iourov et al., 2009). Considering the role of tau in chromosomal stability (Rossi et al., 2008, Rossi et al., 2013), these studies could imply that in AD, increased production of Aβ could lead to tau aberration, such as phosphorylation, preventing it from protecting the genome, as well as stabilising the chromosomes.

Nuclear tau localises to chromosome 13, 14, 15, 21 and 22 (Loomis et al., 1990). Localisation of tau to these chromosomes has been earlier proposed to provide a link between AD and Downs' syndrome (Loomis et al., 1990). Some people with Down

syndrome that live beyond 30 years develop AD later in life (Potter, 1991). The connection could arise due to the overexpression of APP localised on chromosome 21, leading to increased Aβ production, which promotes chromosomal aberration (Granic et al., 2010) and can cause cellular dysfunction through both cytosolic and nuclear tau. Whether tau serves as the link between these two diseases and if aberration in tau contributes to the chromosome 21 specific aneuploidy observed by Ivan *et al.* (Iourov et al., 2009) in the AD brain is something worth investigating.

Tau hyperphosphorylation is an important modification considered to be essential for the development of PHF and tangles (Martin et al., 2011). Some studies showed that hyperphosphorylation of tau reduces its nuclear translocation (Lefebvre et al., 2003) and binding of tau to the DNA (Qi et al., 2015, Camero et al., 2014b). Although several reports showed that nuclear tau could be found in a phosphorylated state (Brady et al., 1995, Greenwood and Johnson, 1995, Rossi et al., 2008, Lu et al., 2013a). For instance, the infection of human neuroblastoma SK-N-MC cells with Herpes simplex virus type 1 led to the hyperphosphorylation and accumulation of tau in the nucleus (Álvarez et al., 2012). Hyperphosphorylation of tau co-occurs with DNA damage in formaldehyde-treated N2a cells, indicating an involvement of the phosphorylated tau in DNA damage (Lu et al., 2013b). *In vitro* evidence suggests that phosphorylated tau can bind and alter the conformation and the integrity of the DNA, and in this way, could change nucleosomal organisation and impact on gene expression (Padmaraju et al., 2010). Recent findings from *Drosophila melanogaster* and a mouse model of tauopathy revealed that downstream changes in tau toxicity include oxidative stress, DNA damage and decompaction of the heterochromatin and aberrant gene dysregulation, especially of genes previously masked in the heterochromatin (Frost et al., 2014, Dias-Santagata et al., 2007, Khurana et al., 2006, Khurana et al., 2012). Findings from Philip

De Jager's Laboratory presented at the recently concluded AD/PD conference 2017 in Vienna, revealed that about 6000 genes that become euchromatic in the brains of people with AD are correlated with tau pathology, suggesting that that the aberration of tau, allows an open chromatin. Although these studies did not investigate the involvement of nuclear tau in the chromatin changes. It has been proposed that in AD, the nuclear tau binding and protecting the DNA (Sultan et al., 2011) or stabilising the heterochromatin (Sjoberg et al., 2006, Mansuroglu et al., 2016) could be altered due to the change in the tau molecule configuration, such as phosphorylation, leading to its detachment from the DNA (Lu et al., 2013b, Qi et al., 2015) and nuclear depletion (Hernandez-Ortega et al., 2015), as a result, causing the alteration of chromatin integrity and aberrant gene regulation (Padmaraju et al., 2010, Frost et al., 2014) and this way promoting neurodegeneration (Bukar Maina et al., 2016).

The work in this thesis was designed to investigate the link between Aβ, the amyloid cascade hypothesis and changes in nuclear tau. Furthermore, considering that oxidative stress, DNA damage, nucleolar stress and heterochromatin alteration are implicated in AD, we tested the hypothesis that stressors associated with AD, such as Aβ and glutamate stress could be associated with these changes in AD. Here, differentiated and undifferentiated SHSY5Y cells and human brain tissue were used to address these questions.

Chapter 2

2. Materials and Methods

2.1 Cell culture

Undifferentiated SHSY5Y neuroblastoma cells were maintained in DMEM/F-12 (Life Technologies, UK), supplemented with 1% (v/v) L-glutamate, 1% (v/v) penicillin/streptomycin and 10% (v/v) Fetal Calf Serum (FCS) at 37°C and 5% CO_2. For experiments involving differentiated cells, SHSY5Y cells were seeded into a culture flask, allowed to adhere overnight, then incubated for five days (with media changes after two days) in a medium containing 1% FCS supplemented with 10 μM trans-retinoic acid. After five days, the medium was replaced with a serum-free media supplemented with 2 nM brain-derived neurotrophic factor (BDNF) (GF029, Merck Millipore). Cells were used two days post-BDNF incubation.

2.2 Preparation of Amyloid beta

Aβ42 (rPEPTIDE, Bogart, GA, USA) was prepared following established procedure (Al-Hilaly et al., 2013). The peptide was solubilised at 0.2 mg/ml in HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) > 99% purity (Fluka, Sigma-Aldrich), vortex-mixed for 60 sec and sonicated for 60 sec in a 50/60 Hz ultrasonic bath (Thermofisher Scientific, FB 15051). The HFIP was removed by bubbling dry nitrogen gas and dried Aβ was dissolved in DMSO (Dimethyl sulfoxide) >99.9% (ACROS Organic) at 0.2 mg/ml. The Aβ-DMSO solution was vortex-mixed for 60 sec, sonicated for 60 sec, then eluted for 2 min at 4°C and 3000 RPM using a 5 ml HiTrap desalting column (GE Healthcare) in 30 μL of Hepes buffer [10 mM Hepes, 50 mM NaCl, 1.6 mM KCl, 2 mM $MgCl_2 \cdot 6H_2O$ and 3.5 mM $CaCl_2 \cdot 2H_2O$ (pH 7.4)]. The oligomeric Aβ was collected in a 4°C-controlled Eppendorf and then used to estimate the Aβ peptide concentration using a Nanodrop spectrophotometer (Thermofisher Scientific) at a wavelength of 280 nm. Using the

extinction coefficient of 1490 m^2/mol for Aβ, the concentration of Aβ42 was determined. An equivalent of 10 μM from the Aβ42 stock was administered to the medium of the differentiated SHSY5Y cells.

2.3 Preparation of Glutamate

L-Glutamic acid (G1251-100G) was solubilised in serum-free DMEM-F12 media to a stock solution of 23.57 mM, from which, 20 mM was used for experiments.

2.4 siRNA transfection

For siRNA transfection, SHSY5Y cells were maintained for 72h in Accell SMARTpool siRNA against Tau (Tau siRNA) or non-targeting pool (NT siRNA) (see Table 2.3) at a concentration of 1.5 μM mixed in Accell siRNA Delivery Media (B-005000-100, Dharmacon).

2.5 Western blotting

SHSY5Y cells treated or untreated with a test compound were fractionated for 15 min on ice using Radioimmunoprecipitation assay (RIPA) (Abcam, ab156034), supplemented with protease (P8340, Sigma) and phosphatase (P0044, Sigma) inhibitors and spun at 16000 x g for 15 at 4°C. Protein concentration was quantified using Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, 23225) and absorbance (562nm) were read using GloMax Multi-Detection plate reader (Promega). A total of 10 μg of protein from each sample was diluted in 4x Laemmli sample buffer (Bio-Rad, 161-0747), supplemented with 10% β-mercaptoethanol (Sigma, M-6250), then loaded to 7.5% Mini-PROTEAN TGX Stain-Free Protein Gels (4561023, Bio-Rad), and run for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100V using 1x running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The proteins were transferred to Polyvinylidene difluoride (PVDF)

membrane (IPVH00010, Merck Millipore) using 1x transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 10% (v/v) methanol) at 100V. The membrane was blocked in 5% (w/v) milk (blocking buffer) dissolved in washing buffer (TBS-Tween Tablet solution) (524753, Merck Millipore), then incubated at 4°C overnight with the different primary antibodies (Table 2.1) diluted in the blocking buffer. The membranes were washed in the wash buffer 5x for 10 min each and probed at room temperature on a shaker for 1h in the corresponding secondary antibodies diluted in blocking buffer. The membranes were washed 5x for 10 min each and subsequently developed in the dark room after incubation in Clarity Western ECL substrate for 1 min (1705060, Bio-Rad). To ensure the specificity of the secondary antibodies, control experiments were run using secondary antibodies, without primary antibodies, and this did not show any specific chemiluminescent signal. For antibodies used as loading control or when sequential analyses of other proteins are required on the same membrane, the membranes were stripped using Restore™ PLUS Western Blot Stripping Buffer (46428, Thermofisher Scientific), then blocked, and probed as described above. The blots were scanned at high resolution, and then bands were quantified using Image J software.

2.6 Immunoprecipitation

SHSY5Y cells were fractionated using RIPA buffer supplemented with protease and phosphatase inhibitors and 1.25 units of Benzonase Nuclease (E1014, Sigma). The pellets were discarded, and supernatants saved at room temperature for 30 min and then kept on ice for at least 2 hours - sufficient time to allow the degradation of nucleic acids by benzonase, then used for immunoprecipitation using Dynabeads protein G (10007D, Lifetechnologies). Dynabeads were resuspended by vortexing and 50 µL bead solution added to 1.5 mL low-bind tubes. The beads were placed on a separation magnet (S1506S, NEB) and the supernatant removed, beads were washed once with

PBS: Tween-20 (0.1%), resuspended in 200 µL antibody solution in PBS: Tween-20 (0.02%) and allowed on a shaker at 700 RPM for 30 min at room temperature. For control preparation, beads were similarly resuspended in 200 µL wash buffer without antibody. The bead solution was then put on the separation magnet and the supernatant discarded, while the beads-antibody complex was resuspended in at least 500 µg of the whole cell extract and incubated on a shaker at 700 RPM at room temperature for 45 min. Next, the supernatant was discarded after separation on the magnet. The beads-antibody-antigen complexes were washed three times using 400 µL wash buffer, each time discarding the supernatant after magnet separation. Next, the beads-antibody-antigen complexes were resuspended in 100 µL wash buffer, then transferred to a new tube, placed on a magnet and the supernatant discarded. Finally, the beads-antibody-antigen complexes were eluted in 30 µL of 50 mM Glycine (pH 2.8) and 15 µL 1x Laemmli Sample Buffer (1610747, Bio-Rad), supplemented with 10% of 2-Mercaptoethanol (Sigma, M-6250), and boiled at 80^{o}C for 10 min. The beads were separated from the magnet and supernatant (containing the eluted protein) and used for SDS-PAGE/Western blotting.

2.7 Immunofluorescence labelling

SHSY5Y cells treated or untreated with test compound, were re-suspended in PBS and spun onto a glass slide at 800 RPM for 3 min using Cytospin Centrifuge (CellSpin I, Tharmac). Cells were fixed for 15 min with 4% Paraformaldehyde (PFA) (Electron Microscopy Sciences) prepared in PBS, slides were next PBS-washed, permeabilised using 0.5% TritonX-100/PBS for 15 min and PBS-washed. The slides were blocked in blocking buffer [4% BSA/PBS/Tween-20 (0.02%)] for 45 min, incubated with primary antibody diluted in the blocking buffer for 45 min, PBS-washed three times, incubated in Alexa fluorophore-conjugated corresponding secondary antibody diluted in the

blocking buffer for 45 min. The slides were PBS-washed three times, incubated in 1/1000 DRAQ5, a far-red fluorescent DNA dye (ab108410, Abcam) diluted in PBS/Tween-20 (0.02%) for 10 min and mounted with coverslips using ProLong® Gold Antifade mountant (P36930, Lifetechnologies). For the labelling of 5-Methylcytosine /(5-mC), cells on the glass slides were fixed at room temperature for 30 min with 2.5% PFA/PBS, next slides were PBS-washed, permeabilised for 1h at room temperature with 0.5% Triton X-100/PBS. The cells were next washed in wash buffer (PBST) [PBS/0.1% Triton X-100] and incubated with 2N HCl for 30 minutes at 37 ˚C to depurinate the DNA, followed by 2x 5 minutes wash with 0.1M borate buffer (pH 8.5). They were then rinsed twice with PBS-T, blocked in blocking buffer (1%BSA/PBS-T) for 1h at room temperature, incubated with the primary antibody diluted in the blocking buffer for two hours at room temperature and washed three times with PBS-T. Then they were incubated with the corresponding secondary antibody diluted in the blocking buffer for 45 minutes at room temperature in the dark and washed three times in PBS-T, then stained with DRAQ5 and mounted

2.8 Confocal microscopy imaging and analysis

Images were taken using a 100x oil objective of LSM510 Meta confocal microscope mounted on Axiovert200M using pinhole size of 1 Airy unit. All images were collected as Z-stacks for all channels using a step size of 1 µm to allow the analysis of the entire signal in the cells. Subsequently, images were Z-projected to sum all signals and then analysed using image J. Five randomly collected images from each experiment and an average of 50 - 70 cells per experiment were subjected to the Image J analysis. For the quantification of nuclear foci/cluster, Image J Procedure presented by the light microscopy core facility of Duke University was used (https://microscopy.duke.edu/HOWTO/countfoci.html). For the quantification of

total nuclear fluorescence intensities, the nuclei were first segmented by thresholding using the DRAQ5 channel, excluding fused nuclei or those at the edges. Subsequently, the multi-measure option on the image J ROI manager was used to measure nuclear fluorescence from all channels in only segmented nuclei. The total corrected nuclear fluorescence (TCNF) was then calculated as TCNF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings) (Boisvert et al., 2007).

2.9 Immunogold labelling transmission electron microscopy (TEM)

Brain tissue from the middle frontal gyrus of the human brain (see Table 2.2) was analysed under local ethics approval and provided by London Neurodegenerative Diseases Brain Bank with informed consent as previously described (Al-Hilaly et al., 2013). The immunogold labelling for these sections and the SHSY5Y cells were performed by minimal, cold fixation and embedding protocols, as previously described (Soura et al., 2012) using an established method that employs PBS+ buffer for dilution of all immunoreagents and washes (Thorpe, 1999). Thin sections were collected onto 300-mesh high transmission hexagonal Nickel grids (Agar Scientific), blocked with 10% normal goat serum for 30 min at room temperature, single or double labelled using antibodies for 12h at 4°C. The grids were washed three times with PBS+ for 2 min each, then incubated with appropriate gold particle conjugated secondary antibodies for 1h at room temperature (see antibody section and results). The grids were next washed three times for 10 min each in PBS+ and four times for 5 min each in distilled water, dried for 5 – 10 min and then post-stained in 0.22 µm-filtered 0.5% (w/v) aqueous uranyl acetate for 1 h in the dark. The grids were finally washed with distilled water five times at 2 min intervals and left to dry for at least 12 hours before TEM observation. This protocol was developed by Dr Julian Thorpe, who also kindly assisted with the tissue preparation in some of the TEM experiments.

2.10 TEM ultrastructural analysis

The TEM ultrastructural processing was previously described (Soura et al., 2012). SHSY5Y cells treated or untreated were pelleted, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate/HCl buffer (pH 7.4) for 2 h at room temperature and then at 4°C on a rotator overnight. The pellets were then post-fixed in 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate/HCl buffer (pH 7.4) for 2 h at room temperature before being dehydrated in an ethanol series. After two 20 min washes in propylene oxide, the pellets were infiltrated over several days, with a few resin changes, in TLV (TAAB low-viscosity) resin before polymerising at 60°C for 16 h. The pellets were then sectioned and stained with 2% (w/v) aqueous 0.22 μm-filtered uranyl acetate at room temperature for 1 h.

2.11 TEM Imaging and analysis

JEOL JEM-1400 Transmission Electron Microscope with a Gatan OneView® camera was used to image the grids at 120V. For colocalisation analysis in the human brain, four nuclei per grid, of medium to large size (>50% of X8000 magnification view), were randomly selected and imaged at X15000-X20000 magnification. Four grids were taken from each case, accounting for one repeat for the two double immunolabelling cases. In all cases, randomised selection was undertaken by identifying nuclei at low magnification (X5000), then imaging at higher magnification. All images were analysed using Image J. For colocalization analysis on brain sections; each observed 15 nm gold particle, signifying a Tau 1 antigen, was checked for colocalisation with 5 nm gold particles, signifying TIP5 antigens. Our definition of colocalisation is; when the number of one antigen (TIP5 particles) within a 50 nm radius of the second antigen (Tau 1) is greater than zero (n>0). Gold particles were included in our analysis if; Tau 1 particles measured between $11 \leq x \leq 19$ nm and TIP5

particles measured between 1≤x≤9 nm. The method of colocalisation analysis was roughly based on the cross-K function; we used the number of gold particles of the first type at distances shorter than a given distance from a typical particle of the second type divided by the area of the 50 nm inclusion circle (Philimonenko et al., 2000).

2.12 CellROX Green Assay

SHSY5Y cells treated or untreated with test compound were incubated with 5 μM CellROX Green Reagent for 1h at 37^{o}C and 5% CO_2 (C10444, Lifetechnologies UK). The cells were next resuspended in PBS and analysed on a FACS using the 488nm excitation laser (BD Accuri 6, BD Biosciences), placing the CellROX Green Reagent signal in the fluorescein (FITC) channel. Intact cells were gated in the Forward Scatter/Side Scatter (FSC/SSC) plot to exclude small debris. A total of 10,000 events were collected and resulting FL1 data were plotted on a histogram.

2.13 Nascent RNA and protein synthesis

Nascent RNA and protein synthesis were visualised respectively using Click-iT RNA Alexa Fluor 488 Imaging Kit (C10329, Lifetechnologies) and Click-iT HPG Alexa Fluor 488 Protein Synthesis Assay Kit (C10428, Lifetechnologies) following the manufacturer's instructions and images were taken using a 100x oil objective of LSM510 Meta confocal microscope mounted on Axiovert200M using pinhole size of 1 AU.

2.14 RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted using TRIzol Plus RNA Purification Kit (12183555, Lifetechnologies, UK). SHSY5Y treated or untreated with test compound, were lysed directly in the fume hood with 1mL TRIzol reagent for 5 min at room temperature. The lysates were resuspended and transferred to separate 1.5 mL tubes, mixed with 200

µL chloroform, agitated by hand vigorously for 15 sec, and incubated for 2-3 min at room temperature. The samples were spun at 12000 x g for 15 min at 4°C to separate the solution into a lower red phenol-chloroform phase, interphase, and colourless upper aqueous phase which contains the RNA. About 450 µL of the top aqueous phase from each sample was transferred to new RNase-free tubes and mixed vigorously with an equal volume of 70% ethanol to obtain 35% ethanol in the mixture and these were transferred to separate spin cartridges (with a collection tube), spun at 12,000 × g for 15 seconds at room temperature and the flow through was discarded. The cartridges were washed with buffer I, spun at 12,000 × g for 15 seconds at room temperature and further washed twice with buffer II at 12,000 × g for 15 seconds at room temperature. The cartridges containing the RNA were dried by additional spin at 12,000 × g for 1 min at room temperature. Using recovery tubes, the RNA from the different cartridges was eluted after a 5 min incubation in 30 µL RNase-free water and spun for 2 min at 16000 x g. The RNA extracts were stored on ice and used for cDNA synthesis. The total RNA extracted was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (4368814, Lifetechnologies, UK). A 20 µL cDNA reaction was prepared for each sample on ice, in PCR tubes, containing 10 µL total RNA and 10 µL 2x Reverse Transcription master mix supplemented with RiboLock RNase Inhibitor at a concentration of 1 U/µL of a reaction mixture (EO0381, Lifetechnologies). All tubes were briefly spun to eliminate bubbles and loaded to the thermal cycler (Biometra), programmed to run at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The cDNA was collected and used for qPCR.

2.15 Quantitative polymerase chain reaction (qPCR)

The synthesised cDNA from all samples were subjected to qPCR using Maxima Probe/ROX qPCR Master Mix (2X) Kit (K0232, Lifetechnologies) and TaqMan gene

expression assays (Life technologies, UK) (See Table 2.4 for the list). A 1X master mix sufficient for a 25 µL-reaction for all samples in duplicates was prepared from the Maxima Probe/ROX qPCR Master Mix (2X), 20X TaqMan gene expression assay and Nuclease-free water, and 20 µL of the mixture were transferred to required wells of a white 96-well semi-skirted PCR plate for Roche Lightcycler (I1402-9909-BC, StarLab, UK). A cDNA serial dilution of 1:1, 1:10, 1:100 and 1:1000 was prepared for standard curve measurement, and 5 µL of cDNA samples were transferred to corresponding wells of the 96-well PCR plate. A fresh master mix and standard curve were prepared for each assay and template negative controls containing only nuclease free H_20 were included in every amplification. Absolute qPCR was carried out on all samples using Roche LightCycler 480 II (Roche Diagnostics, Switzerland). The cycling conditions used were an initial run at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, and 50 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 60 °C for 1 min and finally cooling at 4°C. After the qPCR, transcript levels were automatically determined using the standard curve method by the Roche LightCycler 480 service software. Samples were normalised to TBP and ACTB.

2.16 Restriction digest for DNA methylation assays

To investigate the effect of tau knockdown on CpG methylation of rDNA, whole DNA extract was digested from control or Tau knockdown SHSY5Y cells with 2U/µL of HpaII (R6311, Promega) or MspI (R6401, Promega), or they were mock-digested, following which, we amplified the T0 region (which has 'CpG' sites) using specific primers at Tm 66°C (see Table 2.4) and ran samples on 10% agarose gel for quantitative analysis. For quantification purpose and to avoid loading errors, PCR was performed in a multiplex format, such that, primers against rDNA H41.9 region that does not have 'CpG' sites (so will not be cut by HpaII/Msp1), were also run in the same

reaction with the T0 primers. This enabled normalisation of the PCR product from the T0 region relative to the H41.9 product that is unaltered by the digestion and then compared the normalised values between control and tau knockdown cells. In this way, low levels of the HpaII-cut PCR product would indicate a reduction in methylation in the HpaII sites of the T0 region which allowed the digestion of the full-length product.

2.17 Statistical analysis

Data were first checked whether they passed *Kolmogorov-Smirnov* normality test, data that passed normality check were analysed by unpaired t-test using to establish significance criterion ($p < 0.05$). Data that failed to normality check were analysed by Mann-Whitney test to establish significance criterion ($p < 0.05$). GraphPad InStat software was used for all analyses.

Table 2. 1 Antibodies

Name and Catalogue number	**Dilution**	**Supplier**
Rabbit polyclonal anti-TAU antibody (SAB4501831)	IF: 1/100, TEM IG: 1/10 WB: 1:1000	Sigma-Aldrich
Mouse-Phosphor-Tau (Thr231) Antibody (MN1040)	IF: 1/50	Thermo Fisher Scientific
Mouse Anti-Tau-1 antibody (MAB3420)	IF: 1/200, TEM IG: 1/10, WB: 1/1000, IP: 1/40	Millipore
Rabbit Anti-Tau antibody (phospho T231) (EPR2488)	IF: 1/100, WB: 1:2000	Abcam
Mouse Anti-gamma H2A.X antibody (phospho S139) antibody [9F3]	IF: 1/500	Abcam
Rabbit Anti-HP1 alpha antibody [EPR5777]	IF:1/200, WB:1/2000, IP: 1/40	Abcam
Rabbit Anti-Fibrillarin antibody (ab5821)	IF:1/200, TEM IG: 1/20, WB:1/1000, IP: 1/40	Abcam
Mouse Anti-Histone H3 (tri methyl K9) antibody [ab6001]	IF:1/100	Abcam
Rabbit Anti-trimethyl-Histone H3 (Lys9) antibody (07-442)	IF:1/200	Millipore
Mouse Anti-Histone H3 (di methyl K9) antibody [ab1220]	1/200	Abcam
Mouse- Anti-CENPA antibody [3-19] (ab13939)	IF: 1/200	Abcam
Rabbit- Anti-CENPB antibody (ab25734)	IF: 1/500	Abcam
Rabbit Anti-UBF antibody (H-300) sc-9131	IF:1/200; WB:1/1000	Santa Cruz Biotechnology, Inc
Rabbit Anti-TIP5 Polyclonal Antibody (49-1037)	WB:1/200, TEM IG:1/50, IP: 1/40	Life technologies
Mouse Anti- 5-methylcytosine (5-mC) monoclonal antibody 33D3 (C15200081-100),	IF: 1/500	Diagenode
Rabbit- Anti-EIF2S1 (phospho S51) antibody [E90] (ab32157)	IF: 1/200	Abcam
Rabbit Anti-CENPB (H00001059-D01)	IP: 1/40	Abnova
Mouse Monoclonal Anti-β-Actin antibody (A5316)	WB:1/5000	Sigma-Aldrich
Mouse Anti-GADPH antibody	WB: 1/2000	Novus Biologicals
Normal mouse IgG (sc-2025)	IP: 1/40	Santa Cruz Biotechnology, Inc
Alexa Fluor® 555 Goat Anti-Mouse IgG (H+L) (A31622)	1/500	Invitrogen
Alexa Fluor® 555 Goat Anti-Rabbit IgG (H+L) (A31630)	1/500	Invitrogen

Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L)	1/500	Invitrogen
Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) (A31628)	1/500	Invitrogen
10 nm Gold-particle conjugated Goat anti-Rabbit IgG	1:10	British BioCell
15 nm Gold-particle conjugated Goat anti-Mouse IgG	1:10	British BioCell
5 nm Gold-particle conjugated Goat anti-Rabbit IgG	1:10	British BioCell
Goat Anti-Rabbit IgG H&L (HRP) (ab6721)	1/5000	Abcam
Anti-mouse IgG, HRP-linked Antibody (7076)	1/1000	Cell Signalling
Rabbit TrueBlot®: Anti-Rabbit IgG HRP (18-8816-31)	1/1000	Rockland Immunochemicals Inc.
Mouse TrueBlot® ULTRA: Anti-Mouse Ig HRP	1/1000	Rockland Immunochemicals Inc.

Key: IF – immunofluorescence, IP – Immunoprecipitation, TEM IG – Immunogold Transmission Electron Microscopy, WB – Western blotting

Table 2. 2 Brain tissues

Case	**Age**	**Sex**	**Pathological Diagnosis**
AD 1	77	F	Alzheimer's disease, modified Braak BNE stage 5
AD 2	68	M	Alzheimer's disease HP-tau stage 6 with mild to moderate amyloid angiopathy
Control 1	80	F	Minimal ageing changes
Control 2	66	M	Minimal ageing changes

Table 2. 3 siRNA sequence

siRNA Target name	Target sequence 1	Target sequence 2	Target sequence 3	Target sequence 4
Non-targeting Pool **(**D-001910-10-05**)**	UGGUUUACAUGUCGACUAA	UGGUUUACAUGUUUUCUGA	UGGUUUACAUGUUUUCCUA	UGGUUUACAUGUUGUGUGA
Human MAPT – Tau (E-012488-00-0005)	UGGUGAACCUCCAAAAUCA	CUUGCAAGUCCCAUGAUUU	UUGUGAUCUUAAAUGAGGA	UUAUUGAGUUCUGAAGGUU

Table 2. 4 List of primers used for ChIP, PCR and qPCR

Primer name	**Forward sequence**	**Reverse sequence**
T0	GCTCCCCGGCCCGGCGCT	CCATCGCAGCCACACACG
H42.9	CCCGGGGGAGGTATATCTTT	CCAACCTCTCCGACGACA
H41.9	CCGTGGGTTGTCTTCTGACT	AAGCGAAACCGTGAGTCG
H27	CCTTCCACGAGAGTGAGAAGCG	CTCGACCTCCCGAAATCGTACA
PrimPol	GCAACCCAGTTTTGAAACCA	TCGATGTCCAGCTTTCCTCT
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
TIP5	Taqman Assay (Lifetechnologies)	assay ID; Hs00203782_m1
Fibrillarin	TaqMan Assay (Lifetechnologies)	assay ID; Hs01070449_m1
UBF	Taqman Assay (Lifetechnologies)	assay ID; Hs00610730_g1
RNA18S5	TaqMan Assay (Lifetechnologies)	assay ID; Hs03928985_g1
RNA28S5	Taqman Assay (Lifetechnologies)	assay ID; Hs03654441_s1
TBP	Taqman Assay (Lifetechnologies)	assay ID; Hs00427620_m1
MAPT - Tau	Taqman Assay (Lifetechnologies)	assay ID; Hs00902194_m1
B-actin (ACTB)	Taqman Assay (Lifetechnologies)	assay ID; Hs01060665_g1

	Forward	Reverse	probe
RNA45S	CACCCTCGGTGAGAAAAG	CTACCATAACGGAGGCAG	CTTCTCTAGCGATCTGAGAGGCGTGCC

Chapter 3

3. Molecular mechanism of excitotoxicity: exploring a potential role of nuclear Tau

3.1 Abstract

Glutamate excitotoxicity, oxidative stress, DNA damage, alteration of the chromatin and nucleolar stress are key features of AD. The molecular link between these changes and the role of nuclear tau is unclear. Using differentiated neuroblastoma cells (SHSY5Y), we show that the induction of glutamate stress using 20 mM glutamate for 2h results in a significant induction of oxidative stress, induced a nuclear upsurge of phosphorylated tau and delocalised a species of tau localised in the nucleolus. To understand the importance of these changes, other molecular events occurring within the nucleus and nucleolus alongside the tau dynamics induced by the glutamate incubation were studied. This revealed a significant induction of DNA damage, chromatin instability, and nucleolar stress. Alongside, the glutamate incubation led to altered RNA synthesis and protein synthesis inhibition through the phosphorylation of the eukaryotic initiation factor 2 alpha on serine 51. This study provides a link between nuclear changes observed in AD neurons and altered nuclear and nucleolar tau. It also shows that glutamate toxicity, which is common in many tauopathies, especially AD, could be a contributor to the molecular signatures seen in these diseases. This work has been submitted for publication.

3.2 Chapter Introduction

AD and many tauopathies show signatures of glutamate excitotoxicity, accumulation of oxidative and nucleolar stress, DNA damage and alteration of the chromatin (Markesbery, 1997, Coppede and Migliore, 2009, Crapper et al., 1979, Parlato and Bierhoff, 2015, Frost et al., 2014, Dong et al., 2009). Recent evidence in models of tauopathy revealed a role for tau toxicity in the induction of oxidative stress; DNA damage; heterochromatin relaxation; aberrant gene expression and neuronal apoptosis (Frost et al., 2014, Khurana et al., 2012, Khurana et al., 2006, Dias-Santagata et al., 2007). Heterochromatin relaxation disrupts the integrity of the nucleolus, increase rDNA transcription, and is linked with normal ageing and the pathogenesis of AD (Larson et al., 2012, Frost et al., 2014, Peng and Karpen, 2007).

Although Aβ is one of the primary culprits in AD, glutamate excitotoxicity occurs downstream of Aβ toxicity (Ittner et al., 2010). Glutamate is a non-essential amino acid, and one of the principal excitatory neurotransmitters in the vertebrate brain, essential for many brain functions, such as synaptic plasticity, learning and memory, and maintenance of consciousness. Typically, in the synaptic vesicles, glutamate can reach a concentration of ~ 100 mM/L, while upon release, its concentration can go as high as 5 mM in the synaptic cleft, but this becomes cleared within milliseconds. The abnormal rise or decrease in clearance of glutamate in the synaptic cleft leads to hyperexcitation of postsynaptic neurons, which can result to neurotoxicity (Mark et al., 2001).

Since glutamate excitotoxicity could occur downstream of Aβ toxicity in AD, we set out to investigate the impact of glutamate stress on nuclear tau and whether this stress contributes to some of the changes observed in the AD brain, notably oxidative and

nucleolar stress, DNA damage and heterochromatin alteration. Here, we show that glutamate stress in differentiated SHSY5Y cells results in a significant induction of oxidative stress, the nuclear upsurge of phosphorylated tau alongside DNA damage, heterochromatin loss, nucleolar stress and protein synthesis inhibition. We also found that a nucleolar tau species previously thought to play a role only in undifferentiated cells still retain its nucleolar localisation in differentiated cells and becomes delocalised by the glutamate treatment. This finding links glutamate stress with some of the changes that define the AD brain and exposes a potential pathological involvement of nuclear tau in the pathology.

3.3 Results and Discussion

3.3.1 Tau localises to the nucleolus in differentiated SHSY5Y cells

Nuclear tau was first reported in JC and CG human neuroblastoma cells associated with the nucleolus using the non-phosphorylated tau antibody – Tau 1 (henceforth called nP-Tau) (Loomis et al., 1990). It was subsequently shown that nuclear tau exists in the human brain neurons, but it scarcely localises to the nucleolus, prompting the conclusion that its function is not needed in terminally differentiated neurons (Brady et al., 1995). We chose to use the human neuronal cell line - SHSY5Y neuroblastoma cells, as a model to investigate the soundness of this conclusion. This cell line expresses human tau at normal levels without overexpression in transfected or transgenic primary neurons. They can be differentiated to assume a post-mitotic phenotype that resembles neurons. Therefore, to explore the presence of nucleolar tau in more neuron-like cells, SHSY5Y cells were differentiated using retinoic acid and brain-derived neurotrophic factor (BDNF). This protocol generates terminally differentiated cells that phenotypical and biochemically resemble neurons, with morphologically clearly distinguishable extended neurites (Fig. 3.1A) (Jamsa et al., 2004, Encinas et al., 2000). The nucleolar localisation of tau was investigated after the differentiation using immunofluorescence labelling, revealing that nP-Tau colocalises with fibrillarin – a nucleolar marker, indicating that tau localises to the nucleolus even after differentiation (Fig. 3.1B). Antibodies against other nucleolar proteins were used to confirm the nucleolar localisation of tau and this revealed that nP-Tau similarly colocalises with the upstream binding transcription factor (UBF) and nucleophosmin (B3) in this cells (Fig. 3.1 C & D).

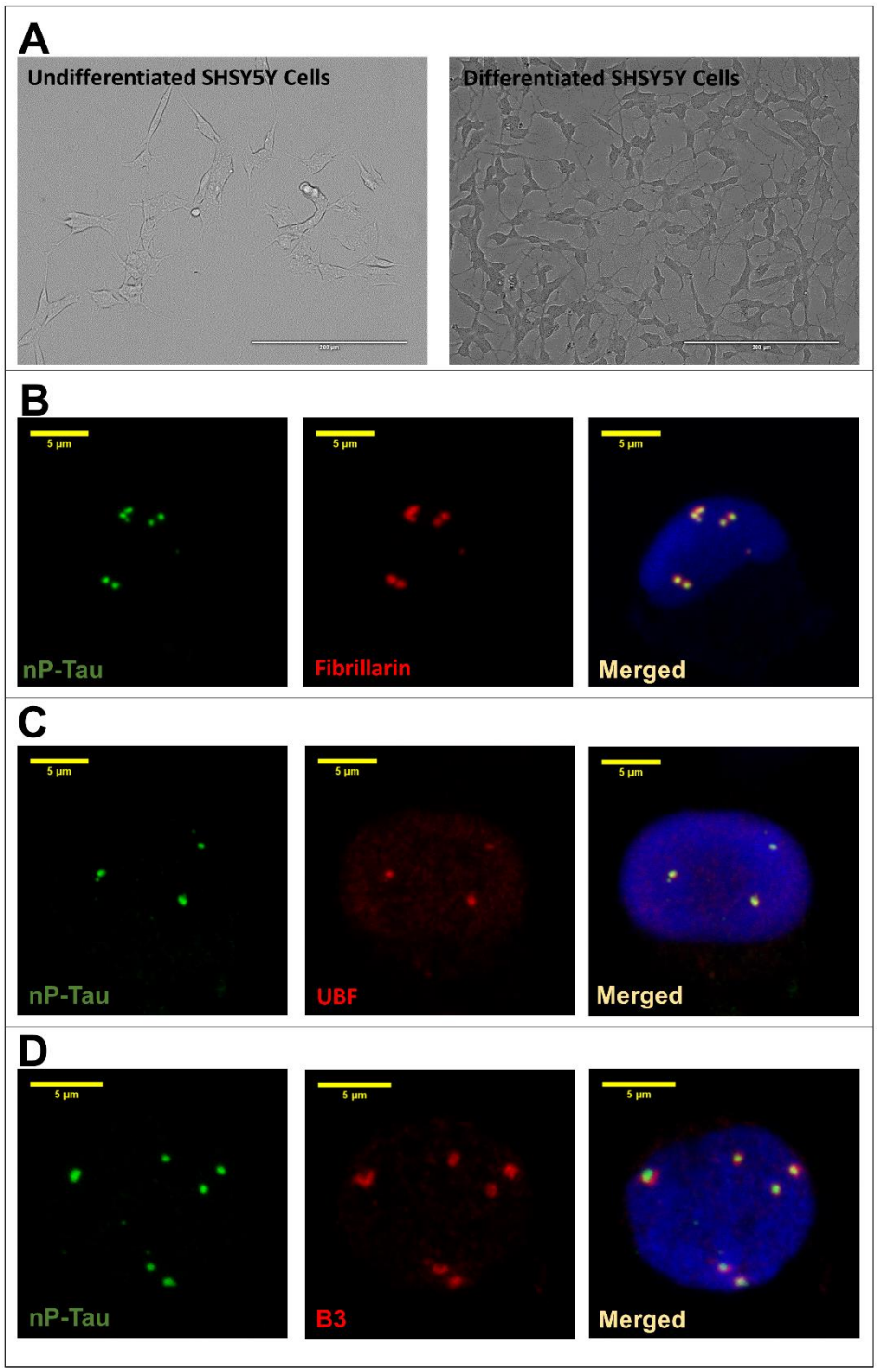

Figure 3. 1 Tau protein localises to the nucleolus in differentiated SHSY5Y cells.
(A) Undifferentiated or differentiated SHSY5Y with extended neurites. Immunofluorescence labelling using Tau 1 antibody (henceforth called nP-Tau) revealed that tau colocalises with fibrillarin (B), UBF (C) and nucleophosmin (B3) (D).

3.3.2 Cellular stress impact on different species of Tau in the nucleus

In light of the above findings suggesting a role for tau in the nucleolus of terminally differentiated neurons, the impact of cellular stress on nuclear tau was next investigated using high concentrations of glutamate to induce stress. Glutamate toxicity has been shown to occur in SHSY5Y cells via a ROS-dependent mechanism (Ha et al., 2010). Up to 80 mM glutamate in differentiated or undifferentiated cells causes a concentration-dependent toxicity at 48h (Nampoothiri et al., 2014). Therefore, the capacity of 20 mM glutamate to induce oxidative was next investigated in the differentiated SHSY5Y cells. CellROX Green reagent is widely used to quantify the level of oxidative stress. It is a cell permeant DNA dye fed to live cells that is weakly fluorescent in a reduced state. However, upon oxidation due to the accumulation of ROS, it binds the DNA and exhibits bright green fluorescence with absorption/emission maxima of ~ 485/520. Using this reagent with flow cytometry revealed that the glutamate incubation for 2h resulted in significant oxidative stress (increase to 191.8%), compared to the untreated control (87.4%) Fig. 3.2A). Early response to cellular stress in primary neurons has been shown to be associated with a rapid decrease in tau phosphorylation on Thr231, concomitant with an increase in nP-Tau (Galas et al., 2006), likely to prevent tau hyperphosphorylation (Bulbarelli et al., 2009). We wondered whether the glutamate incubation would also exert a similar effect on the tau molecule in the differentiated SHSY5Y cells. Using western blotting of whole cell extracts from control and glutamate-treated cells revealed an increase in nP-Tau, concurrent with a decrease in phosphorylated tau (Thr231) (henceforth called P-Tau), with no changes to the total cell levels of total tau (T-Tau) (Fig. 3.2B).

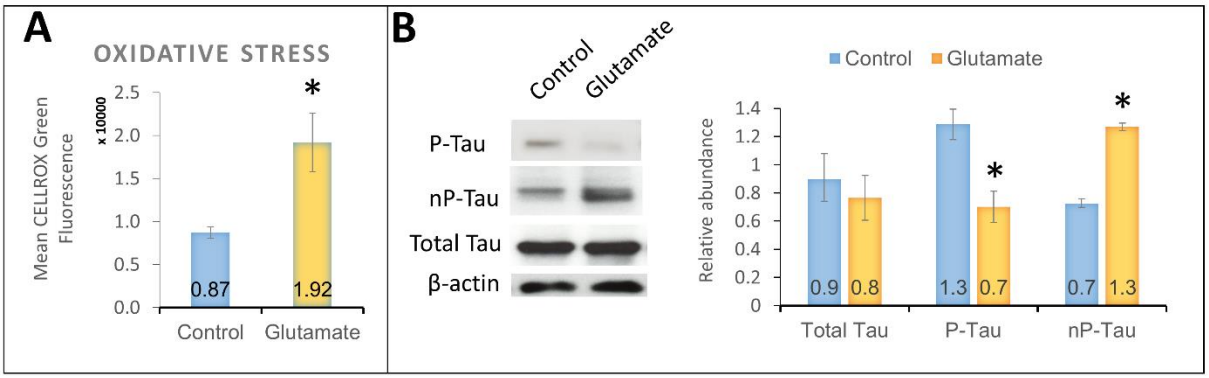

Figure 3.2 Glutamate induces oxidative stress and impact on tau phosphorylation.
(A) Flow cytometry experiments with CellROX Green revealed an increase in oxidative stress in 20 mM glutamate-treated cells. (P=0.0013). (B) Western blotting on whole cell extracts showed a significant decrease in P-Tau (P=0.003), concomitant with an increase in non-phosphorylated tau (nP-Tau) (P<0.0001), with no changes in total tau (T-Tau) levels (P=0.47). Experiments repeated five independent times.

The accumulation of nuclear phosphorylated tau has been shown to occur following cellular stress (Noel et al., 2016, Lu et al., 2013a). Interestingly, by focusing on the nucleus using immunofluorescence, we found that the glutamate incubation also led to a nuclear increase in total tau (Fig. 3.3A), which appears to be mainly tau phosphorylated on Thr231 (Fig. 3.3B). In fact, we observed very low levels of nuclear P-Tau in untreated cells, consistent with findings showing that nuclear tau is mainly non-phosphorylated (Lu et al., 2014). Therefore, there may be an overall decrease in total cell P-Tau. However, glutamate stress selectively leads to its accumulation in the nucleus.

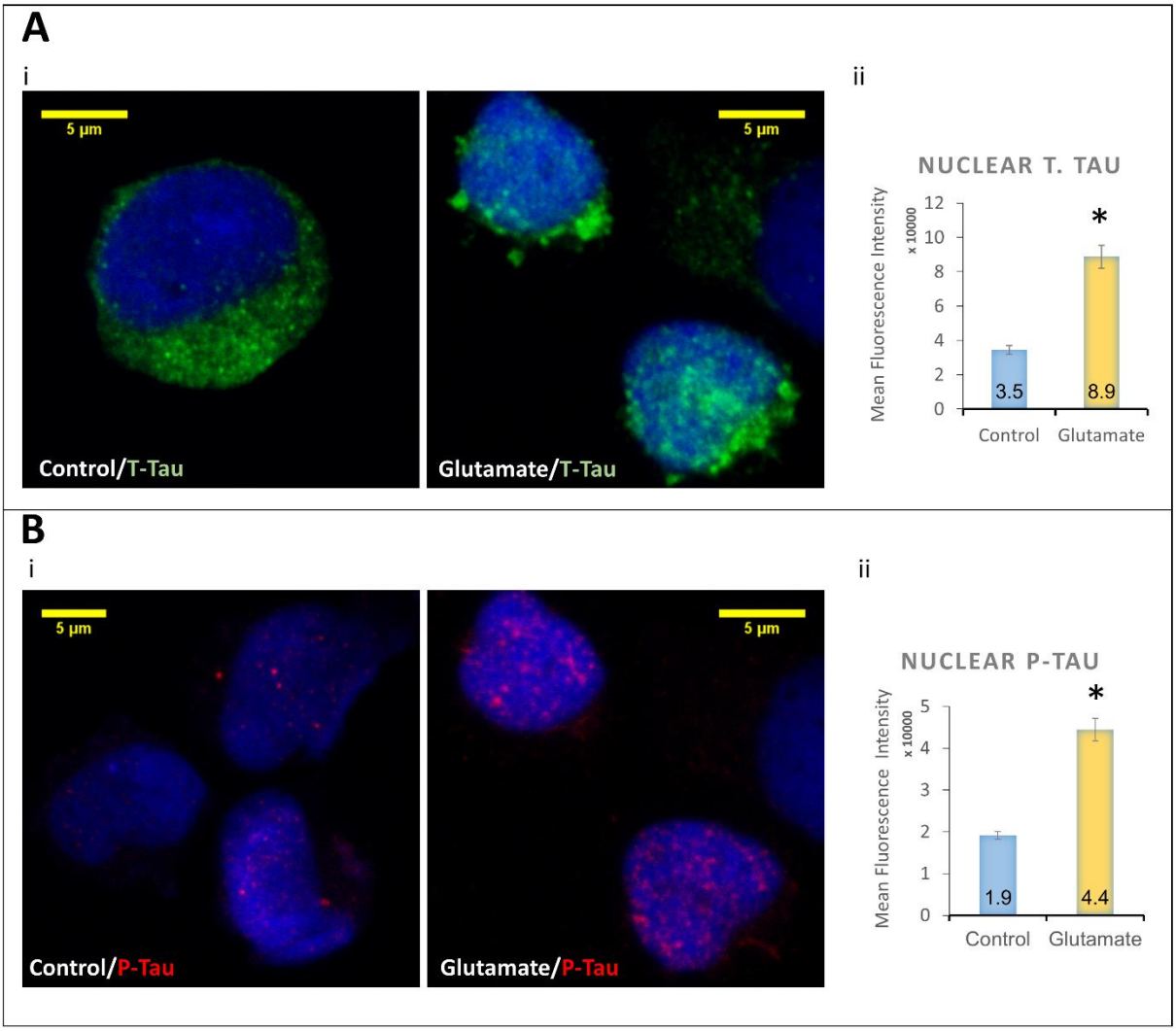

Figure 3.3 Cellular stress induces the accumulation of nuclear phospho-tau.
Immunofluorescence microscopy showed a significant increase in nuclear levels of total tau (T. Tau) ($P<0.0001$) (Ai-ii), which seemed to be phosphorylated on the Thr231 (P-Tau) ($P<0.0001$) (Bi-ii). Experiments repeated five independent times.

Considering that the nP-Tau shows mainly nucleolar localisation, it is not clear whether when P-Tau accumulates in the nucleus, it localises to the nucleolus. Double immunofluorescence labelling for both untreated and glutamate-treated cells showed that P-Tau does not colocalise with fibrillarin (Fig. 3.4A) and does not colocalise with nP-Tau in stressed cells (Fig. 3.4B). The result potentially indicates that the nuclear phosphorylated tau induced by glutamate accumulates in a different nuclear compartment, other than the nucleolus.

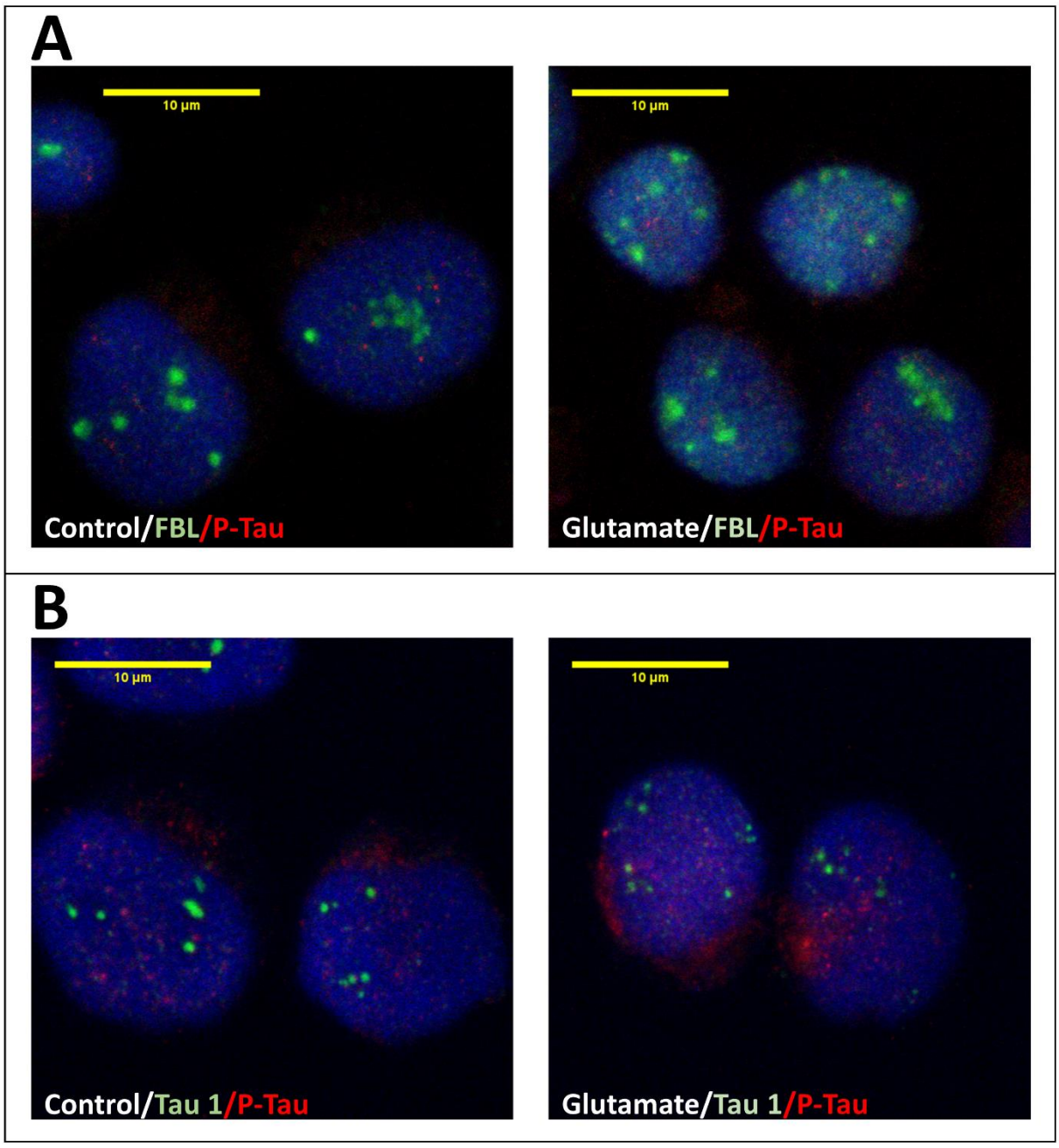

Figure 3.4 Nuclear phospho-tau does not colocalise with nucleolar proteins.
(A) Double labelling revealed that the nuclear P-Tau does not colocalise with fibrillarin in control and glutamate-treated cells. (B) Similarly, it localises to separate locations with nucleolar Tau 1 (nP-Tau). Cells in the glutamate group were treated for 2h with 20 mM glutamate which was dissolved in serum free media. For the cells in the control group, their media was replaced with serum free media for the 2h duration of the experiment.

Previously, the accumulation of nuclear phosphorylated tau has been reported in Aβ-treated SHSY5Y cells (Noel et al., 2016), formaldehyde-treated N2A neuroblastoma cells, primary neurons and mouse brain (Lu et al., 2013a). In N2A cells, the nuclear phosphorylated tau was coincident with DNA damage and reduction of cell viability,

thus suggesting a role for this tau species in these changes (Lu et al., 2013a, Lu et al., 2013b). Indeed, nuclear-hyperphosphorylated tau appears to be a feature of neurodegeneration, as it has been previously localised in the nucleus, but not nucleolus, of a patient with presenile dementia with motor neuron disease (Papasozomenos, 1995). Therefore, the absence of co-localisation between P-Tau and fibrillarin found here may indicate a physiological relevance of our findings.

A standard feature of nucleolar stress is the reorganisation of the nucleolus, which often leads to the redistribution of nucleolar proteins, like fibrillarin (Kodiha et al., 2011, Boulon et al., 2010). The distribution of fibrillarin was quantified using immunofluorescence labelling, revealing that 33% of the glutamate-treated cells showed fibrillarin redistribution to the nucleoplasm or cytoplasm (Fig. 3.5).

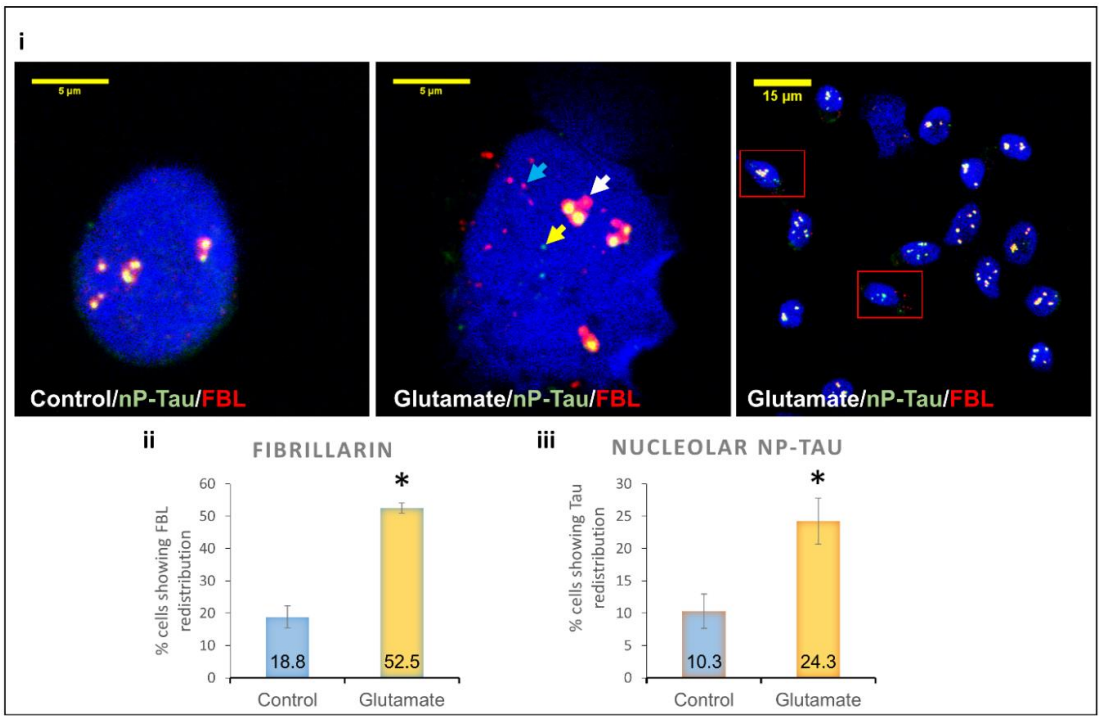

Figure 3. 5 Cellular stress causes the redistribution of nucleolar nP-Tau.
Glutamate administration resulted in the redistribution of fibrillarin (blue arrows) and nucleolar nP-Tau (yellow arrows) (i), and a significant increase in the number (33%) of cells showing fibrillarin redistribution (P=0.02) (ii). Immunofluorescence labelling showed that some of these cells show cytoplasmic relocalisation of nucleolar nP-Tau and fibrillarin (i, see red box). Quantification revealed that 14% of glutamate-treated cells showed nucleolar nP-Tau redistribution (P=0.02) (iii). Experiments repeated four independent times.

We also examined whether nucleolar nP-Tau showed similar stress-induced redistribution. Interestingly, although to a lesser extent to the fibrillarin redistribution, approximately 14% of the glutamate-treated cells also showed nucleolar nP-Tau redistribution to the nucleoplasm or cytoplasm (Fig. 3.5 iii). All cells that showed nucleolar nP-Tau redistribution exhibited fibrillarin redistribution, while 19% of the cells showed only fibrillarin redistribution and some showed a diffuse and decreased fibrillarin signal that is non-punctate, indicating the fibrillarin may have degraded. This may suggest that the fibrillarin is more sensitive to the stress than the nucleolar nP-Tau. However, it also suggests that nucleolar nP-Tau can be redistributed following cellular stress and may lead to its reduction in the nucleolus, similar to other nucleolar proteins (Kodiha et al., 2011). Overall, these results revealed that glutamate-induced stress impacts on P-Tau and nucleolar nP-Tau in different ways, indicating that the altered distribution of tau species in the nucleus induced by the stress may have consequences in the nucleus.

3.3.3 Nuclear P-Tau and redistributed nucleolar nP-Tau occur with DNA damage, heterochromatin loss and nucleolar stress

To understand the consequence of the increase in nuclear P-Tau and the changes in nucleolar nP-Tau, the nuclear and nucleolar events that are associated with both changes were next examined (Fig. 3.6 & 3.7). Transmission electron microscopy reveals apoptotic cells characterised by chromatin condensation and nuclear fragmentation (Ziegler and Groscurth, 2004). With assistance from Dr Julian Thorpe who performed embedding protocols and cut thin sections and processed the differentiated cells for ultrastructural analysis, we observed no obvious changes in nuclear morphology typical of dying cells following the glutamate incubation (Fig. 3.6A). However; several lines of evidence showed that various toxic stimuli, such as

cellular stress, lead to the phosphorylation of the histone variant H2Ax on serine 139, called γH2Ax, serving as a marker for DNA damage (Valdiglesias et al., 2013). By immunofluorescence labelling, γH2Ax forms foci or pan-nuclear staining in the nucleus indicating the site of DNA damage (Valdiglesias et al., 2013). Consistent with this, immunofluorescence labelling for γH2Ax showed that the glutamate incubation resulted in a significant degree of DNA damage (Fig. 3.6B). In support of this finding, previous findings demonstrated that DNA damage in primary cerebellar cultures occurs in the early stage of excitotoxicity before the cells enter an irreversible stage of injury (Didier et al., 1996). Taken together, this suggests that glutamate stress leads to the co-occurrence of the nuclear accumulation of P-Tau, redistributed nucleolar nP-Tau and DNA damage before the apparent sign of cell death.

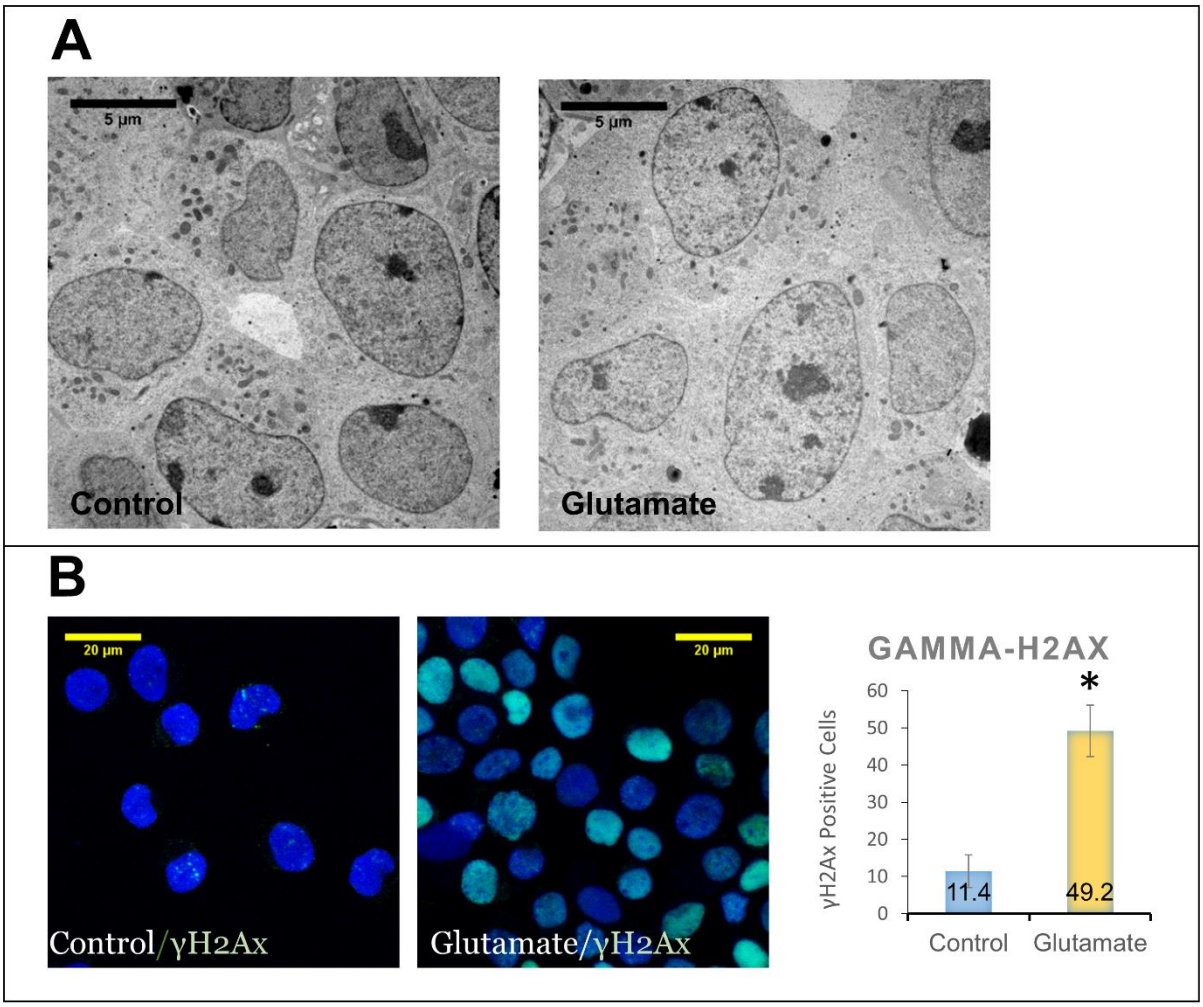

Figure 3.6 Altered nuclear tau species co-occur with DNA damage.
Incubation of the differentiated SHSY5Y cells with glutamate did not cause noticeable morphological changes to the nucleus at 2h when compared to untreated cells (A). However, it resulted in a significant increase in γH2Ax foci-positive cells compared to the control (P=0.02) (B). Experiment in A repeated once, and B, repeated four independent times.

Tau has been identified associated with the pericentromeric heterochromatin (Sjoberg et al., 2006). Pathological tau species, identified by both TG-3 and Alz-50 antibodies (See Fig. 1.6), have also been localised to the heterochromatin in AD brain (Luna-Munoz et al., 2005). Although DNA damage is known to induce relaxation of the heterochromatin (Cann and Dellaire, 2011), heterochromatin relaxation has been shown to occur downstream of aberrant tau phosphorylation (Frost et al., 2014). Therefore, we next investigated whether the increased nuclear P-Tau and redistributed nucleolar nP-Tau are associated with heterochromatin relaxation following glutamate-

induced stress. The heterochromatin is a chromatin domain that is compact and less permissive to transcription, comprised of impermissive epigenetic markers such as HP1α, H3K9me2, and H3K9me3, constituents of both nuclear and nucleolar heterochromatin (Bártová et al., 2010, Chen et al., 2011). Immunofluorescence labelling for HP1α and H3K9me3 showed that they concentrate in foci that form constitutive heterochromatin foci (Fig. 3.7A), while labelling for H3K9me2 showed a pan-nuclear staining (Fig. 3.7Bi). This is not surprising because H3K9me2 shows more preference to the facultative heterochromatin, while HP1α and H3K9me3 are enriched in constitutive heterochromatin (Trojer and Reinberg, 2007). The impact of glutamate incubation for 2h on the heterochromatin was next examined, revealing a significant decrease in the markers for constitutive heterochromatin, as indicated by the decrease in H3K9me3 and HP1α foci (Fig. 3.7C & D), without changes in H3K9me2 levels (Fig. 3.7Bii). The facultative heterochromatin is dynamic, modifiable temporarily (e.g. during cell cycle), spatially (e.g. changes in nuclear localisation) and/or through inheritance (e.g. monoallelic gene expression) (Trojer and Reinberg, 2007). Thus, the lack of noticeable changes in the levels of H3K9me2 indicates that the facultative heterochromatin is unaffected by the glutamate stress.

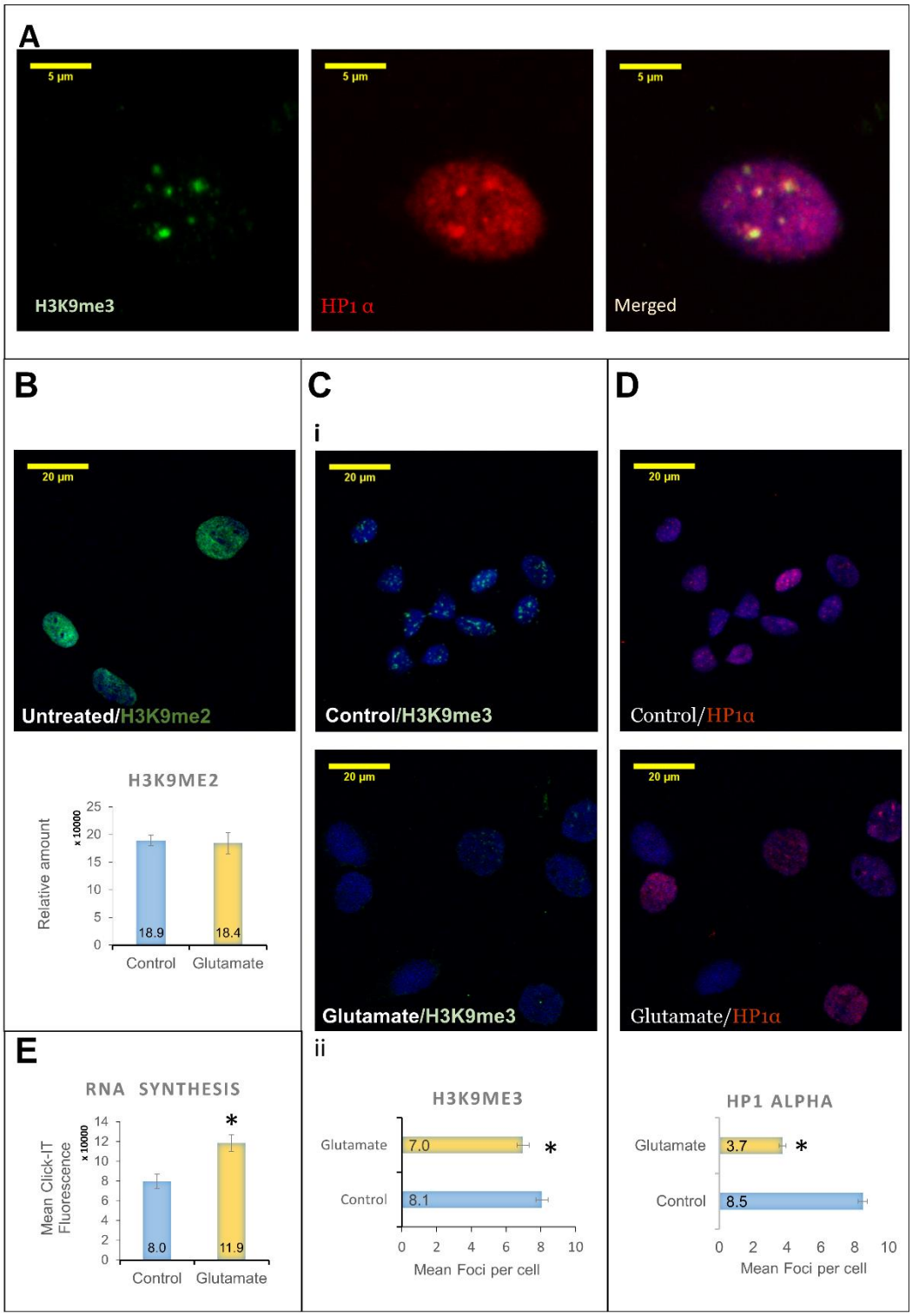

Figure 3.7 Altered nuclear tau species co-occur with heterochromatin loss.
(*A*) Double immunofluorescence staining with mouse anti-H3K9me3 and Rabbit anti-HP1α showed that they colocalise to form constitutive heterochromatin foci. Staining for H3K9me2 showed pan-nuclear staining, which didn't change following glutamate treatment (P=0.76) (B). Incubation of the cells with glutamate resulted in a significant decrease in clusters for H3K9me3 (P=0.03) (Ci & ii) and HP1α (P<0.0001) (Di & ii). (E) Quantitative Click-iT RNA Alexa Fluor 488 immunofluorescence labelling showed a significant increase in global nascent RNA synthesis in the glutamate-treated cells (P<0.0001). Experiments repeated four independent times.

The loss of the constitutive heterochromatin following the glutamate treatment suggests an alteration in the transcriptional state of the chromatin. Evidence from *Drosophila*, mouse and human tauopathy models showed widespread constitutive heterochromatin relaxation concomitant with an increase in transcription of heterochromatic genes (Frost et al., 2014). Therefore, we next examined whether the loss of the heterochromatin observed is associated with changes in RNA production using Click-iT RNA Imaging assay. The assay detects the global level of nascently synthesised RNA based on the incorporation of an alkyne-modified nucleoside, 5-ethynyl uridine (EU), to RNA during RNA synthesis. EU is fed to live cells, and following its incorporation to newly synthesised RNA, the RNA is detected with an azide-containing dye by utilising a "click" reaction between an azide and the alkyne on the EU. The results from the Click-iT RNA assay revealed that the glutamate stress led to a significant increase (increase to 118.5%) in RNA synthesis compared to untreated (79.9%) (Fig. 3.7E), suggesting that the heterochromatin loss allows an aberrant increase in nuclear transcription.

Since the glutamate treatment led to the redistribution of fibrillarin and nucleolar nP-Tau, we next investigated whether this affected other nucleolar functions by looking at the key proteins involved in silencing (TIP5) or activating (UBF) rDNA transcription. TIP5 mediates the silencing of a fraction of rDNA, leading to heterochromatin formation and transcriptional silencing, while UBF is a nucleolar transcription factor that drives the transcription of rDNA (Grummt, 2010). Western blotting revealed a significant decrease in TIP5, UBF and fibrillarin (FBL) in glutamate-treated cells (Fig. 3.8A). We expected an inverse change between TIP5 and UBF since they are antagonistic of one another (Grummt, 2010). Therefore, the overall decrease in these

nucleolar proteins indicates that the glutamate treatment directly affected the nucleolus causing its reorganisation.

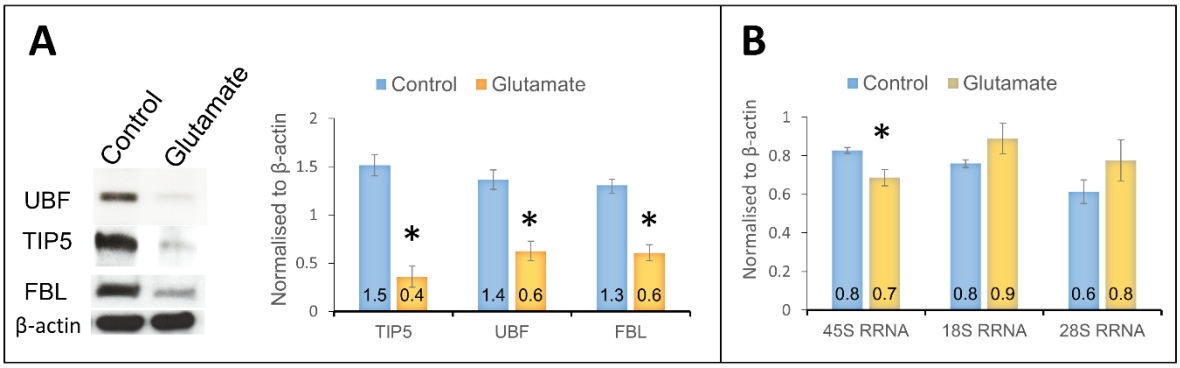

Figure 3.8 Altered nuclear tau species co-occur with nucleolar stress.
(A) Western blotting analysis revealed that the glutamate treatment led to a significant decrease in TIP5, UBF, and FBL. (TIP5 P<0.0001; UBF P=0.0004; FBL P=0.0002). (B) qPCR analysis of rDNA transcription and processing showed that the glutamate incubation resulted in a significant decrease in 45S pre-rRNA synthesis with no changes in the processing of 18S rRNA and 28S rRNA. (45S pre-rRNA P=0.008; 18S rRNA P=0.16; 28S rRNA P=0.32). Experiments in A repeated five independent times and B repeated four independent times.

Different cellular stress feeds into the nucleolus, leading to the regulation of the energy consuming process of ribosome biogenesis through the inhibition of rDNA transcription, in this way allowing for the regulation of energy expenditure during stress. This process is accompanied by a rapid mislocalisation and degradation of nucleolar proteins (Cohen et al., 2008, Boulon et al., 2010). Therefore, we next investigated whether glutamate stress alters rDNA transcription, which is also normally blocked when cells are under nucleolar stress (Boulon et al., 2010). Typically, rDNA transcription produces long 45S pre-rRNA, which is subsequently processed to 18S and 28S rRNA that ultimately contributes to ribosome formation and protein synthesis (See Fig. 1.7, Chapter 1). qPCR revealed that the glutamate treatment led to reduction in rDNA transcription, as revealed by a decrease in 45S pre-rRNA (82.8% in control, compared to 68.7% in Glutamate –treated cells (Fig. 3.8B).

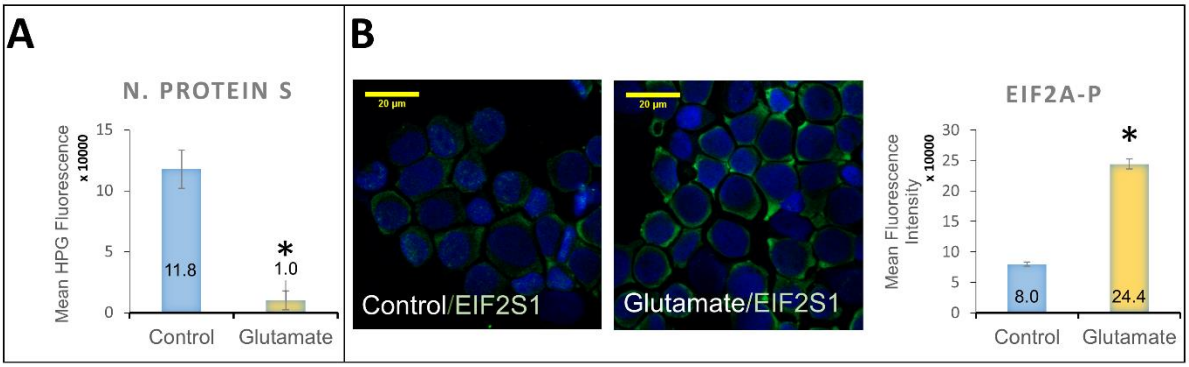

Figure 3.9 Glutamate inhibit protein synthesis through eIF2α phosphorylation.
(A) Quantitative Click-iT HPG Alexa Fluor 488 immunofluorescence labelling showed a global decrease in nascent protein synthesis in the glutamate-treated cells ($P<0.0001$). (B) Quantitative immunofluorescence labelling for phosphor S51 eukaryotic translation initiation factor 2A (eIF2α-P) showed a significant increase in the glutamate-treated cells ($P<0.0001$). Experiments repeated three independent times.

Increased global RNA synthesis induced by the glutamate treatment (Fig. 3.7) would be expected to lead to increased protein synthesis. Therefore we used Click-iT HPG assay to quantify the level of nascently synthesised proteins. The assay utilises HPG (L-homopropargylglycine) - an amino acid analogue of methionine with an alkyne moiety that can be fed to live cultured cells and become incorporated into proteins during active protein synthesis. The newly synthesised proteins are detected via a "click" reaction between an azide and alkyne, where the alkyne-modified protein is detected with azide-containing dye. Interestingly, the Click-iT HPG imaging assay revealed a marked reduction in global protein synthesis (118% in control to 10.2% in control) (Fig. 3.9A). This suggests that although the glutamate treatment induces a global increase in cellular RNA in the cells, the RNA were not translated into protein. This seems to posit a paradox. One would expect the increased global RNA synthesis induced by the glutamate treatment to turn to increased protein synthesis. Although, the decreased rDNA transcription should normally translate to a drop in protein synthesis since the level of rDNA transcription reflects the degree of protein synthesis. The absence of significant changes in the levels of 18S and 28S rRNA products ruled

out that possibility since their contribution to ribosome formation may not have been altered (Fig. 3.8B). However, cells exposed to stress inhibit protein synthesis to reduce cellular energy expenditure and the production of unwanted proteins that could interfere with the stress response. They achieve this by partly initiating the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) at Serine 51 which results in the inhibition of protein synthesis (Holcik and Sonenberg, 2005). The phosphorylation of eIF2α has been reported in many tauopathies that show excitotoxic neurodegeneration, such as AD (Hoozemans et al., 2009, Hoozemans et al., 2005). Indeed, quantitative immunofluorescence labelling for eIF2α revealed a marked increase in its fluorescence following the glutamate treatment indicating an increase in its phosphorylation on serine 51 (Fig. 3.9B). This provides a plausible explanation for the paradox since even if there is an increased level of RNA, phosphorylation of eIF2α can inhibit the downstream protein synthesis.

3.4 Conclusion

Overall, our results provide evidence for a nucleolar role for tau in terminally differentiated cells. It also suggests that glutamate stress could contribute to some of the changes that occur in the AD brain, notably DNA damage, heterochromatin instability and nucleolar stress. The increase in nuclear P-Tau observed here may indicate a deleterious role for P-tau in the nucleus of stressed cells, in support of findings which linked nuclear phosphorylated tau with DNA damage (Lu et al., 2013a, Lu et al., 2013b). Importantly, the redistribution of nucleolar nP-Tau observed here suggests a novel nucleolar role for tau following cellular stress, likely contributing to alteration of the stability and function of the nucleolus. The heterochromatin and nucleolus share protein pools important for the stability of one another (Bártová et al., 2010, Guetg et al., 2010). Given the localisation of tau to these compartments (Sjoberg et al., 2006, Mansuroglu et al., 2016, Luna-Munoz et al., 2005), the upsurge in nuclear P-Tau and delocalisation of nucleolar nP-Tau observed here suggests that these changes could contribute to the heterochromatin alteration and nucleolar stress observed. Given that tau is mostly known for its role in microtubule destabilisation and tangle formation in AD, this finding calls for research into the pathological involvement of tau in nuclear dysfunction in AD.

The next step would be to uncover the pathway via which glutamate induces the cellular stress and associated changes observed. Normally, an excessive amount of glutamate induces stress via over activation of its receptors. It is not clear whether glutamate receptors in the SHSH5Y cells are the mediators of the glutamate stress (Kritis et al., 2015). Therefore it would be interesting for future experiments to use glutamate channel blockers to investigate whether the changes observed here would be prevented. The glutamate concentration used was very high, future studies should

titrate the lowest concentration needed to induce changes in the SHSY5Y cells and investigate whether at such concentration, the glutamate could still induce the changes observed here or whether a different pathway would be impacted upon instead. Several kinases are known to induce eIF2α phosphorylation, while not much is known about kinases that induce nuclear tau phosphorylation. Therefore, it would be interesting for future studies to investigate the upstream kinases that induces the phosphorylation of eIF2α and nuclear P-Tau observed in this work.

Chapter 4

4.0 Amyloid beta oligomers alter nucleolar Tau localisation and drive protein synthesis dysfunction

4.1 Abstract

It is believed that Aβ42 oligomers, rather than fibrils, are the neurotoxic species and their levels in the AD brain correlate with the severity of dementia, suggesting that they play a critical role in the pathogenesis of the disease. Here, we show that the incubation of differentiated neuroblastoma cells (SHSY5Y) with freshly prepared Aβ42 oligomers initially induced oxidative stress and subtle nucleolar stress without significant DNA damage or cell viability loss, which worsen over time to inhibit protein synthesis by decreasing rRNA synthesis and processing and global level of newly synthesised RNA. Aβ toxicity has been linked to tau phosphorylation and localisation, here, we show that Aβ42 oligomers also altered tau phosphorylation and its abundance in the nucleolus. This finding provides direct evidence for the involvement of Aβ42 in nucleolar and protein synthesis machinery alteration *in vitro*, which replicates what is observed in mild cognitive impairment and early AD in the absence of mass neuronal death. It also supports findings in Chapter 3 implicating altered distribution of nucleolar tau in nucleolar stress. This work has been submitted for publication.

4.2 Chapter Introduction

The reformulated amyloid cascade hypothesis posits that soluble, oligomeric Aβ species are the mediators of neuronal toxicity (Selkoe and Hardy, 2016). These species have been found to correlate with the severity of dementia in the disease (McLean et al., 1999, DaRocha-Souto et al., 2011). Moreover, Aβ, especially Aβ42, has been found to cause excitotoxicity (Ittner et al., 2010), synaptic aberration (Lacor et al., 2007), disrupt long-term potentiation (Lambert et al., 1998), and cause memory dysfunction (Zhang et al., 2014), indicating that the Aβ42 oligomers cause a gradual disturbance in cell function before neuronal loss (Pedersen et al., 1996).

Chapter 3 described results showing that glutamate treatment induces oxidative stress, DNA damage, heterochromatin loss and nucleolar stress, implicating it in some of the changes that occur in the AD brain (Chapter 3). Although studies have shown that glutamate excitotoxicity occurs downstream of Aβ toxicity (Ittner et al., 2010), both can influence one another (Molinuevo et al., 2005). However, several studies indicate that Aβ toxicity can occur via several other mechanisms. To dissect this, work in the Serpell Lab has previously shown that Aβ42 oligomers enter SHSY5Y cells before cell death leading to lysosomal damage (Soura et al., 2012). In rat primary hippocampal neurons, the Serpell Lab has also demonstrated that Aβ42, but not its non-toxic variant, become internalised and alter synaptic vesicle recycling properties (Marshall et al., 2016), suggesting a critical role for Aβ42 in driving the neurochemical changes in AD (Benilova et al., 2012). The high concentration of glutamate used in Chapter 3 induced several changes, some of which may not be widespread in people with MCI and early AD. To investigate the culprit and pathway that drives some of the changes that occur in MCI and early AD, here we used the post-mitotic neuron-like model; differentiated SHSY5Y cells, to examine the hypothesis that short-term exposure to

Aβ42 could mimic the early changes that occur in the disease when no widespread neuronal loss is visible. To this end, we show that the incubation of differentiated SHSY5Y with Aβ42 oligomers causes oxidative stress, with gradual accumulation of nucleolar stress, which leads to altered transcription and processing of 45S rRNA, heterochromatin compaction and a decrease in RNA and protein synthesis, without significant loss of cell viability and DNA damage. This provides evidence of the involvement of Aβ42 toxicity in nucleolar and protein synthesis machinery alteration that were reported in MCI and early AD (Hernandez-Ortega et al., 2015, Ding et al., 2005). The Aβ42 oligomers incubation also led to alteration of the phosphorylation state and localisation of nuclear tau protein, which has also been observed in AD progression in the human hippocampus (Hernandez-Ortega et al., 2015). This demonstrates that the early changes in Aβ42 levels that occur decades before full-blown AD (Jack et al., 2013) could contribute to the ribosome and protein synthesis machinery alteration that occurs at the early stage of the disease.

4.3 Results and Discussion

4.3.1 Aβ42 induces oxidative stress and alters Tau phosphorylation and localisation without loss of cell viability and DNA damage

In AD, changes in Aβ42 levels appear decades before the onset of dementia (Jack et al., 2013). Hence it is thought that the Aβ causes subtle changes in neuronal function that gradually leads to cell death. Therefore, the capability of Aβ42 oligomers to impact on the cell viability of differentiated SHSY5Y cells was first investigated after 24h exposure. Consistent with previous findings from human cortical slices treated with Aβ oligomers for 24h (Sebollela et al., 2012), the incubation of differentiated SHSY5Y cells with freshly prepared 10 μM Aβ42 oligomers for 24h did not significantly affect their viability (Fig. 4.1A). We next examined whether the Aβ causes DNA damage using a well-known DNA damage marker - γH2Ax foci (Valdiglesias et al., 2013). The Aβ-treated differentiated SHSY5Y showed no significant increase in γH2Ax foci formation (Fig. 4.1B). In contrast, it has been previously shown that undifferentiated SHSY5Y cells treated with $A\beta_{25-35}$ for 24h accumulate oxidative stress and DNA damage (Martire et al., 2013). In primary neurons, Aβ42 treatment was shown to induce the accumulation of ROS (De Felice et al., 2007). Therefore, CellROX Green flow cytometry assay was used to investigate whether the Aβ incubation causes oxidative stress. Interestingly, this induced a significant oxidative stress (142% in control to 197% in Aβ-treated cells) (Fig. 4.1C), albeit lower to the stress induced by glutamate which increased to 191.8% from control value of 87.4% (Chapter 3). Therefore, unlike glutamate, the findings suggest that Aβ42 can selectively cause oxidative stress, without exerting significant cell viability loss or DNA damage over the 24 h time frame of the experiments. Indeed, several lines of evidence revealed that Aβ42 could induce oxidative stress, which is thought to play a critical role in AD progression (Butterfield et al., 2007, Butterfield et al., 2013).

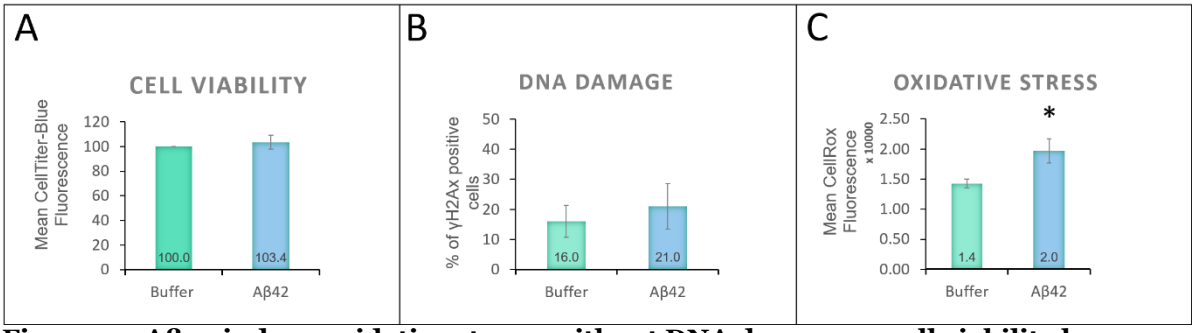

Figure 4.1 Aβ42 induce oxidative stress, without DNA damage or cell viability loss
(A) CellTiter-Blue experiment showed that the 24h Aβ incubation did not affect cell viability. (P= 0.103). (B) Quantification of γ-H2Ax foci-positive cells showed the absence of DNA damage following the 24h Aβ incubation. (p= 0.13). (C) Flow cytometry experiment with CellROX Green showed that the 24h Aβ incubation induces a significant level of oxidative stress. (P=0.03). Experiments repeated five independent times.

Aβ toxicity is widely believed to aberrantly impact on tau protein. Likewise, several studies suggest that tau modifications, such as phosphorylation/dephosphorylation, may be a sign of general cellular stress (Zambrano et al., 2004, Kátai et al., 2016, Egaña et al., 2003, Galas et al., 2006). The results in Chapter 3 showed that cellular stress induced by glutamate treatment alters tau phosphorylation on Thr231 (P-Tau) and Ser 195, 198, 199, and 202 identified by the Tau-1 antibody (nP-Tau) (Maina et al., 2017, *submitted*). Therefore, western blotting was used to investigate whether the Aβ treatment impacts on tau phosphorylation state. Whole cell lysates of differentiated SHSY5Y cells incubated with Aβ42 for 24h showed a significant decrease in P-Tau and the associated reduction in nP-Tau (Fig. 4.2). This occurred without any change in the whole cell total tau levels (T-Tau) (Fig. 4.2). A decrease in nP-Tau means an increased phosphorylation on Ser 195, 198, 199, and 202.

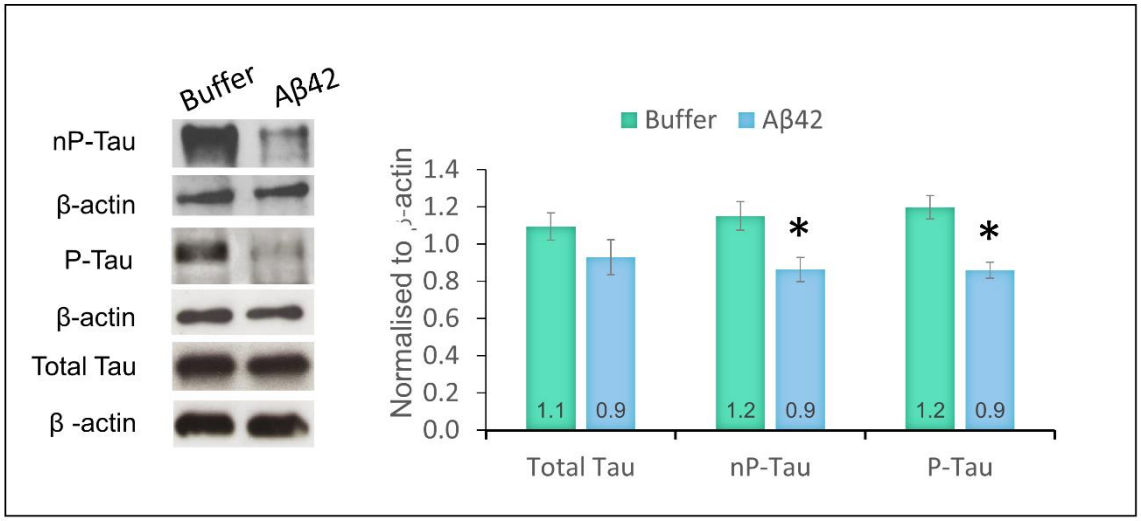

Figure 4.2 Aβ42 alter the phosphorylation of tau epitopes.
Western blotting on whole cell extracts showing the levels of Tau phosphorylated on Thr231 (P-Tau) (P= 0.003), Tau-1 (nP-Tau) (P= 0.017) and total tau (T-Tau) (P= 0.103) following Aβ administration for 24h. Normalised to β-actin. Experiments repeated five independent times.

Considering the several epitopes on the tau molecule that can be post-translationally modified (Martin et al., 2011), these changes suggests a dynamic phosphorylation of different epitopes of tau due to the Aβ stress. Consistent with this, it has previously been shown that incubation of primary neurons with Aβ42 for up to 8h leads to a Pin1-mediated dephosphorylation of tau on Thr231, Ser199, Ser396, Ser400, and Ser404, with a progressive increase in its phosphorylation on Ser262 (Bulbarelli et al., 2009). This has been suggested to serve as an early response to prevent Aβ-induced tau hyperphosphorylation (Bulbarelli et al., 2009), which can be influenced critically by its phosphorylation at Thr231 (Lin et al., 2007). Furthermore, a recent study revealed that Aβ induces the phosphorylation of tau on Threonine 205, as an early mechanism for neuroprotection against excitotoxicity (Ittner et al., 2016). Thus, it seems that different stress signals or kinases could change the cellular activity and behaviour of the tau molecule (Lu et al., 2013b, Liu et al., 2007, Pooler et al., 2012, Lu et al., 2013a).

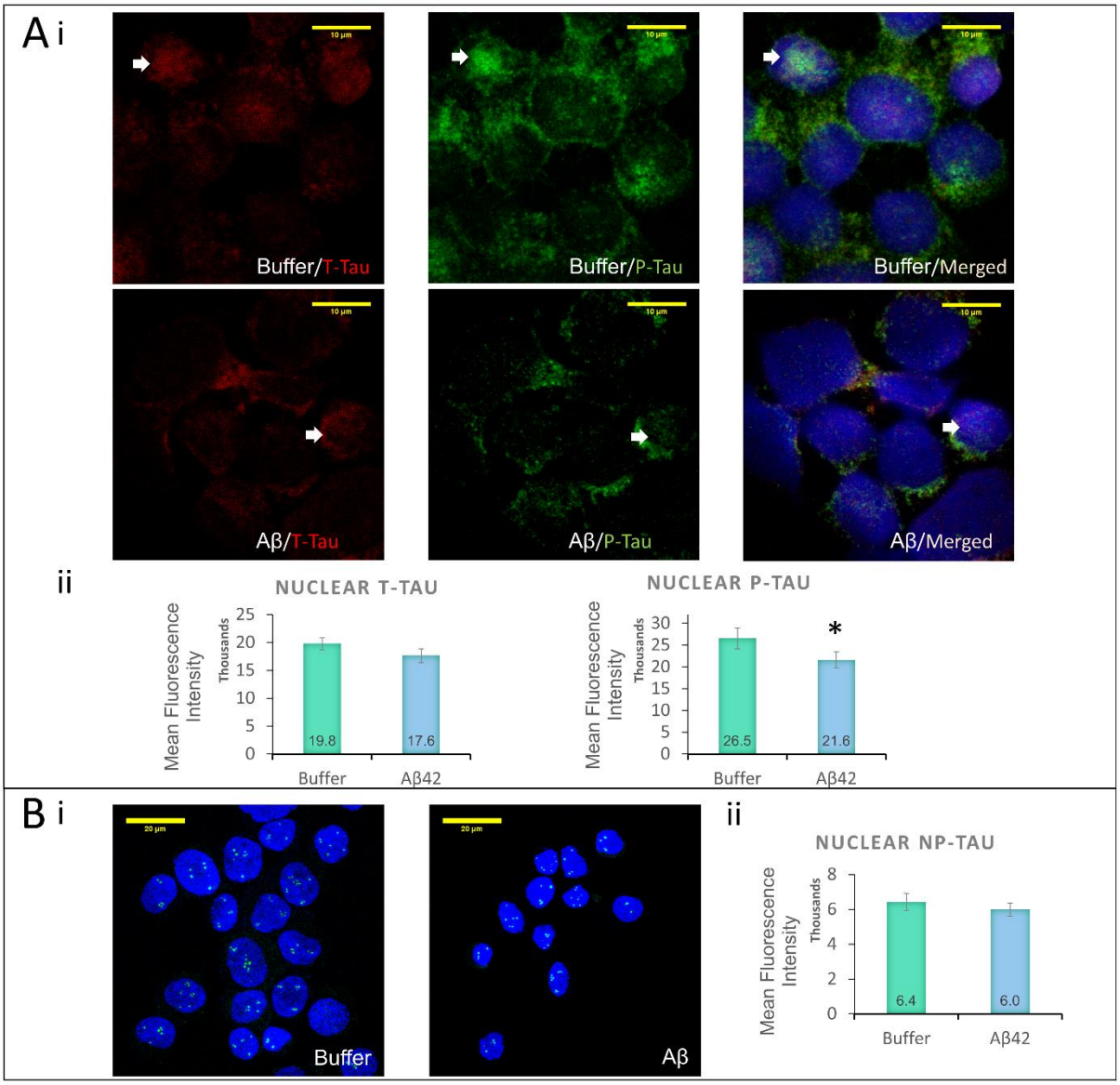

Figure 4.3 Impact of Aβ42 on nuclear tau localisation.
(A) Immunofluorescence labelling indicating the presence of nuclear phosphorylated tau (Ai), which significantly decreases following the 24h Aβ treatment, without changes in total nuclear tau (Aii) or nuclear nP-Tau (B). (ii). (T-Tau, P= 0.081; P-Tau, P= 0.014; nP-Tau, P= 0.49). Experiments in A repeated three independent times, and B repeated five independent times.

The results of Chapter 3 showed that even though cellular stress in differentiated SHSY5Y cells induces a decrease in total cell P-Tau, it selectively induced its accumulation in the nucleus, redistributed nucleolar nP-Tau and these changes co-occurred alongside nucleolar stress, DNA damage and heterochromatin loss. The incubation of differentiated SHSY5Y cells with Aβ42 for 8h (Noel et al., 2016) or N2a cells with formaldehyde for 2-4h (Lu et al., 2013a), has also been shown to induce the

accumulation of phosphorylated tau in the nucleus. Therefore, the differentiated SHSY5Y cells were next examined to investigate whether the Aβ incubation also causes nuclear changes in tau species (Fig. 4.3). Buffer treated differentiated SHSY5Y showed mild immunoreactivity to nuclear P-Tau. However, this decreased significantly following the Aβ incubation with no changes in the nuclear level of total tau (Fig. 4.3). This is consistent with the total cell reduction in the P-Tau observed by Western blotting following the Aβ incubation (Fig. 4.2).

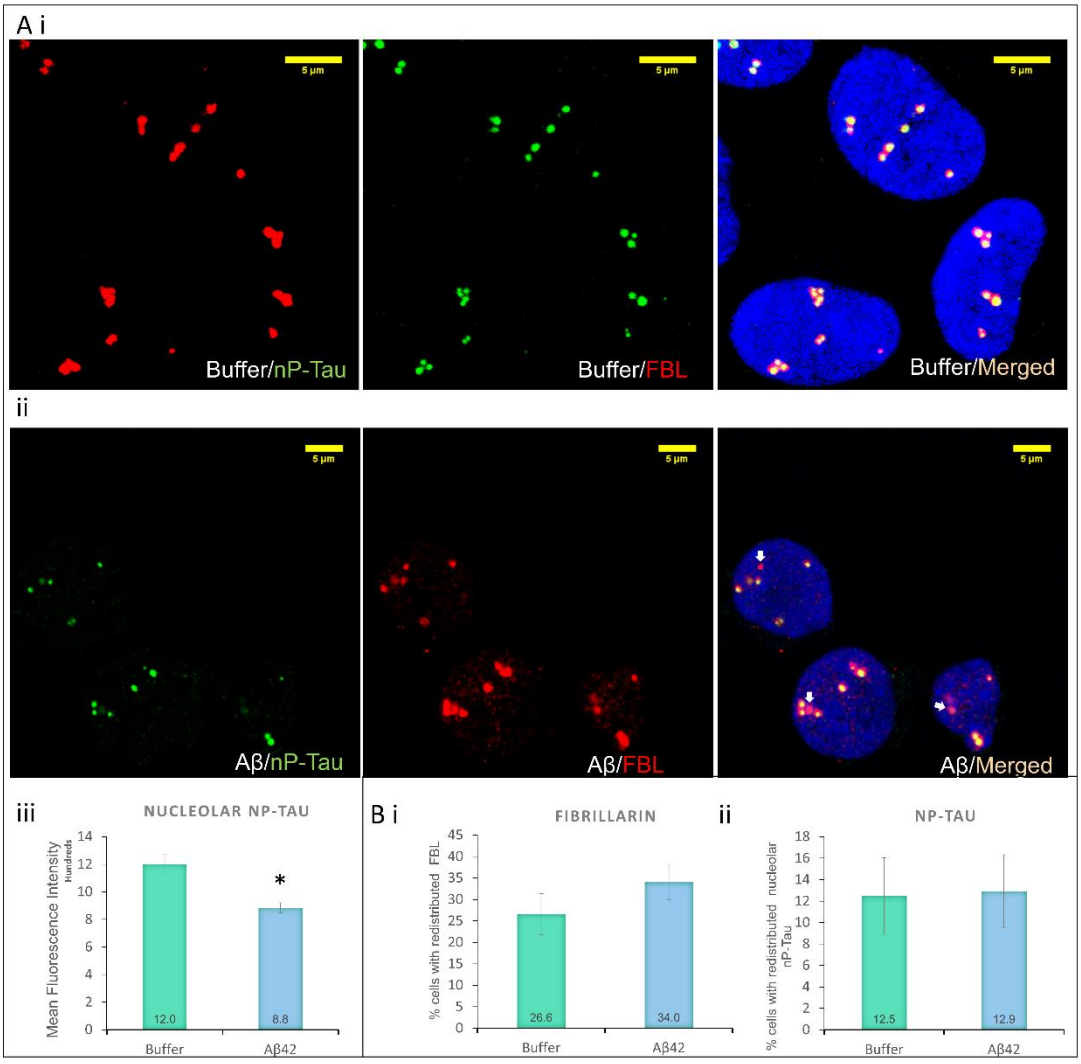

Figure 4.4 Aβ42 alter the nucleolar level of tau.
(A) Immunofluorescence labelling showed a reduction in nP-Tau colocalisation with fibrillarin (FBL) following the Aβ administration. Quantification showed that this altered colocalisation of nP-Tau with FBL is significant (P= 0.014). (B) The Aβ treatment did not change the overall nuclear distribution of FBL (P=0.27) or nP-Tau (P=0.93). Experiments repeated five independent times.

The level of nP-Tau in the whole cell extract showed a significant decrease (Fig. 4.2), no change in its nuclear abundance was observed using immunofluorescence (Fig. 4.3B). Chapter 3 demonstrated that glutamate stress induces the cytoplasmic and nucleoplasmic redistribution of fibrillarin and nucleolar nP-Tau. Here, fibrillarin and nP-Tau immunofluorescence were examined to check whether they were redistributed by the Aβ42 treatment, revealing no such redistribution (Fig. 4.4). Hence, using fibrillarin punctate fluorescence to segment the nucleolus, we quantified the levels of nucleolar nP-Tau, and this showed a significant reduction in the nucleolar-nP-Tau following the 24h Aβ incubation (Fig. 4.4). Since no difference in the total nuclear levels of nP-Tau was observed (Fig. 4.3B), the decrease in nucleolar nP-Tau suggests changes in its nuclear/nucleolar ratio, which has been reported for fibrillarin (Kodiha et al., 2011). Overall, these results showed that the Aβ incubation reduces total cell P-Tau and nP-Tau levels and primarily decreases the levels of nucleolar nP-Tau in the differentiated SHSY5Y cells.

This points to a potential early mechanism of Aβ toxicity, during which it selectively induces oxidative stress in the absence of significant cell death, and alters the phosphorylation level of tau epitopes, changing tau's localisation within the nucleolus.

4.3.2 Aβ42 induces nucleolar stress and inhibits RNA and protein synthesis

Cellular stress is known to disrupt the integrity of the nucleolus (Boulon et al., 2010). Therefore, the decrease in nucleolar nP-Tau prompted us to investigate whether the nucleolus was under stress. In Chapter 3, we showed that glutamate-induced nucleolar stress causes a reduction in the level of fibrillarin, UBF and TIP5. Given that nucleolar stress causes the degradation of nucleolar proteins, Western blotting was used to investigate the presence of nucleolar stress by quantifying the levels of these proteins,

revealing that UBF and TIP5 but not fibrillarin, become significantly decreased following Aβ incubation (Fig. 4.5A). To examine whether these changes were specifically at the protein or gene expression level, their transcripts levels were quantified using qPCR, revealing that Aβ incubation led to a significant decrease in the RNA levels of fibrillarin, UBF and TIP5 (Fig. 4.5B). This indicates that the decrease in gene expression of these proteins likely contributes to the reduction observed at the protein level. The absence of a difference in the protein level of fibrillarin despite the decrease in its transcript may be due to a longer half-life for fibrillarin, as some proteins have a longer half-life than others (Greenbaum et al., 2003, Vogel and Marcotte, 2012).

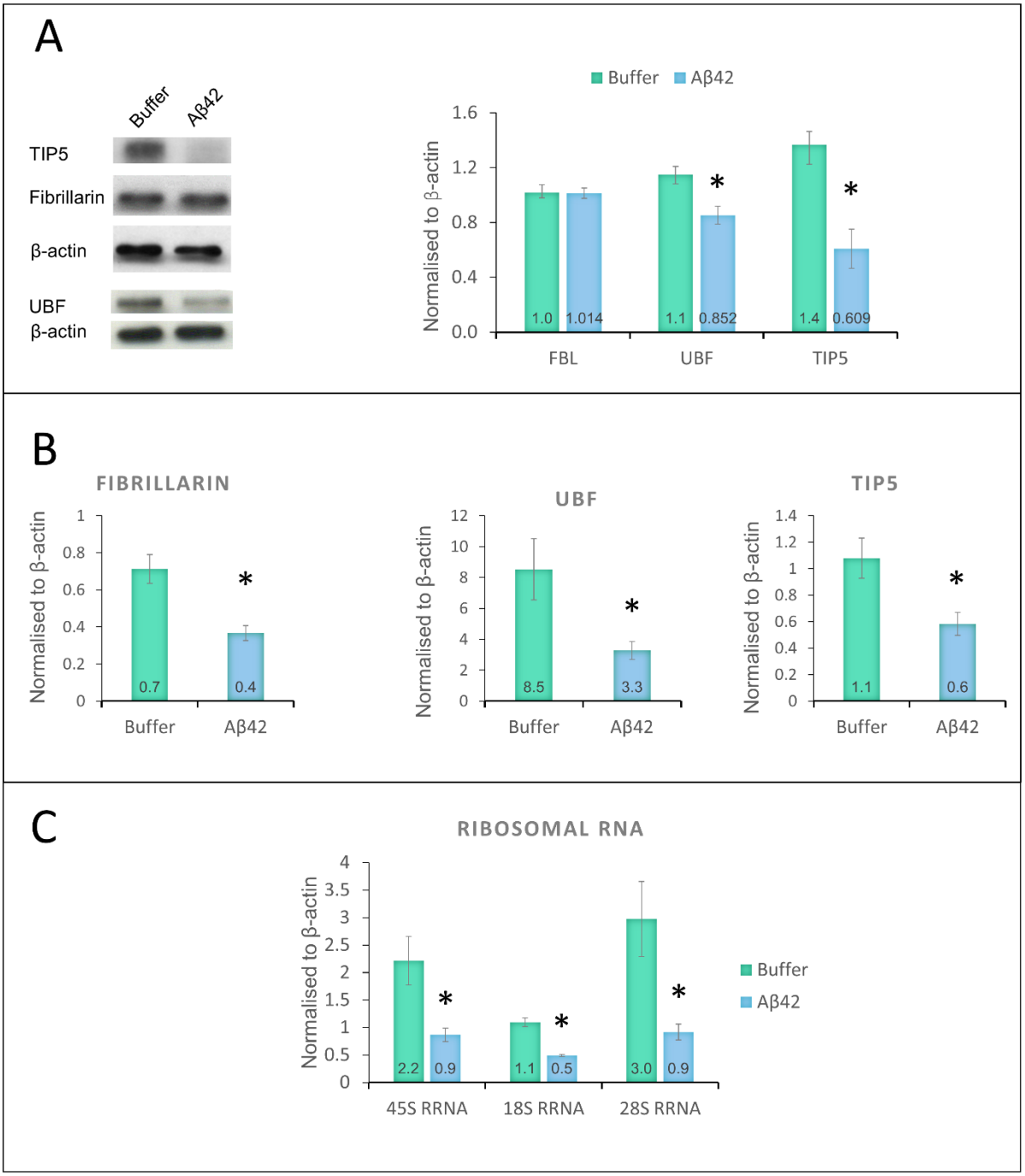

Figure 4.5 Aβ42 induces nucleolar stress and inhibit RNA Synthesis.
(A) Western blotting revealed that the Aβ treatment led to a significant decrease in UBF (P=0.007), TIP5 (P=0.001), but not fibrillarin (P=0.967). Normalised to β-actin. (B) qPCR analysis of gene expression showed a significant reduction of UBF (P=0.018), TIP5 (P=0.013) and fibrillarin (P=0.0015) transcripts. Normalised to β-actin or TBP. (C) qPCR analysis of rDNA transcription and processing showed that the Aβ incubation resulted in a significant decrease in 45S pre-rRNA synthesis (P=0.01) and processing of 18S rRNA (P=0.0001) and 28S rRNA (P=0.01). Normalised to β-actin or TBP. Experiments in A repeated five independent times, and B & C repeated four times.

As indicated in Chapter 3, glutamate-induced nucleolar stress led to the reduction in rDNA transcription. The reduction in transcription or maturation of the 28S and 18S rRNA or their degradation has been suggested to contribute to nucleolar dysfunction in AD (da Silva et al., 2000). Hence, to assess further the presence of nucleolar stress in the differentiated SHSY5Y cells following the Aβ incubation, the transcription of 45S pre-rRNA and its processing was investigated. qPCR analysis showed that the Aβ induced a significant reduction of 45S pre-rRNA and its processing to 18S and 28S rRNA (Fig. 4.5C). Unlike with the glutamate, the reduction in 18S and 28S rRNA observed here indicate that the Aβ may impact on ribosome formation since 18S and 28S rRNA contribute to the formation of the 40s and 60S ribosomal subunits, respectively. Collectively, these findings reaffirm that Aβ incubation induced nucleolar stress with an associated inhibition of rRNA production and its processing and this can impact on the integrity of the protein translation machinery since rRNA are required for the assembly of functional ribosomes (van Riggelen et al., 2010). Further, findings from this differentiated SHSY5Y cell model links the rRNA deficits reported in the AD brain with Aβ toxicity.

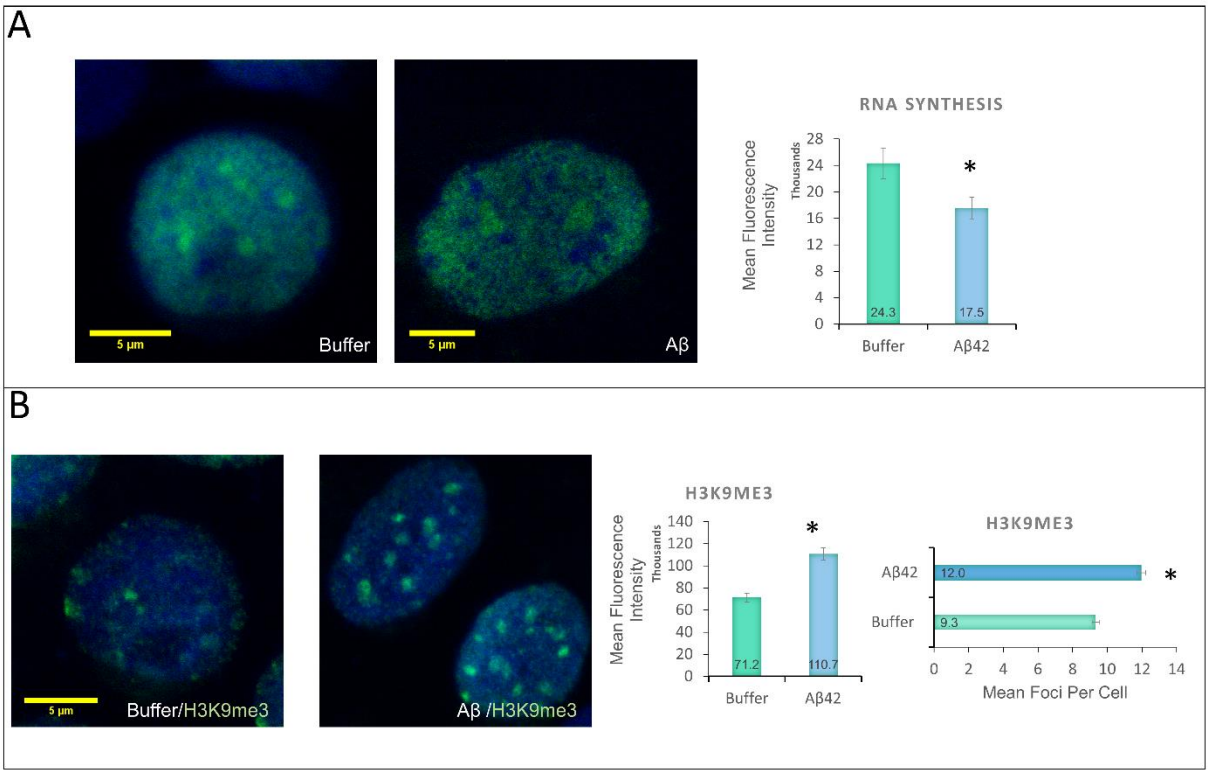

Figure 4.6 Aβ42 decrease RNA synthesis and increase chromatin compaction.
Quantitative Click-iT RNA immunofluorescence labelling showed that the Aβ causes a global reduction in newly synthesised RNA (P= 0.0054) (A), which is associated with a nuclear increase in H3K9me3 intensity (P=0.0001) and foci (P= 0.0001) (B). Experiments repeated five independent times.

Given that we found a significant decrease in the transcript levels of fibrillarin, UBF and TIP5, in addition to a drop in rDNA transcription, we examined whether the Aβ incubation also impacts on global RNA synthesis using the Click-iT RNA imaging assay. Accordingly, the Aβ treatment led to a significant reduction in nascent RNA synthesis (Fig. 4.6A). The epigenetic marker - H3K9me3 is known for its role in transcriptional repression, and it accumulates to form foci at constitutive heterochromatin and can be used as a readout of the constitutive heterochromatin levels of a cell (Saksouk et al., 2015). Immunofluorescence labelling revealed a significant increase in the nuclear level of H3K9me3 and its foci (Fig. 4.6B). Therefore, this finding complements the qPCR and Click-iT RNA labelling data, which revealed a decline in RNA levels (Fig. 4.5

& 4.6), such that an increased level of H3K9me3 would be expected to promote heterochromatin formation and transcriptional silencing, and therefore a reduction in RNA synthesis. Interestingly, as far back as the 1970s, a widespread AD-associated reduction in RNA and increase in heterochromatin formation was reported in human cortical neurons (Mann et al., 1977, Mann and Sinclair, 1978, Mann et al., 1980, Lewis et al., 1981, Crapper et al., 1979, McLachlan et al., 1991). Consistent with this, a microarray analysis of human cortical neurons challenged with Aβ oligomers had previously shown that it causes 70% downregulation of gene expression of 345 genes (Sebollela et al., 2012).

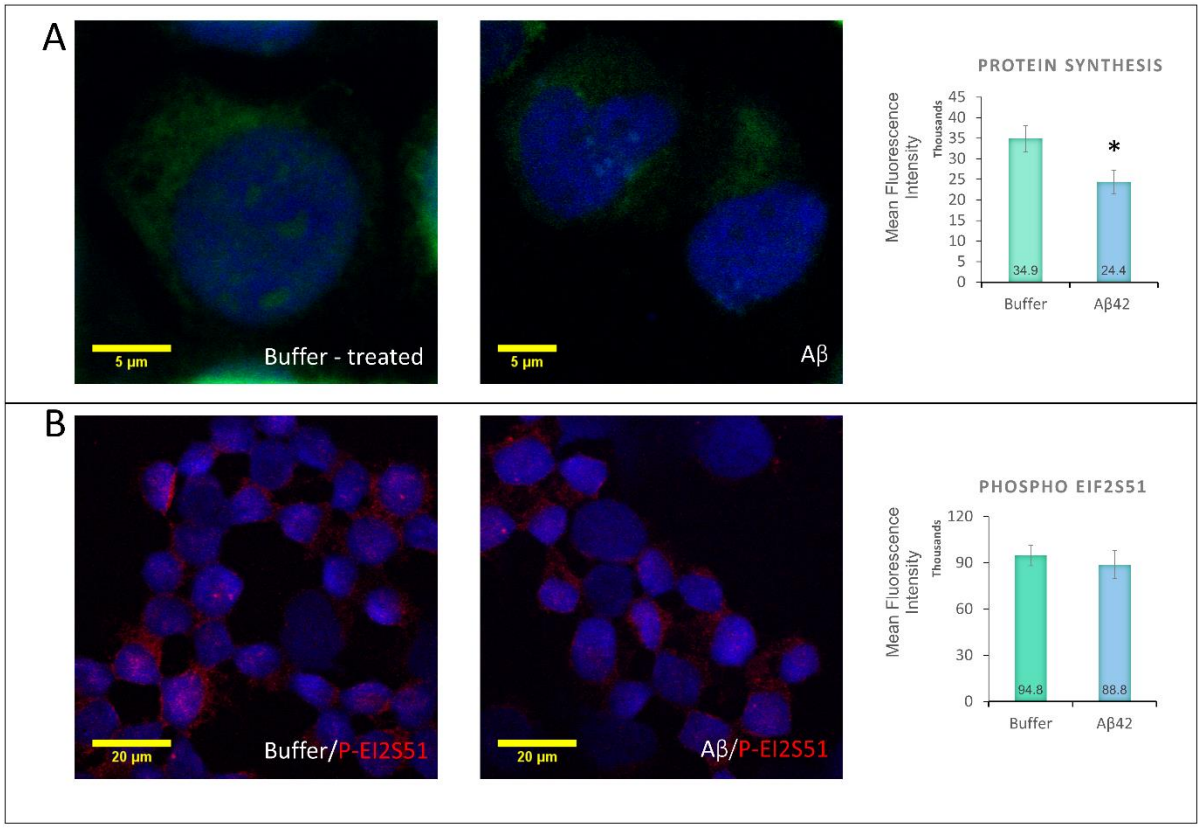

Figure 4.7 Aβ42 inhibit Protein Synthesis.
(A) Quantitative Click-iT HPG Alexa Fluor 488 immunofluorescence labelling showed a significant decrease in nascent protein synthesis following the Aβ treatment (P= 0.0003). (B) Quantitative immunofluorescence labelling for phosphor S51 eukaryotic translation initiation factor 2A (eIF2α -P) showed no changes following the Aβ treatment (P=0.37). Experiments were repeated five independent times.

Synthesised RNAs are translated into proteins through the recruitment and assembly of many factors, such as ribosomes (Henras et al., 2015, van Riggelen et al., 2010). Depending on the metabolic activity of cells, rDNA transcription in mammalian cells accounts for ~35 to 65% of total cellular transcription (Strohner et al., 2004). Since we observed a global increase in heterochromatin, a decrease in RNA synthesis and rDNA transcription, we next asked whether this culminates in a reduction in synthesised proteins. Click-iT HPG protein synthesis assay following the Aβ incubation led to a significant decrease in the global levels of newly manufactured proteins, from 34.9% in control to 24.4% in Aβ-treated cells (Fig. 4.7). This reduction in protein synthesis could be due to a collective low availability of the RNA and rRNA. With the glutamate, the reduction in protein synthesis observed was partly due to the serine 51 phosphorylation of eIF2α rather than rRNA deficits (Chapter 3). An increased level of eIF2α phosphorylation has been associated with the pathogenesis of AD (Ohno, 2014), as well as other neurodegenerative diseases (reviewed in Halliday and Mallucci, 2015). Therefore, we investigated whether the reduction of protein synthesis caused by the Aβ incubation is also associated with the eIF2α pathway. However, using quantitative eIF2α immunofluorescence analysis, we observed no difference in eIF2α phosphorylation between control and Aβ-treated cells (Fig. 4.7B).

Overall, these findings indicate that in our model, nucleolar stress, associated with a deficit in rDNA transcription may occur earlier than eIF2α phosphorylation in Aβ-induced pathogenesis. This links Aβ to the early mechanism of protein synthesis machinery alteration in AD (Ding et al., 2005, Ding et al., 2006, Hernandez-Ortega et al., 2015). Consistent with early studies on chromatin and RNA changes in AD (Mann et al., 1977, Mann and Sinclair, 1978, Mann et al., 1980, Lewis et al., 1981, Crapper et al., 1979, McLachlan et al., 1991) and recent findings with Aβ (Sebollela et al., 2012),

our results also indicate that the increased heterochromatin formation and reduction in the transcripts could contribute to the decrease in synthesised proteins induced by Aβ. Although recent evidence on gene expression in AD shows variability between brain regions and proteins, AD pathology has been associated with differential changes in gene expression, where, some pathways show decreased gene expression, while others show an increase (Sebollela et al., 2012, Liang et al., 2008a, Dunckley et al., 2006). Data from laser-capture microdissected neurons previously revealed that some of the regions affected early in AD show underexpression of genes involved in energy metabolism (Liang et al., 2008b). Therefore, the decrease in RNA transcripts observed here reiterates the importance of Aβ in the early process of the disease.

4.3.3 Early responses to Aβ42 exposure are oxidative stress and subtle nucleolar stress

The findings thus far indicate that without causing significant DNA damage or viability loss, Aβ induces oxidative stress and a reduction in the levels of protein synthesis. To identify the earliest event induced by the Aβ incubation, we studied the changes that result from a short exposure to Aβ42 (Fig. 4.8). Differentiated SHSY5Y cells showed no impact of 2-hour Aβ treatment on cell viability loss me, DNA damage or tau phosphorylation and localisation (Fig. 4.8A-C). However, a significant increase in oxidative stress was observed by CellROX Green flow cytometry assay (Fig. 4.8D). The levels of fibrillarin, UBF and TIP5 were quantified by Western blotting of whole cell lysates following Aβ treatment for 2h to examine whether the cells were suffering from nucleolar stress. Notably, while fibrillarin and TIP5 remain unchanged, the short exposure to Aβ led to a modest, but significant, reduction in UBF (Fig. 4.8E). Considering the critical role of UBF in rDNA transcription (Bártová et al., 2010), this decrease would result in the reduction of rDNA transcription, which occurs in

response to cellular stress (Boulon et al., 2010). We next examined whether the short Aβ exposure impacts on RNA synthesis and heterochromatin configuration but observed no effects on the levels of global RNA synthesis, H3K9me3 or protein synthesis (Fig. 4.8F-H).

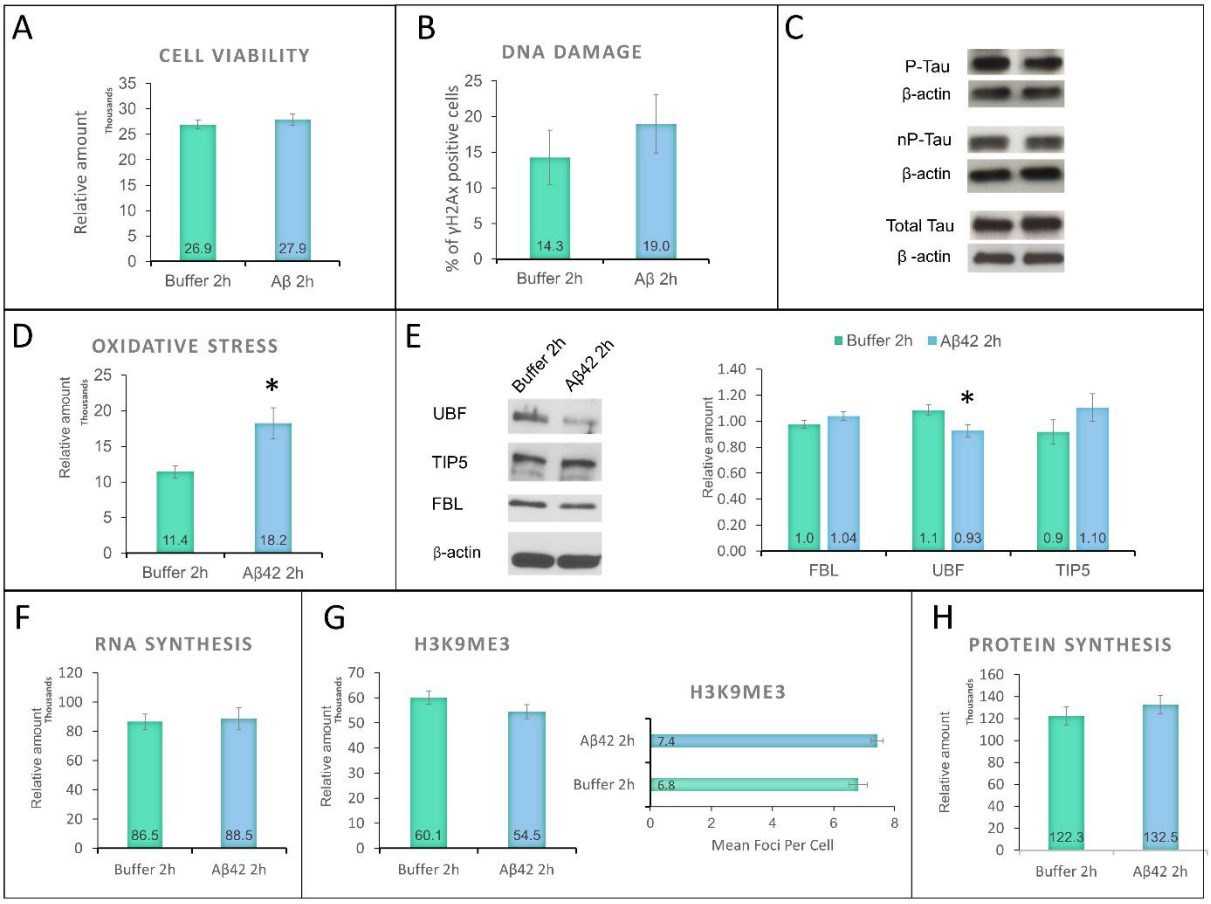

Figure 4.8 Early responses to Aβ42 exposure are oxidative and nucleolar stress.
(A) CellTiter-Blue viability assay showed no difference between 2h buffer and Aβ-treated cells (P=0.48). (B) Quantification of γ-H2Ax foci-positive cells showed the absence of significant DNA damage following the Aβ incubation for 2h (P= 0.12). (C) The Aβ incubation for 2h also didn't change tau phosphorylation or its nuclear levels (P-Tau, P= 0.88; nP-Tau, P= 0.83; T-Tau, P= 0.2). (D) Flow Cytometry experiment with CellROX Green showed that Aβ incubation for 2h induces a significant level of oxidative stress (P= 0.02). (E) Western blotting revealed that at the 2h time point, Aβ causes a reduction of only UBF (P= 0.02), not FBL (P= 0.3) or TIP5 (P= 0.13). Quantitative Click-iT RNA immunofluorescence labelling showed no change in newly synthesised RNA (P= 0.39) (F); H3K9me3 (P= 0.85) (G); or in newly synthesised proteins (P=0.39) (H).

Hence, the findings from the exposure to Aβ for 2h and 24h reveal that early consequences of Aβ incubation in the differentiated SHSY5Y cells are oxidative stress and subtle nucleolar stress. These become exacerbated over time, into a robust

nucleolar stress that negatively impacts on the levels of RNA and protein synthesis within the cells.

4.4 Conclusion

The amyloid cascade hypothesis places Aβ as the primary culprit for the pathogenesis of AD (Hardy and Higgins, 1992), even though evidence has subsequently emerged to indicate that Aβ and non-Aβ factors could both serve to trigger or promote the disease (Pimplikar, 2009). Our findings here are consistent with the previous investigation showing a non-toxic, suppressive effect of Aβ42 on the cholinergic system (Pedersen et al., 1996), and gene expression (Sebollela et al., 2012), learning and memory (Ford et al., 2015), and supports a role for Aβ in altering the protein synthesis machinery observed in this disease (Ding et al., 2005, Ding et al., 2006, Hernandez-Ortega et al., 2015). The fact that the changes we observed were in cells that showed no overt neurodegeneration (e.g. loss of cell viability), make this finding particular exciting since deficits in protein synthesis machinery have been observed in MCI, a time-point in the progression of AD, when there is no evidence of overt neuronal loss (Ding et al., 2006, Ding et al., 2005). This work links Aβ in nucleolar and protein synthesis dysfunction and points it as a culprit for the early cellular changes that occur, and impact on the progression of AD, decades before full-blown AD (Jack et al., 2013). These findings also implicate Aβ as a culprit for the heterochromatinisation and decrease in RNA levels that are reported to occur during the disease (Mann et al., 1977, Mann and Sinclair, 1978, Mann et al., 1980, Lewis et al., 1981, Crapper et al., 1979, McLachlan et al., 1991). The next key step is to identify the mechanisms by which Aβ influences these changes and especially the role of tau, which we recently identified to play a role in heterochromatin stability and rDNA transcriptional silencing (Chapter 5).

Given the importance given to Aβ in AD and its widely reported toxicity in various models, future studies should use other markers of DNA damage to examine the

toxicity of Aβ. It would be important to investigate these findings in another relevant model, like iPSC neurons. Future studies should also employ proper peptide control (e.g. scrambled or reverse Aβ) or anti-Aβ antibodies to validate these findings. Lower Aβ concentration should also be examined to investigate if findings are specific to the concentration used in this work. We can not entirely conclude that eIF2α phosphorylation would not be involved in the Aβ toxicity studied here, since as misfolded proteins accumulate, ER stress would become activated, resulting in eIF2α phosphorylation. Therefore, other sensitive approaches are required to confirm the eIF2α phosphorylation status following the Aβ treatment and also to investigate the involvement of eIF2α kinases.

Chapter 5

5.0 Tau protein is required for heterochromatin stability and rDNA transcriptional silencing

5.1 Abstract

The work in chapter 3 & 4 showed the nucleolar localisation of tau and its redistribution by cellular stress. Here, we reveal that tau localises to both the nucleolus and heterochromatin in neuroblastoma cells (SHSY5Y), where it associates with TIP5, a key player in heterochromatin stability and rDNA transcriptional repression. Depletion of tau results in heterochromatin loss, a decrease in DNA methylation and an increase in rDNA transcription, suggesting that tau is required for silencing of the rDNA and promoting the stability of repressive marks in the nucleus similar to TIP5. Using quantitative Immunogold labelling, we showed that tau associates with TIP5 in the human brain and this association increases in the AD brain. We hypothesise that tau belongs to the machinery that maintains the stability of rDNA and heterochromatin and may cooperate or independently to promote the methylation of rDNA and vulnerable genes in AD brain. The findings from this work have been submitted for publication.

5.2 Chapter Introduction

In previous chapters, we have shown that the nucleolar localisation of tau is retained in SHSH5Y cells even after differentiation, contrary to early conclusions that tau's function may not be required in the nucleolus in terminally differentiated cells (Brady et al., 1995). The nucleolus is the major hub for rRNA gene metabolism and a compartment for repetitive DNA sequences (Bukar Maina et al., 2016). The rRNA genes are organised in tandem repeats of the rDNA, a subset of which are kept transcriptional silent, associated with DNA methylation patterns and other proteins that promote heterochromatin stability (Guetg et al., 2010, Akhmanova et al., 2000). The nucleolar remodelling complex, comprised of TIP5 and the ATPase SNF2h, emerged as in important player for heterochromatin formation and silencing of the rDNA (Santoro et al., 2002). TIP5, the larger subunit of this complex has been shown to be indispensable for the stability of rDNA, major and minor satellites and heterochromatin formation at constitutive heterochromatin, comprised of centromeric and telomeric domains (Fig. 5.1) (Guetg et al., 2010, Postepska-Igielska et al., 2013). At the centromeric chromatin, TIP5 interacts with centromere protein A (CENP-A) (Postepska-Igielska et al., 2013). CENP-A is the histone H3 variant found in centromeres and forms complex with CENP-B at the CENP-A nucleosome (Fujita et al., 2015). CENP-B has been shown to be essential for centromeric heterochromatin formation (Nakagawa et al., 2002, Okada et al., 2007).

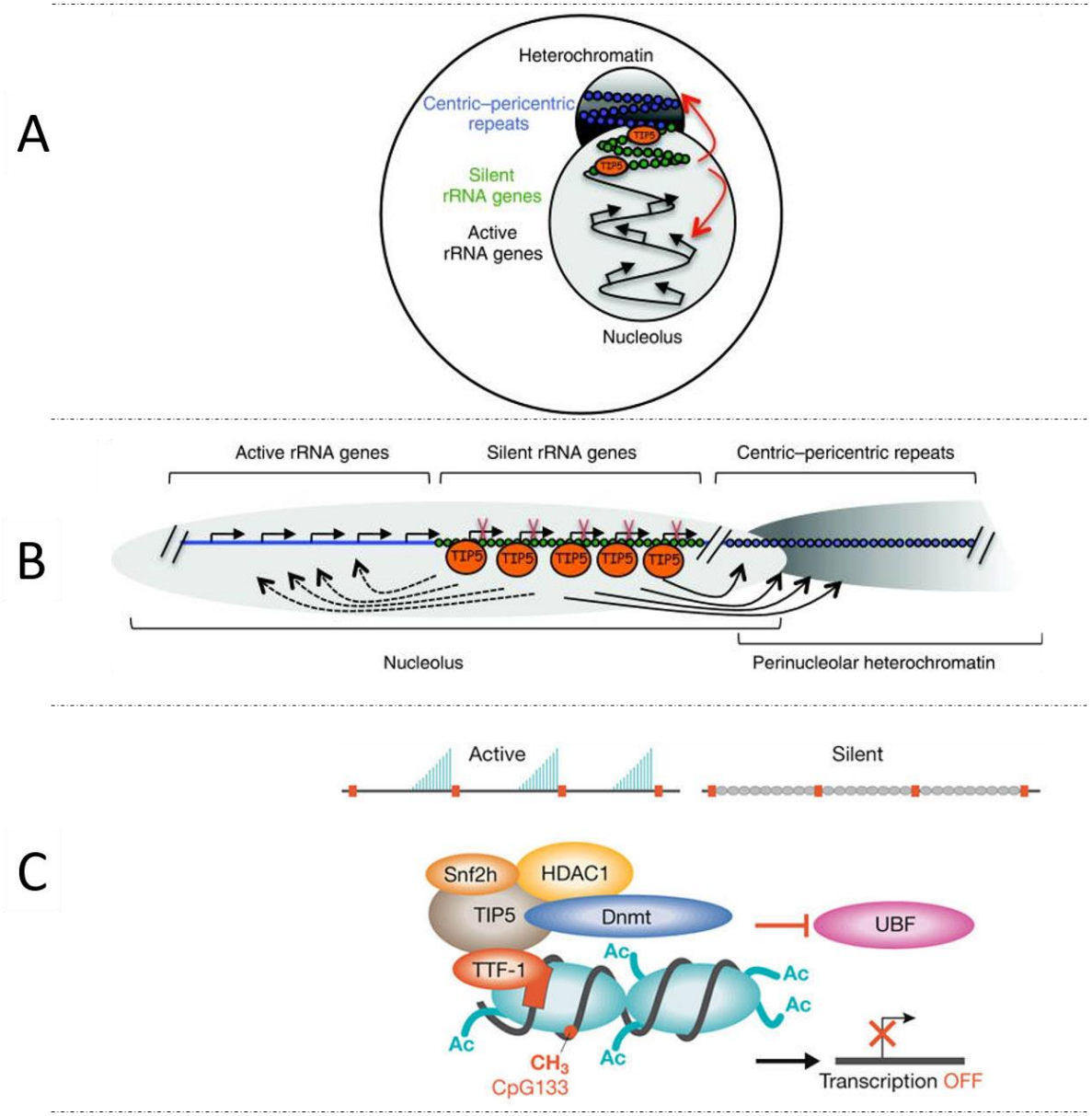

Figure 5. 1 TIP5 mediated heterochromatin formation.
(A) In a transient association model of TIP5-mediated heterochromatin formation, TIP5 from its stable binding site at rDNA in the nucleolus interacts transiently and/or weakly with nearby localised chromatin domains (centric-pericentric repeats) to spread heterochromatin to these domains, as well as to other rDNA regions (red arrows). (B) A second model showing that the spread of heterochromatin from silent rRNA genes or formation of nucleolar/perinucleolar compartment enriched in chromatin repressor complexes (e.g. TIP5) may affect the perinucleolar heterochromatin and active rRNA genes. (C) The mechanism of silencing and heterochromatin formation by TIP5 at rDNA involves the association of TIP5 with the termination factor TTF-1 at the To element upstream of the rDNA promoter, leading to the recruitment of histone deacetylase (HDAC1) and DNA methyltransferases (Dnmt), leading to silencing of the rDNA from histone H4 deacetylation, H3K9 methylation and DNA methylation. This prevents the transcription factor UBF from associating with the promoter and transcription is blocked. Nucleosomes (turquoise ovals) contain histone acetyl groups (Ac) and DNA methylated (CH_3) at CpG 133. A & B taken from (Guetg et al., 2010), and C taken from (Matthews and Olson, 2006).

Nuclear tau has been shown to interact with the perinucleolar heterochromatin, indicating it may play a role in gene regulation (Sjoberg et al., 2006). Indeed, evidence from tau KO mice showed that it can enhance the expression of many genes (Oyama et al., 2004), such as pericentromeric heterochromatin transcription (Mansuroglu et al., 2016) and the smarce1 gene, whose product is part of the large ATP-dependent chromatin remodeling complex SWI/SNF involved in chromatin remodelling (Gómez de Barreda et al., 2010). Recent evidence indicated that tau KO mice harbour pericentromeric instability, which was partly corrected by tau overexpression to the nucleus (Mansuroglu et al., 2016). All these indicate that its nucleolar localisation and chromatin association may play a role in heterochromatin formation and gene repression.

To better appreciate the role of tau in the nucleolus and heterochromatin, here we uncovered a novel interacting partner and function for tau in silencing of the rDNA. We show that nuclear tau associates with TIP5 in the nucleolus and heterochromatin in SHSY5Y cells. Accordingly, we show that tau knockdown destabilises the heterochromatin and increases rDNA transcription similar to findings following TIP5 depletion (Guetg et al., 2010, Postepska-Igielska et al., 2013). Co-localisation electron microscopy analysis in human brain tissue revealed that tau localises with TIP5 in the nucleolus and the heterochromatin, confirming the potential physiological significance of our findings in the neuroblastoma cells. We also show that the association between tau and TIP5 increases in the AD brain. Our work revealed that tau is essential for silencing of the rDNA and heterochromatin stability in cultured cells and also points to a physiological relevance of these findings in human brain tissue.

5.3 Results and Discussion

5. 3.1 Tau localises to the nucleolus and heterochromatin and interacts with TIP5

Recently, tau has been reported to translocate to the nucleus upon cellular stress, where it is thought to play a role in DNA protection or response to damage (Sultan et al., 2011, Lu et al., 2013a). Cellular stress is also known to induce nucleolar stress, a shared feature of many tauopathies (Yang et al., 2016, Parlato and Kreiner, 2013). In contrast, tau toxicity has been shown to induce DNA damage and heterochromatin relaxation, a feature found in the hippocampi of the AD brain (Frost et al., 2014). To better understand these phenomena, we set out to investigate the link between tau, the nucleolus and chromatin changes associated with cellular stress. Building on previous findings (Chapter 3 & 4), here we found that similar to the differentiated SHSY5Y cells, non-phosphorylated tau (nP-Tau) co-localised with fibrillarin in the undifferentiated cells (Fig. 5.2), indicating that the undifferentiated and differentiated SHSY5Y cells show similar nuclear tau localisation.

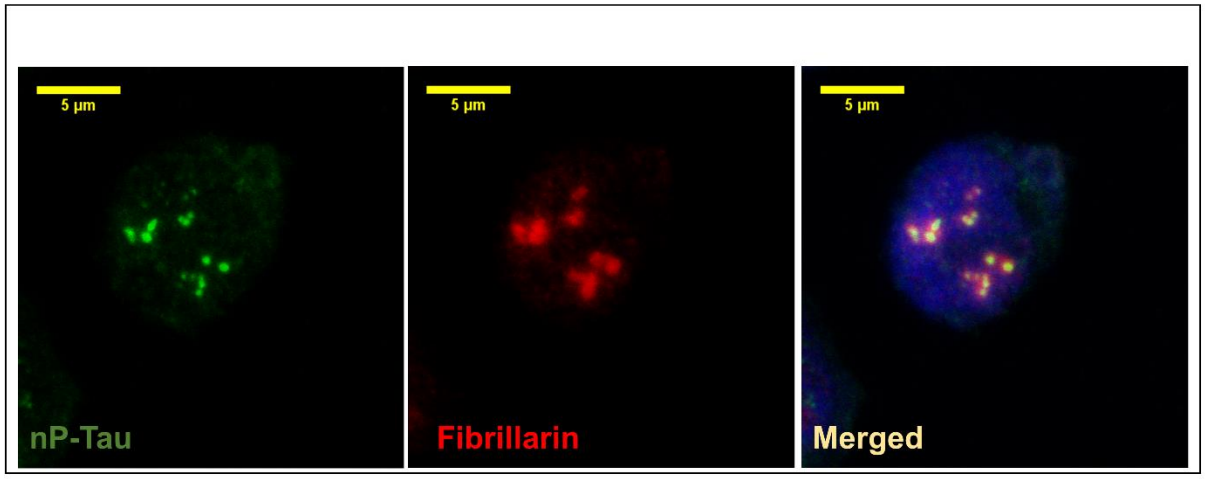

Figure 5. 2 Tau protein localises to the Nucleolus in undifferentiated SHSY5Y cells. Immunofluorescence labelling in the undifferentiated cells revealed that nP-Tau colocalises with fibrillarin.

The distribution of tau in the SHSY5Y cells was next examined using immunogold electron microscopy, which is ideal for identifying the cellular distribution of proteins due to its high resolving power. With the assistance of Dr Julian Thorpe who performed embedding protocols and cut thin sections for labelling, immunogold labelling using an antibody against total tau revealed tau localisation in the nucleus within the nucleolus and heterochromatin in the undifferentiated SHSY5Y cells (Fig. 5.3A). Heterochromatin proteins, like HP1α and H3K9me3, localise to the nucleolus and constitutive heterochromatin, such as centromeric heterochromatin, where they play roles in transcriptional silencing (Harničarová Horáková et al., 2010, Chen et al., 2011, Saksouk et al., 2015). This is also true for TIP5 which localises to both nucleolus and centromeric and telomeric heterochromatin (Postepska-Igielska et al., 2013). Given that tau also localises to both nucleolus and heterochromatin, we next examined the association between tau and the nucleolar and constitutive heterochromatin complexes, particularly centromeric chromatin. Immunoprecipitation using an antibody against nP-Tau showed that tau interacts with TIP5, CENP-B and HP1α in

the undifferentiated cells (Fig. 5.3B), indicating that tau interacts with the nucleolar heterochromatin and centromeric heterochromatin.

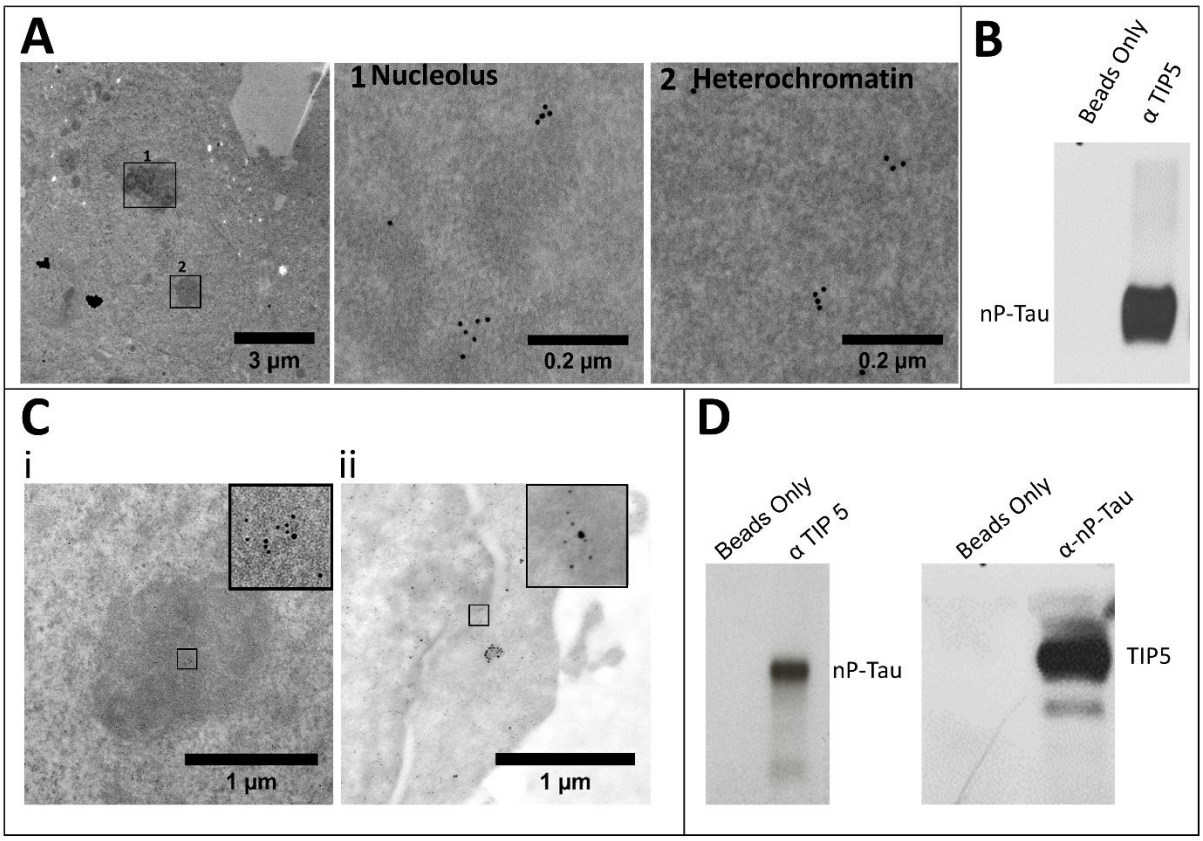

Figure 5. 3 Tau interacts with nucleolar and heterochromatin proteins.
(A) Immunogold labelling of undifferentiated SHSY5Y cells with anti-total tau antibody using 10nm gold particles conjugated secondary antibody showed tau gold particles within the nucleolus and heterochromatin. (B) Immunoprecipitation experiments on whole cell lysates from undifferentiated cells showed that nP-Tau associates with TIP5, CENP-B and HP1α. (C). Immunogold labelling of differentiated cells revealed tau within the nucleolus and heterochromatin, associated with TIP5 (Tau 15 nm and TIP5 5 nm). Nucleolus (*i,* see insert) and heterochromatin (*ii*, see insert). (D) Immunoprecipitation experiments on whole cell lysates from differentiated cells also showed that tau associates with TIP5. All experiments, except C, repeated three times. Experiment in C repeated twice.

To examine whether tau colocalises with TIP5, double immunogold labelling was employed. This revealed that tau also localises to the nucleolus and heterochromatin, colocalising with TIP5 in the differentiated cells (Fig. 5.3C). Immunoprecipitation using an antibody against nP-Tau or TIP5 revealed that similar to the undifferentiated cells, tau associates with TIP5 in the differentiated cells (Fig. 5.3D). The association between tau and TIP5 is particularly intriguing as TIP5 is known to be crucial for the

silencing and stability of rDNA and heterochromatin stability (Postepska-Igielska et al., 2013, Santoro et al., 2002), suggesting a potential role for tau in these processes. Together, these findings establish that nuclear tau in the undifferentiated and differentiated cells show similar localisation and interaction and it is distributed in the nucleolus and heterochromatin, where it associates with the major subunit of NoRC - TIP5.

5. 3.2 Tau knockdown alters the integrity of the heterochromatin

To dissect the specific role of tau in the nucleolus and heterochromatin, RNA interference to transiently deplete tau was employed, then the functional consequence of its down-regulation was investigated. The SHSY5Y neuroblastoma cells are known for their impermissivity to transfection strategies (Martin-Montanez et al., 2010). We found the differentiated cells even harder to transfect after employing several strategies, even with Accell siRNA which does not require transfection reagents and believed to offer efficient knockdown of proteins in neurons (Nakajima et al., 2012). Since the undifferentiated and differentiated SHSY5Y cells show very similar distributions and interactions of nuclear tau forms (Fig. 5.2 & 5.3), undifferentiated SHSY5Y cells were incubated for 72h with 1.5 μM Accell siRNA which caused a reproducibly significant loss of tau at both the protein and mRNA levels (Fig. 5.4).

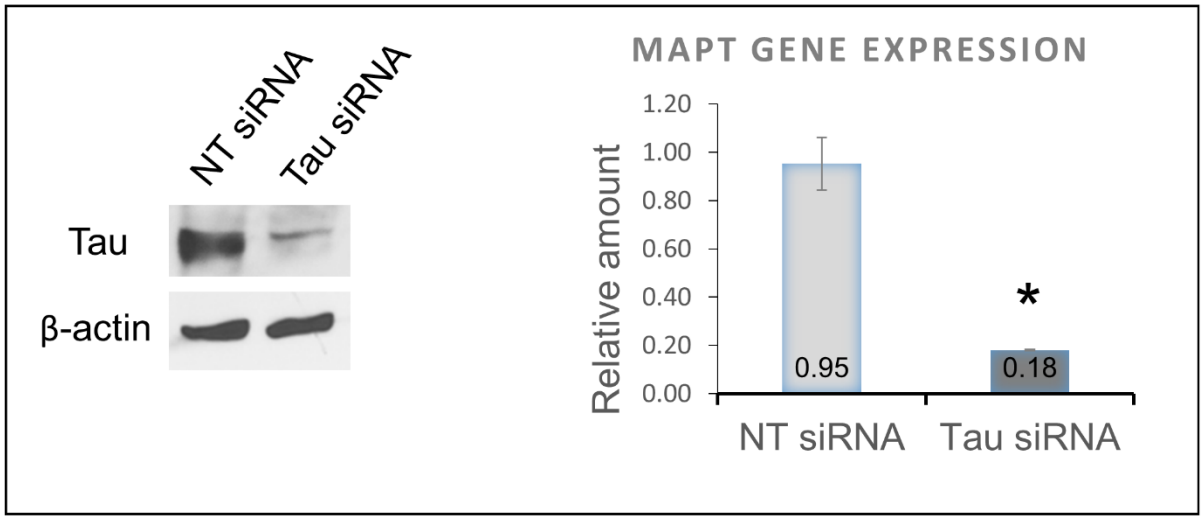

Figure 5. 4 Tau knockdown.
Western blotting and qPCR revealed a successful and reproducible tau knockdown in the undifferentiated SHSY5Y cells. P<0.0001 for both Western blot and qPCR. Experiments repeated four times.

Following the depletion of tau, we next investigated whether the loss of tau alters the integrity of the heterochromatin. Tau's interacting partner – TIP5, has been shown to be indispensable for heterochromatin formation at constitutive heterochromatin and rDNA (Postepska-Igielska et al., 2013, Guetg et al., 2010). Therefore, we speculated that tau could play a similar role in heterochromatin stability. Quantitative immunofluorescence labelling for HP1α, H3K9me2 and H3K9me3 showed that the tau knockdown significantly reduced constitutive heterochromatin foci (HP1α and H3K9me3 foci) in the cells, with an accompanying decrease in the total nuclear intensities of H3K9me2 (Fig. 5.5). Given that H3K9me2 show more preference to the facultative heterochromatin (See Chapter 1), this suggests that the presence of tau in the heterochromatin plays a vital role in maintaining heterochromatin stability at both constitutive and facultative heterochromatin domains.

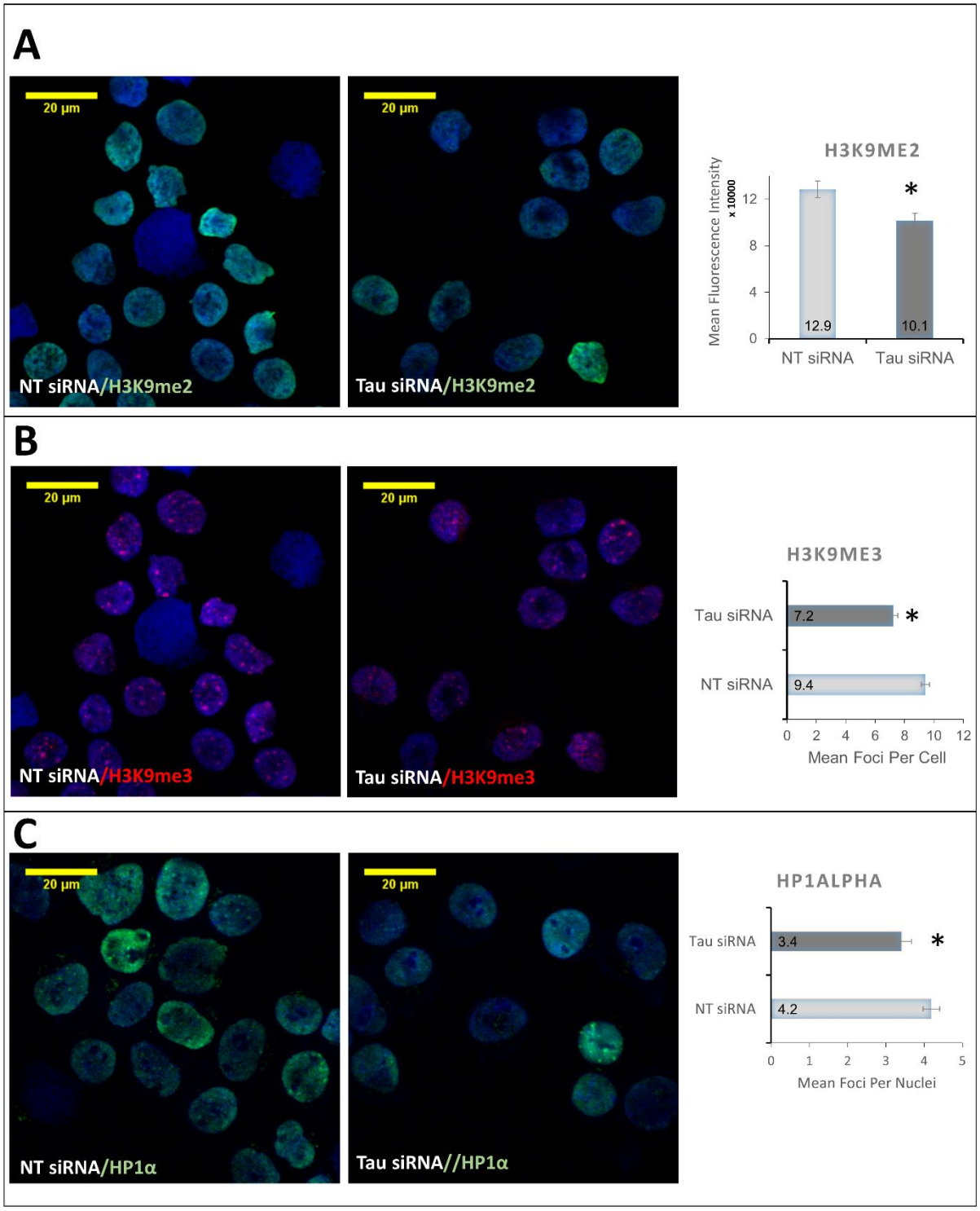

Figure 5. 5 Tau knockdown alters the integrity of the heterochromatin.
(A) Quantitative immunofluorescence labelling showed that the tau knockdown caused a significant reduction in the levels of H3K9me2 ($P<0.0001$). The knockdown significantly reduced the number of H3K9me3 foci ($P<0.0001$) (C), and HP1α foci ($P=0.0022$) (D). Experiments repeated four times.

H3K9me2, H3K9me3, and HP1α are enriched in centromeric heterochromatin coexisting with CENP-A and CENP-B – key centromere proteins (Pidoux and Allshire,

2005, Dunleavy et al., Saksouk et al., 2015) (See Chapter 1, Fig. 1.8). The centric heterochromatin also surrounds the nucleolus, in this way indicating a close relationship between the nucleolus and nuclear heterochromatin domain (Padeken and Heun, 2014, Guetg and Santoro, 2012). Indeed, the loss of H3K9 methylation has been shown to alter repetitive DNA stability (e.g. rDNA and satellite DNA) (Peng and Karpen, 2007). Since the tau knockdown altered heterochromatin stability, CENP-A/B foci and nuclear intensity were quantified following tau knockdown to investigate whether the loss of the heterochromatin integrity at centromeres was associated with loss of CENP-A/B. Although no changes were detected in CENP-A or CENP-B foci numbers, a significant decrease in total CENP-B nuclear intensity was observed (Fig. 5.6).

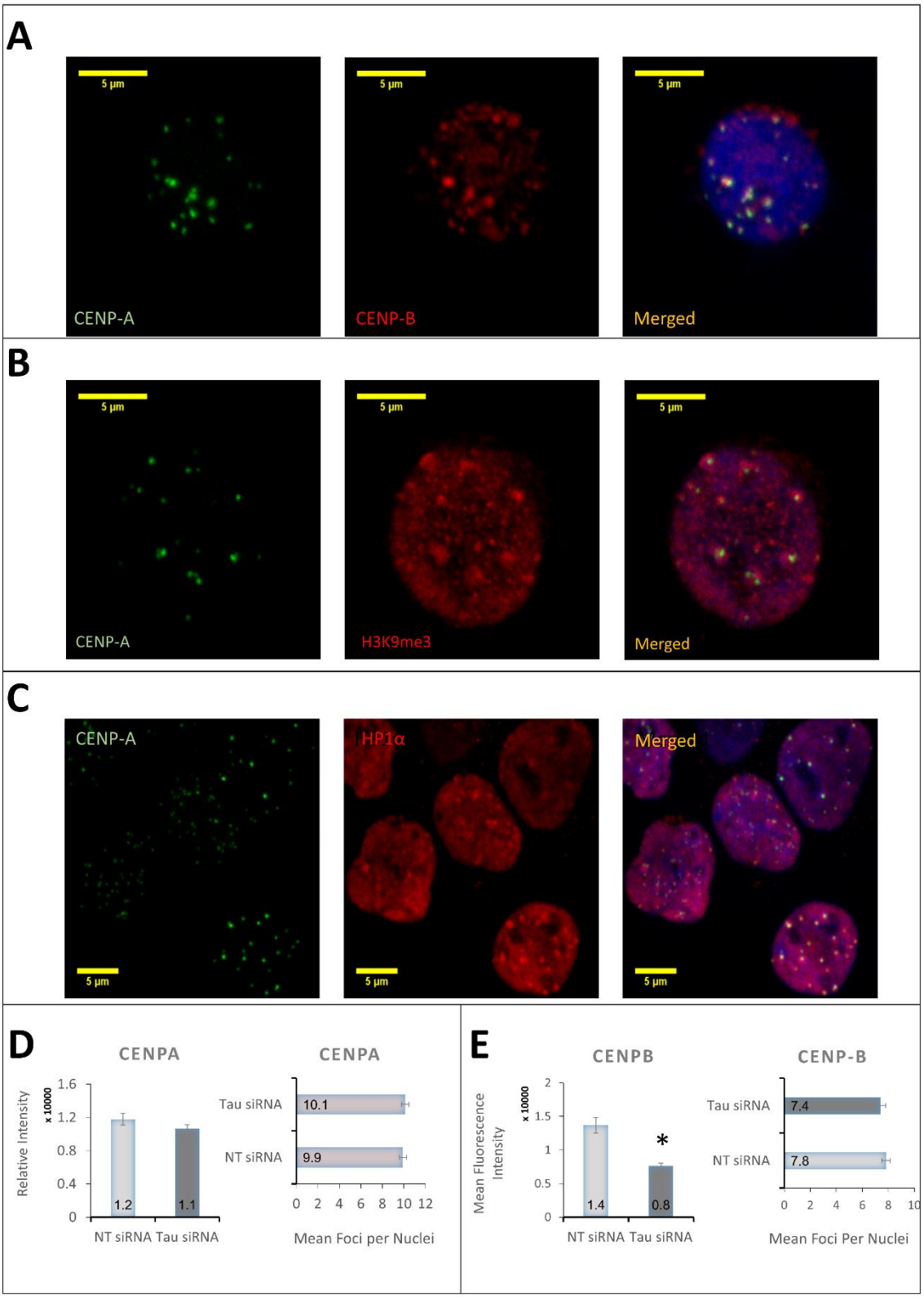

Figure 5. 6 Impact of Tau knockdown on the centromere
Double immunofluorescence labelling of the undifferentiated SHSY5Y cells showed that CENP-A and CENP-B colocalise to form foci (A); CENP-A colocalises with H3K9me3 (B) and HP1α (C). (D) Labelling for CENP-B revealed that the tau knockdown caused a significant reduction in its nuclear intensity but not foci (CENPA-B intensity P<0.0001; CENPA-B foci P=0.296). The tau knockdown does not alter the nuclear levels or foci of CENP-A (CENP-A intensity P= 0.575; CENP-A foci, P= 0.573). Experiments repeated four times.

CENP-B interacts with DNA through a 17-bp CENP-B box and has been shown to be essential for the enhancement of H3K9me3 methylation and DNA methylation, thus assisting centromeric heterochromatin formation on satellite DNA sequences (Okada et al., 2007). In yeast, reduction of the human CENP-B homologues leads to a decrease in centromeric heterochromatin (Nakagawa et al., 2002). The recruitment of HP1α to H3K9me2 or H3K9me3 upstream of a promoter is known to trigger silencing through the recruitment of DNA methyltransferases that leads to the heterochromatinisation of the gene (Chen et al., 2011). Considering the above findings, we reasoned that the tau knockdown could also have a consequence on DNA methylation. Indeed, using 5-methylcytosine (5-mC) quantitative immunofluorescence labelling, we observed a significant reduction in 5-mC DNA methylation following the depletion of tau (Fig. 5.7). Since we showed that tau associates with centromeric heterochromatin (Fig. 5.3), it is conceivable that tau plays a vital role in this complex, such that its absence affects the integrity of the complex. In strong support of this model, a recent study reported that loss of tau protein in mice affects the integrity of the pericentromeric heterochromatin by altering the HP1α-mediated recruitment of H3K9me3 to heterochromatin (Mansuroglu et al., 2016). Considering the role of CENP-B in H3K9me3 and DNA methylation in the centromeric heterochromatin (Okada et al., 2007), the reduction observed in its nuclear levels could alter the DNA methylation and recruitment of the H3K9me3 to heterochromatin, thus explaining the findings reported here. Overall, these results reveal that, similar to TIP5; tau plays a vital role in maintaining heterochromatin stability.

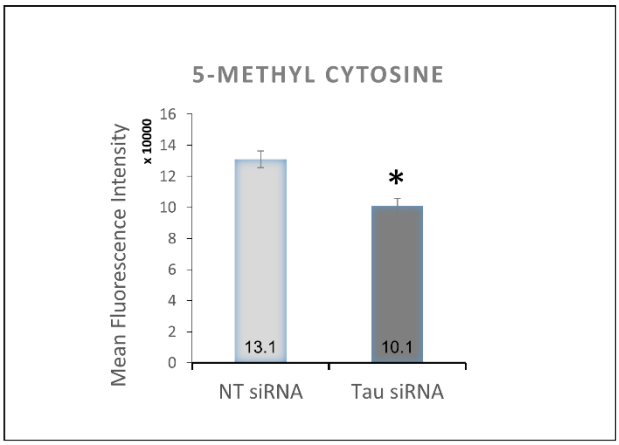

Figure 5. 7 Tau knockdown reduces DNA methylation.
Labelling for 5-Methylcytosine (5-MC) showed that the tau knockdown caused a significant reduction in the nuclear levels of 5-mC methylation ($P<0.0001$). Experiment repeated four times.

5. 3.3 Tau knockdown increases rDNA transcription

Chapter 3 and 4 revealed that glutamate and Aβ administration induce nucleolar stress and nucleolar tau delocalisation. Whether depletion of tau would result in nucleolar stress was therefore investigated. Western blotting revealed no difference in the levels of UBF, fibrillarin and TIP5 following the knockdown of tau (Fig. 5.8A). Whether the rDNA transcription is altered was investigated as an additional marker to study the presence of nucleolar stress. Surprisingly, the qPCR analysis showed that tau knockdown resulted in a significant increase in rDNA transcription and processing (Fig. 5.8B). Unlike with the glutamate or Aβ, these findings revealed that the nucleolus was likely not under stress following the depletion of tau, but the increase in rDNA transcription and processing suggests a role for tau in transcriptional silencing, which is consistent with our findings on tau's role in heterochromatin stability. Indeed, heterochromatin remodelling has been demonstrated to modulate rDNA transcription (Larson et al., 2012). To investigate if changes in CpG methylation are associated with the impact of tau knockdown on rDNA transcription, a restriction digest was performed on extracted DNA, following which the DNA was amplified using primers against the To region of the rDNA (which has "CpG" sites). The To element is a

proximal terminator sequence that lies upstream of the rDNA promoter and binding of this element by TIP5 through its interaction with Transcription termination factor I (TTF-I) initiates silencing of the rDNA promoter linked to the T0 element (Fig. 5.8C) (Grummt and Pikaard, 2003). Consistent with findings on the 5-mC labelling, Msp1/HpaII restriction digest showed that the tau knockdown reduced the methylation in the T0 region (Fig. 5.8D). Together, these findings suggest that tau plays a vital role in the stability of constitutive and nucleolar heterochromatin, such that its depletion resulted in heterochromatin loss and transcription permissive environment that allowed for an increased rDNA transcription.

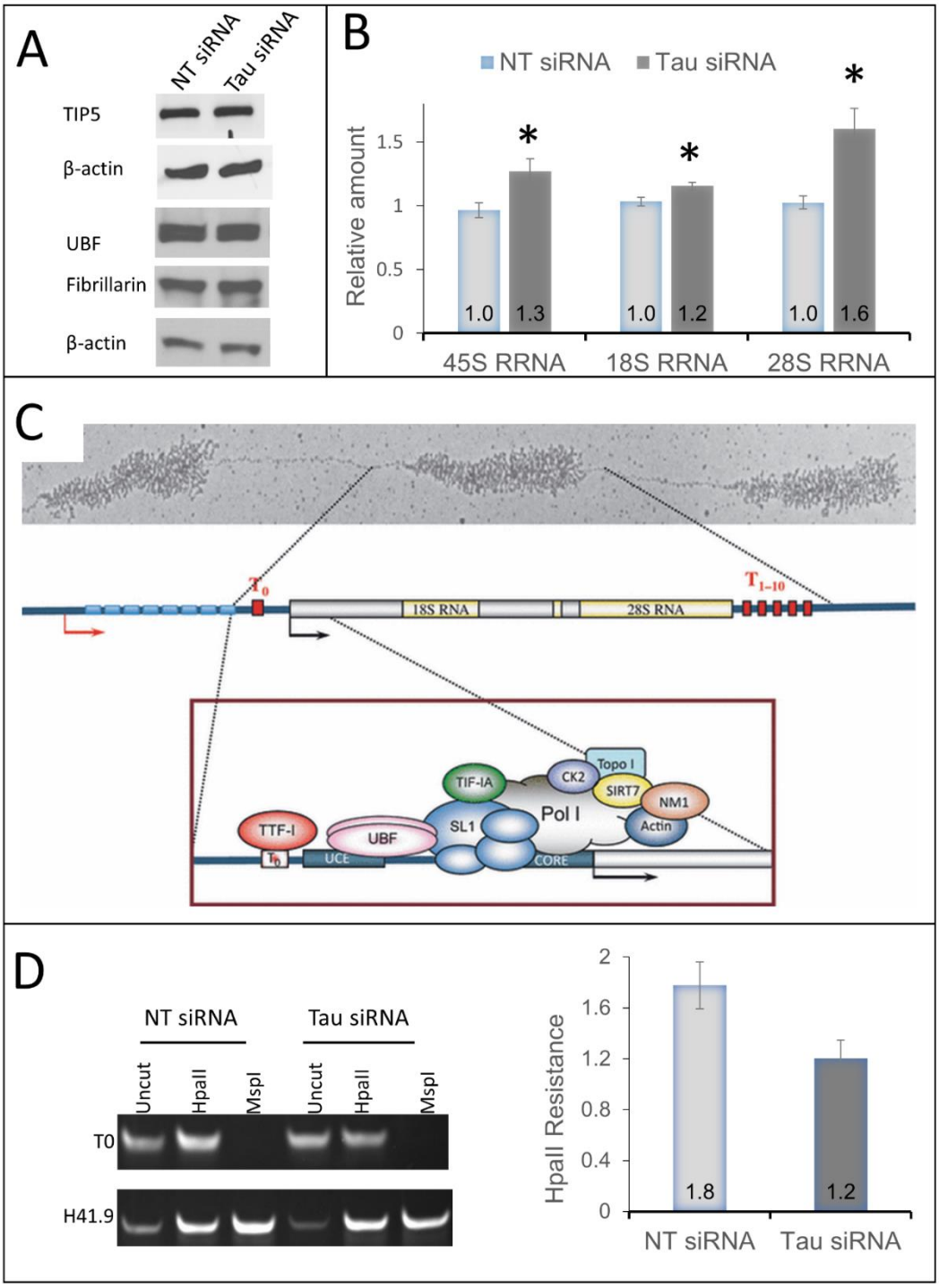

Figure 5.8 Tau knockdown Increases rDNA transcription.
(A) The tau knockdown does not change the protein levels of TIP5 (P= 0.72), UBF (P= 0.33), and fibrillarin (P= 0.29). (B) qPCR on samples from the knockdown cells showed a significant increase in 45S-pre-rRNA synthesis and 18S rRNA and 28S rRNA processing. (45S pre-rRNA P=0.017; 18S rRNA P=0.018; 28S rRNA P=0.0038). (C). Mammalian rDNA repeat showing the TTF-I bound To element upstream of the gene promoter. Taken from (Grummt, 2010). Restriction digest for "CCGG" sites using the methylation-insensitive (Msp1) and methylation-sensitive (HpaII) enzymes or mock digest on DNA extracted from NT or Tau siRNA treated undifferentiated SHSY5Y cells, amplified for the To region (which has Msp1/HpaII sites) and H41.9 region (which has no Msp1/HpaII sites) in a multiplex PCR. The tau knockdown reduces the methylation on the To element. Experiments repeated four independent times.

Protein synthesis operates downstream of rDNA transcription and depending on the metabolic activity of cells, rDNA transcription in mammalian cells accounts for ~35 to 65% of total cellular transcription (Strohner et al., 2004). Given the likely role of tau in heterochromatin stability and rDNA transcriptional silencing, the levels of global RNA and protein synthesis in cells depleted of tau were examined, however unlike with glutamate or Aβ; we found no changes (Fig. 5.9 A, B). However, this may not mean that there were no changes in the transcription rate of some genes, as a slight and consistent increase in the TIP5 transcripts without similar changes at the protein level was observed (Fig. 5.9C).

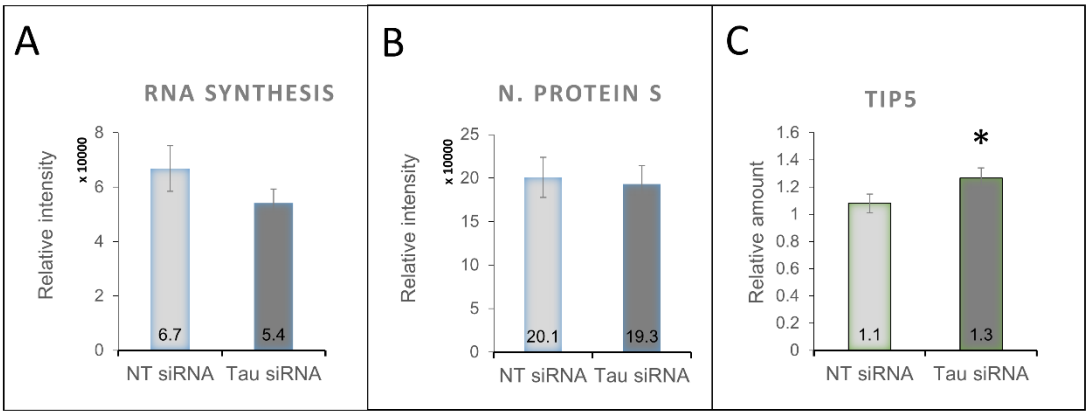

Figure 5. 9 Tau knockdown does not alter global RNA and protein synthesis.
Quantification of nascent RNA synthesis (P= 0.73) (A) or protein synthesis (P= 0.5738) (B) showed no changes following the tau knockdown. qPCR for TIP5 transcripts levels showed that the tau knockdown increases TIP5 mRNA (P= 0.039). Experiments in A and B repeated three independent times, experiment in C repeated four independent times.

Recently, tau KO neurons were found to have increased transcription of pericentromeric heterochromatin non-coding antisense RNA (Mansuroglu et al., 2016). Here, we cannot rule out that the production of some proteins was selectively enhanced due to the knockdown of tau. It was previously shown that tau-deficient mice showed increased transcription of the smarce1 gene, with an associated increase in the protein levels of its product BAF57 (Gómez de Barreda et al., 2010). Equally, tau has been found to associate with the translation machinery in normal brains, suggesting it

may play a role in protein translation (Meier et al., 2016). Therefore, we cannot rule out whether the lack of noticeable changes in the global protein synthesis observed following tau knockdown was due to lack of association between tau and the translation machinery.

5. 3.4 Nuclear Tau in the human brain

To explore the physiological relevance of these findings in the human brain, Immunogold electron microscopy on middle frontal gyrus tissue sections of the human brain was performed. Under my supervision, labelling using an antibody against total tau conducted by Saskia Pollack showed that tau localises in the nucleus; within the heterochromatin, and the nucleolus in the normal human brain (Fig. 5.10A). To study whether tau associates with TIP5 in the human brain similar to the SHSY5Y cells, double Immunogold labelling for nP-Tau and TIP5 was performed with the assistance of Dr Julian Thorpe. This revealed that nP-Tau associates with TIP5 within the nucleolus and the heterochromatin (Fig. 5.10B & C). Co-localisation analysis of gold particles revealed that tau associates with TIP5 as close as 11 nm apart, and approximately 30% of nuclear nP-Tau are associated with TIP5 within a 50 nm radius. Interestingly, in some cases, we observed nP-Tau/TIP5 gold particles localised to ring-like formations at the nuclear border, which may indicate they are transported into the nucleus together (Fig. 5.10Ci). This prompted us to revisit the Immunogold labelled SHSY5Y cells to investigate whether similar colocalisation patterns could be observed. Notably, this revealed similar nP-Tau/TIP5 gold labelled structures localised to the nuclear border in this cells (Fig. 5.10Cii).

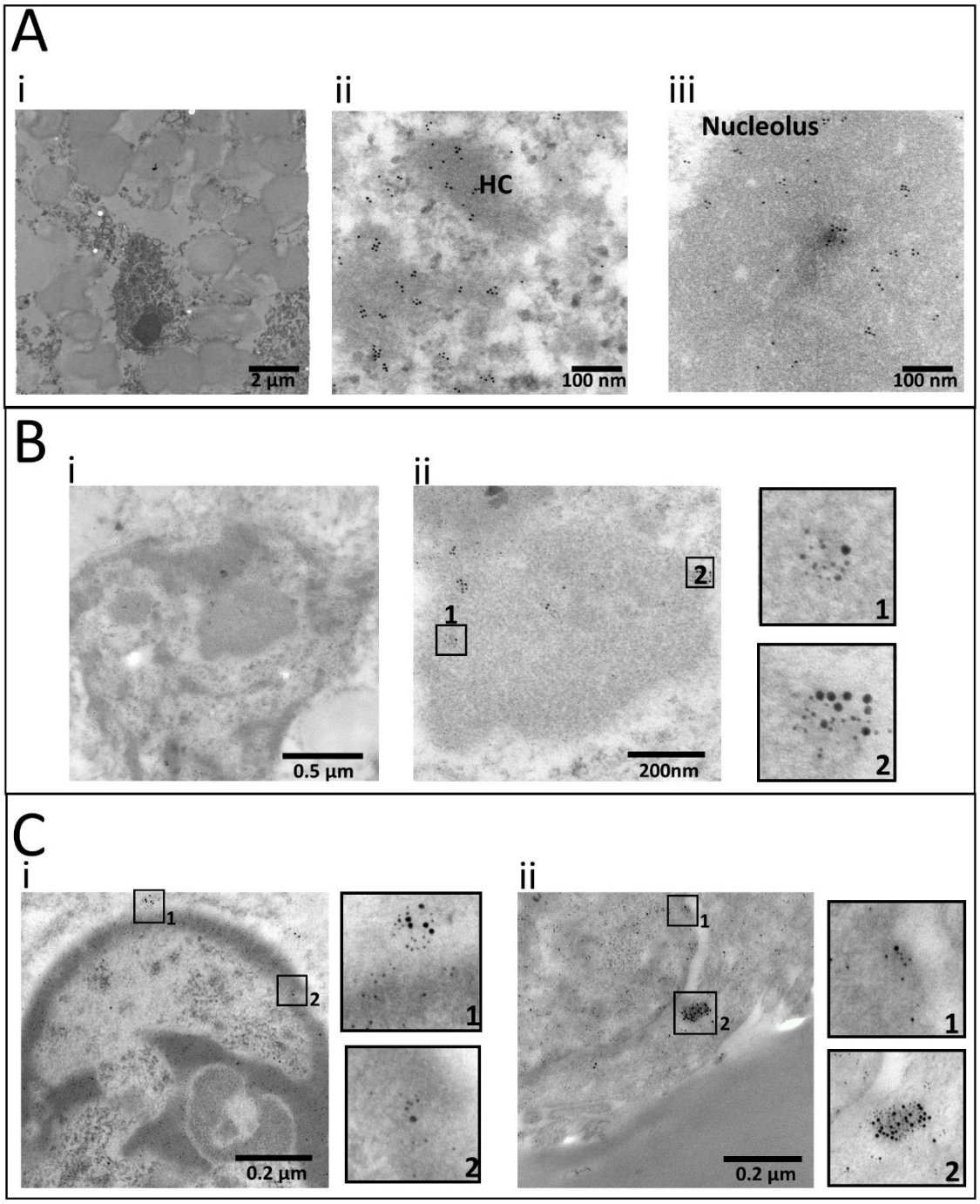

Figure 5. 10 Localisation of tau in the human brain neuronal nucleus.
Immunogold electron microscopy on brain sections labelled with Rabbit Anti-Tau (total) antibody and detected with 10nm gold conjugated anti-Rabbit secondary antibody showed the presence of tau in the human neuronal cell nucleus (Ai) Heterochromatin (HC) (Aii) and nucleolus (Aiii). Double Immunogold labelling for nP-Tau (15nm) and TIP5 (5nm) showed that they associate in the human brain neuronal nucleolus (Bi zoomed in Bii, see insert for labelling in the nucleolus and nucleolar border). It also showed that tau associated with TIP5 in the cytoplasm, bordering the nucleus (Ci, see insert 1) and heterochromatin (Ci, see insert 2). Double Immunogold labelling in SHSY5Y cells for nP-Tau (15nm) and TIP5 (5nm) showed that tau associates with TIP5 in the heterochromatin (Cii, see insert 1) and in the cytoplasm, bordering the nucleus (Cii, see insert 2). See methods for the quantification of nP-Tau/TIP5 association. Experiments in A repeated twice. Experiments in B-Ci repeated three times. Immunogold labelling in SHSY5Y cells Cii repeated twice.

Overall, these findings suggest an intimate relationship between tau and TIP5 in cell models and human brain tissue, which may have a functional relevance. Although tau in the human brain was previously visualised in the nucleolus using immunofluorescence microscopy, because the staining was weak, it was thought that it might not be present in terminally differentiated cells, such as neurons (Brady et al., 1995). These results demonstrate that tau can now also be considered a bona fide nucleolar and heterochromatin protein.

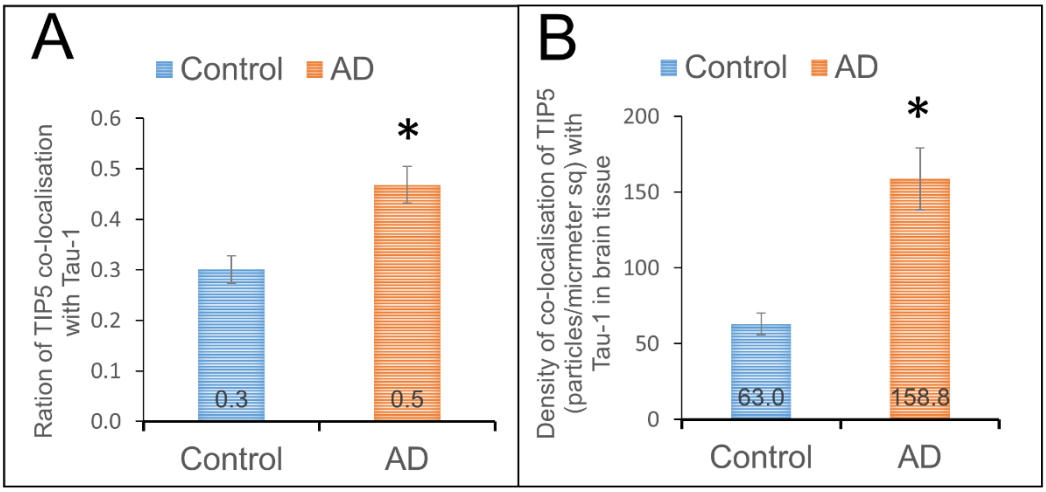

Figure 5. 11 The association of tau and TIP5 increases in AD brain.
Quantitative Immunogold labelling showed that Tau-1 and TIP5 associate more in the AD brain compared to the control (P= 0.0001) (A). The density of TIP5 particles around nP-Tau is also significantly higher in the AD brain (P= 0.0001) (B). Experiments repeated twice. Control sample used showed minimal ageing, and the AD samples were from Braak stage 5 & 6, see Materials and Methods for more information about the donors.

To understand if our findings have any relevance to AD and begin to identify possible involvement of TIP5 in the disease, under my supervision, Sherin Wagih and Luca Biasetti examined tissue sections from two stage six AD cases and two age-matched controls using double immunogold labeling for nP-Tau and TIP5, imaged the cells and quantified the level and density of colocalisation between these proteins (See Chapter 2, Materials and Methods). Interestingly, this revealed a significant increase in the degree of nP-Tau/TIP5 colocalisation and an increase in the density of TIP5 gold

particles around nP-Tau particles in the AD brains compared to the control (Fig. 5.11 A, B). Although the analysis was done on a small sample, it provides preliminary data that indicate the potentially important role of tau TIP5 interaction in Alzheimer's disease.

5. 4 Conclusions

In this study, we revealed a novel association for tau and TIP in the heterochromatin and nucleolus and showed that our findings from SHSY5Y are supported by investigation of human brain tissue. Considering the role of tau uncovered in this study, its association with TIP5, and the indispensable role of TIP5 in heterochromatin formation, we postulate that the Tau/TIP5 association may function to stabilise the repressive epigenetic marks on the rDNA and constitutive heterochromatin. The rDNA has been reported to become hypermethylated in AD (Pietrzak et al., 2011, Lee et al., 2012), and this can lead to the deficit in rRNA production that occurs in the disease (da Silva et al., 2000, Ding et al., 2005). Moreover, studies have linked AD pathology with hypermethylation of many genomic regions compared to control brains (Smith et al., 2016). This may suggest that the increased association between Tau/TIP5 in the AD brains, function to promote rDNA methylation and hypermethylation of vulnerable genes in AD. Although future studies will address the relationship between Tau and TIP5 in heterochromatin stability and rDNA transcription, this study revealed that tau alone is important for these functions. Although neurons are mostly post-mitotic, and nuclear tau localises to neuronal and non-neuronal cells, the implication of our study and others (Rossi et al., 2013, Rossi et al., 2008, Mansuroglu et al., 2016) implicates a role for tau in genome stability and/or transcriptional repression in nervous and non-nervous tissues.

The role uncovered for tau in this study would be strengthened by the overexpression of tau to investigate whether it would enhance heterochromatin formation and suppress rDNA transcription. The analysis of tau and TIP5 association in the human brain described here was from two cases. Therefore, more cases need to be studied to arrive at a more sound conclusion regarding its likely functional role.

Chapter 6

6.0 Discussion

It is now over 110 years since Alois Alzheimer first reported AD. Breakthrough came with the identification of Aβ42 as the main constituent of plaques and tau protein as the main constituent of tangles. The amyloid cascade hypothesis puts forward Aβ as the culprit in AD (Selkoe and Hardy, 2016, Hardy and Higgins, 1992). Indeed, a substantial amount of research in the AD field and particularly, mechanism of Aβ toxicity, has demonstrated its impact on the synapse (Selkoe, 2002), neuroinflammation (Heppner et al., 2015) and oxidative stress (Butterfield et al., 2013). The effect of AD on the synapse is substantial, and occurs early in the disease, as a result of which AD has been described as a disease of synaptic failure (Selkoe, 2002). In this thesis, I have described work to explore other mechanisms contributing to neurodegeneration in AD and found that glutamate or Aβ toxicity induce chromatin alteration, nucleolar stress and protein synthesis inhibition. This work also discovered that tau protein, mainly known for its role in microtubule binding and stability, is involved in heterochromatin stability and transcriptional silencing of the rDNA.

6.1 Glutamate and Aβ: Common neurotoxins, different paths, to common goal

Substantial evidence indicates that AD-related impairment of protein synthesis (Langstrom et al., 1989, Mann et al., 1981a), could occur early in the disease before full-blown AD (Ding et al., 2005, Keller, 2006). This impairment may result from multiple pathways, such as a reduction in rRNA production, processing, and ribosome formation. It could also arise from the phosphorylation of eIF2α on serine 51 which leads to the inhibition of protein translation. EIF2α can be phosphorylated by several kinases that are mostly activated by specific stimuli. For instance, nutrient deprivation

leads to the activation of general control nonderepressible 2 kinase (GCN2); accumulation of misfolded proteins leads to the activation of unfolded protein synthesis response (UPR) due to which the Pancreatic endoplasmic reticulum kinase (PERK) becomes activated; viral infection or double-stranded RNA activates Protein kinase RNA-activated kinase (PKR); and haem deficiency or oxidative stress activate haem-regulated inhibitor kinase (HRI). The phosphorylation of eIF2α on serine 51 prevents it from forming a ternary complex with GTP and the initiator Met-tRNAi required for protein translation, leading to the inhibition of protein synthesis (Holcik, 2015, Holcik and Sonenberg, 2005). The impairment of protein synthesis capability in primary neurons treated with Aβ25-35 or glutamate has been demonstrated to occur before overt neurodegeneration (Gordon et al., 2012, Shan et al., 2007). Protein synthesis is a process downstream of gene expression, which depends on chromatin configuration. Supporting this, several studies reported chromatin alteration in AD, linking deficits observed in the levels of RNA and proteins in the disease to upstream changes in the chromatin of diseased neurons (Mann et al., 1977, Mann and Sinclair, 1978, Mann et al., 1980, Lewis et al., 1981, Crapper et al., 1979, McLachlan et al., 1991). This thesis showed that glutamate stress in neuroblastoma cells results in a significant induction of oxidative stress, DNA damage, heterochromatin loss, nucleolar stress and protein synthesis inhibition which occurs via eIF2α phosphorylation, rather than a reduction in rRNA levels. Likewise, Aβ42 oligomers induced oxidative stress, with gradual accumulation of nucleolar stress, which culminated to full-blown nucleolar stress and protein synthesis impairment, without either DNA damage or eIF2α phosphorylation. How glutamate induces eIF2α phosphorylation in the SHSY5Y cells has not been investigated, but it may be via the induction of oxidative stress, which has been previously shown to be responsible for glutamate-induced neuronal injury

associated with UPR activation in rat cortical neurons (Chen et al., 2012). Consistent with this, oxidative stress in SHSY5Y cells has been shown to induce eIF2α phosphorylation via PKR not PERK (Mouton-Liger et al., 2012). Therefore, this would suggest that the eIF2α phosphorylation observed here following glutamate treatment likely resulted from oxidative stress-induced PKR activation. Overall, findings from both glutamate and Aβ treatment reveal that protein synthesis inhibition is the common path towards neurodegeneration induced by both Aβ and glutamate. This is interesting since previous studies showed that Aβ toxicity could cause glutamate excitotoxicity (reviewed in Esposito et al., 2013). Likewise, activation of NMDA receptors exerts a reciprocal effect by enhancing Aβ and tau protein production (Molinuevo et al., 2005). In AD, an increase in PKR and phosphor-eIF2α has been reported in the brain cortices (Mouton-Liger et al., 2012). The levels of BiP/GRP78 and phosphor-PERK – both components of the UPR pathway, also increase in the AD brain but do not colocalise with AT8-positive tangles, suggesting that UPR activation is an early event that occurs before tangle deposition (Hoozemans et al., 2009, Hoozemans et al., 2005). Several other neurodegenerative diseases, such ALS, Parkinson's disease, progressive supranuclear palsy, and FTD have also been reported to show protein synthesis impairment, particularly via UPR-PERK-eIF2α pathway (reviewed in Halliday and Mallucci, 2015). Restoration of protein synthesis in animal models of prion disease and FTD has been demonstrated to reverse neurodegeneration, thus raising the hopes that the restoration of protein synthesis could be the holy grail for the treatment of neurodegenerative diseases (Halliday et al., 2017). However, our findings revealed that glutamate but not Aβ activates the eIF2α pathway. Accordingly, a previous finding using Aβ42 oligomers failed to find UPR activation in differentiated SHSY5Y cells even after four days of incubation (Chafekar et al., 2007). UPR activation

was only observed in the non-neuronal HEK293 cells engineered with a fluorescent ER-stress reporter (Chafekar et al., 2007). Incubation of cells with a mixture of oligomeric and fibrillar Aβ also failed to activate the UPR in rat cultured cortical neurons (Yu et al., 2006). The work in this thesis revealed that the incubation of differentiated SHSY5Y cells with Aβ does not induce eIF2α phosphorylation, but instead leads to the reduction of rDNA transcription and global RNA synthesis, which could have directly contributed to the decrease in protein synthesis observed (Fig. 6.1). Howbeit, the UPR can be activated by misfolded proteins (Rao and Bredesen, 2004). Therefore it is possible that at high concentration of Aβ or later time when Aβ aggregates accumulate, the UPR- eIF2α phosphorylation would become activated.

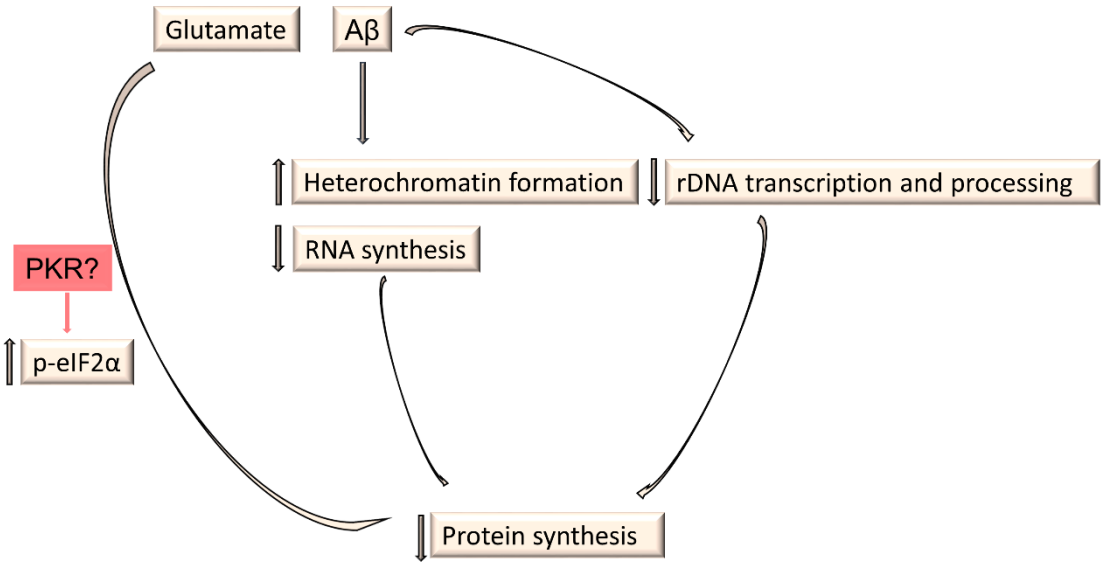

Figure 6. 1 Pathways for protein synthesis inhibition by glutamate and Aβ
Glutamate and Aβ administration both lead to inhibition of protein synthesis, albeit via separate mechanisms. Glutamate administration results in a decrease in protein synthesis inhibition via eIF2α phosphorylation. Aβ incubation also results in a reduction in protein synthesis by reducing rDNA transcription and processing, which would affect ribosome formation. It also increases heterochromatin formation and thus decreases RNA synthesis. Collectively, both would result in decreased protein synthesis.

The work in this thesis highlights a difference between glutamate and Aβ toxicity in the induction of cellular dyshomeostasis, particularly in the period studied. Indeed, excitotoxicity induced by glutamate or kainic acid has been shown to activate the UPR in primary rat astrocytes and neurons (Kim et al., 2010, Sokka et al., 2007, Zhang et al., 2016). The UPR activation occurs before the onset of neurodegeneration (Sokka et al., 2007). This is consistent with our finding using glutamate, which induced DNA damage and protein synthesis inhibition through UPR activation before apparent cell death. Our results using Aβ specifically support deficits in rRNA production reported in MCI (Ding et al., 2005, Keller, 2006, Ding et al., 2006), as an early event in AD, compared to UPR activation that occurs in the later stage of the disease (Hoozemans et al., 2009). Since glutamate excitotoxicity is a secondary mechanism of toxicity in AD, downstream of Aβ toxicity (Dong et al., 2009, Molinuevo et al., 2005), early effects of Aβ on the protein synthesis machinery in AD might be the induction of nucleolar stress mainly affecting the rRNA pathway. Subsequently, the accumulation of misfolded proteins and glutamate excitotoxicity induced by Aβ could accumulate to activate the UPR, thereby facilitating neurodegeneration.

6.2 Glutamate relaxes, while Aβ compacts, the chromatin

Chapter 3 & 4 showed that both glutamate and Aβ ultimately decreased protein synthesis in the SHSY5Y cells (Fig. 6.1). However, the Aβ incubation led to an increase in heterochromatin formation without DNA damage. In contrast, glutamate treatment resulted in heterochromatin relaxation with DNA damage, pointing to slightly different pathways to neurodegeneration induced by the Aβ and glutamate toxicity. Inactivation of the histone-lysine N-methyltransferase - suv39h1 has been shown to cause a widespread reduction in H3K9me3 and an increase in pericentromeric satellite 2 and centromeric α – satellite transcription, indicating that heterochromatin

relaxation enhances transcription of previously masked genes (Wang et al., 2013). Consistent with this, tau toxicity has been shown to induce heterochromatin decompaction, followed by the enhancement of the transcription of previously silenced genes (Frost et al., 2014). This work also found that the heterochromatin relaxation induced by glutamate is accompanied with enhanced transcription of global RNA. Likewise, the chromatin compaction induced by Aβ is accompanied with reduced transcription of global RNA. However, the key question is why does glutamate relax, while Aβ compacts the chromatin since they both ultimately reduce protein synthesis? A possible explanation could be that this difference results from the DNA damage observed following the glutamate treatment. DNA damage response could induce heterochromatin decompaction through a mechanism involving the phosphorylation of Kruppel-associated Box (KRAB)-associated Co-repressor KAP1 (Price and D'Andrea, 2013). KAP1 is a transcriptional repressor that associates with other repressors, such as HP1 to produce a compact heterochromatin. DNA damage response induced phosphorylation of KAP1 at Ser824 and Ser473 has been shown to induce its dissociation from HP1 (White et al., 2012, Hu et al., 2012, Goodarzi et al., 2008), It may also cause the removal of HP1β from the heterochromatin when phosphorylated at Ser473 (Ayoub et al., 2008, Bolderson et al., 2012). These, lead to heterochromatin decompaction and accessibility of DNA repair proteins to the sites of damage (Price and D'Andrea, 2013). Therefore, the relaxation of the heterochromatin induced by the glutamate treatment but not Aβ may arise from the DNA damage induced by the glutamate, but not Aβ.

6.3 The non-microtubular side of Tau protein

The involvement of pathological tau protein in various neurodegenerative diseases has made it a major interest in neurodegeneration research. Recently, interest in other

cellular locations of tau and their functional role has increased (Bukar Maina et al., 2016). To investigate the impact of Aβ or glutamate-induced cellular stress on nuclear tau, this work discovered a novel role for tau protein in the silencing of rDNA transcription and heterochromatin stability. Immunogold electron microscopy showed that tau localises to the heterochromatin and the nucleolus in neuroblastoma cells and the human brain. Nuclear tau has been previously revealed in the human brain, although it rarely localises to the nucleolus (Brady et al., 1995). Evidence presented here confirms the nucleolar localisation of tau and suggests that tau can be considered a bonafide nucleolar and heterochromatin protein.

Cellular distress has been shown to induce the nuclear translocation of non-phosphorylated (Sultan et al., 2011, Violet et al., 2014) or phosphorylated (Noel et al., 2016, Lu et al., 2013a, Lu et al., 2013b) tau into the nucleus, where it may play a role in genome stability or damage, respectively. This work showed that cellular stress induced by glutamate causes nuclear accumulation of phosphor-tau in the nucleus co-occurring with widespread DNA damage and heterochromatin loss (Fig. 6.2). The incubation of cells with Aβ did not induce nuclear phosphorylated tau and was neither associated with DNA damage or heterochromatin loss. Recent work showed that oxidative stress, DNA damage, heterochromatin loss and aberrant gene expression occur downstream of tau toxicity (Frost et al., 2014, Khurana et al., 2012, Khurana et al., 2006, Dias-Santagata et al., 2007). Therefore, the accumulation of nuclear phosphor-tau induced by the glutamate but not Aβ could have contributed to the chromatin decompaction observed here.

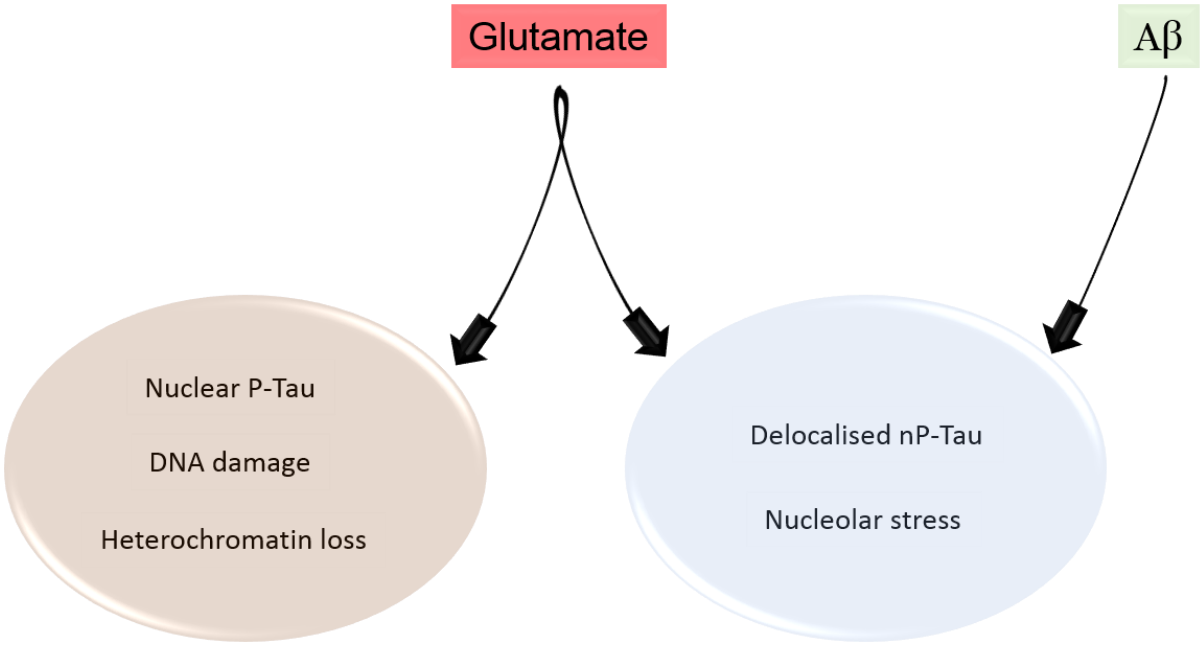

Figure 6. 2 Similarities and difference in glutamate and Aβ – induced nuclear insults. Glutamate and Aβ administration both lead to the delocalisation of nP-Tau from fibrillarin and nucleolar stress. However, glutamate administration alone results in DNA damage, heterochromatin loss and accumulation of nuclear phosphorylated tau (P-Tau). Accumulation of nuclear P-Tau is associated with both DNA damage, heterochromatin loss and nucleolar stress, while delocalisation of tau is only associated with nucleolus stress. This suggests a deleterious role for the P-Tau and indicates that different tau species could be involved in nuclear stress response.

This work also found that nucleolar stress co-occurs with delocalised nucleolar non-phosphor tau (nP-Tau) following the glutamate treatment. Aβ treatment is associated with nucleolar stress and delocalised tau, without DNA damage. The absence of DNA damage in cells having only delocalised nP-Tau following the Aβ treatment may indicate that the delocalisation occurs as a stress response, similar to other nucleolar proteins like fibrillarin which also become redistributed following cellular stress (Kodiha et al., 2011). Just as many neurodegenerative diseases share glutamate excitotoxicity as a common feature (Colantuoni et al., 2011, Dong et al., 2009), nucleolar stress is a common feature of many of such diseases (da Silva et al., 2000, Ding et al., 2005, Dönmez et al., 2005, Garcia-Esparcia et al., 2015, Hernandez-Ortega et al., 2015, Parlato and Bierhoff, 2015). Interestingly, in a similar fashion to a previous

report in a human patient with presenile dementia with motor neuron disease (Papasozomenos, 1995), following the glutamate distress, the nuclear phosphor-tau did not show obvious colocalisation with nucleolar non-phosphor tau or fibrillarin. This indicates that different tau species could be involved in driving the nuclear pathology induced by the glutamate. Future studies are required to address the specific involvement of the different tau species in the nuclear stress response and examine whether the accumulated nuclear phosphor-tau observed here impacts on the heterochromatin and DNA damage and how.

6.4 Tau meets the nuclear and nucleolar heterochromatin

A very good relationship exists between the nucleus and nucleolus in the maintenance of nuclear architecture, such as chromosomal localisation (Fig. 6.3) (van Koningsbruggen et al., 2010). This relationship is more apparent between the nucleolus and nuclear heterochromatin domains, such that both influence one another and share protein pools that are particularly involved in gene repression, such as TIP5, HP1, H3K9me3 and H3K9me2 (Németh and Längst, 2011, Bártová et al., 2010). Heterochromatin instability alters the integrity of the nucleolus and rDNA transcription, indicating an impact on nucleolar heterochromatin (Peng and Karpen, 2007, Larson et al., 2012). Likewise, nucleolar functions such as rDNA stability regulate heterochromatin stability and genome gene expression (Paredes and Maggert, 2009, Paredes et al., 2011, Gibbons et al., 2014). For instance, deletion of rDNA affects gene expression and cause heterochromatin relaxation in *Drosophila* (Paredes and Maggert, 2009). The nucleolus is in contact with many chromosomal loci such as telomeric and centromeric DNA (Fig. 6.3) (van Koningsbruggen et al., 2010), pointing to a bi-directional relationship between nuclear and nucleolar

heterochromatin, the maintenance of which is important for nuclear function and stability.

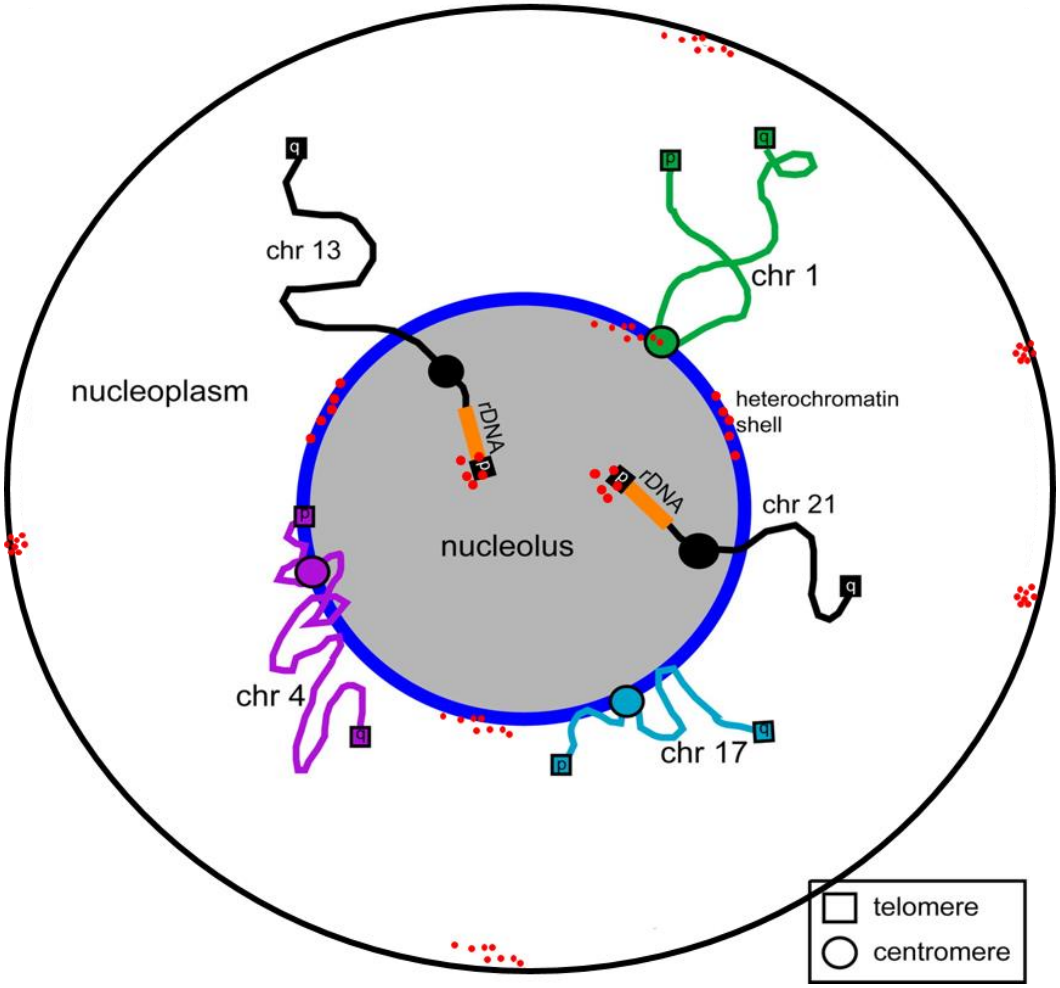

Figure 6. 3 Nuclear tau and nucleolar-associated chromatin regions.
Modified from (van Koningsbruggen et al., 2010). Schematic depicting some chromosome loci, such as telomeres and centromeres that show a preferential nucleolar association. Although in humans nucleolar organiser regions form around the short arm of acrocentric chromosomes (13, 14, 15, 21 and 22), here only chromosome 13 and 21 are depicted. All the chromosome regions that show preferential nucleolar association have AT-rich sequence elements within the nucleolar-associated loci. Tau (red dots) localises to the short arm of the acrocentric chromosomes (Loomis et al., 1990), show affinity for AT-rich sequences (Sjoberg et al., 2006), localises to both the nucleolus, perinucleolar heterochromatin and laminar associated heterochromatin, indicating that it interacts with both chromosome domains localised to the nucleolus and heterochromatin domains at nucleolar and nuclear periphery. The arrangement of the heterochromatin around the nucleolus and different loci of the chromosomes illustrate a potential role for the nucleolus in the global organisation of chromosomes within the nucleus. The distinct pattern of tau localisation may also illustrate its importance to the chromosome organisation and chromatin stability within the nucleus. Note that the depiction of tau close to chromosome one is only to indicate that tau could associate with centromeric DNA (circle) around the nucleolus (Sjoberg et al., 2006, Mansuroglu et al., 2016), not specifically those from chromosome 1.

The relationship between the nucleolus and several nuclear domains is partly why some proteins have emerged as key players for the maintenance of heterochromatin domains within the nucleus and nucleolus. This is the case for TIP5, the larger subunit of the NoRC which plays an indispensable role in heterochromatin formation at centromeres, telomeres and rDNA (Postepska-Igielska et al., 2013, Santoro et al., 2002). Interestingly, the first finding to document species of nuclear tau revealed that tau localises to the acrocentric chromosomes (Loomis et al., 1990) – a region enriched with heterochromatin (Prakhongcheep et al., 2013, Hughes and Hawley, 2009). Tau has subsequently been shown to associate with the pericentromeric heterochromatin (Sjoberg et al., 2006). Here, we revealed that tau localises to the heterochromatin at the nucleolar and nuclear periphery associated with TIP5 in both SHSY5Y cells and the human brain. It also associates with TIP5 in the nucleolus, indicating that it may localise to the nucleolar heterochromatin. The nucleolus-associated heterochromatin contains repetitive satellite sequences which might nucleate the assembly of perinucleolar heterochromatin (Németh and Längst, 2011). The maintenance of such repeats by TIP5 has been shown to be important for genome stability (Guetg et al., 2010). Studies revealed that tau could bind the pericentromeric α-satellite DNA sequences in human fibroblasts, lymphoblasts and HeLa cells (Sjoberg et al., 2006). It specifically forms protein:DNA complex by interacting directly with the human α and murine γ satellite AT-rich sequences (Sjoberg et al., 2006, Qi et al., 2015). It has been observed that silent rDNA repeats are segregated from active repeats by extranucleolar localisation to the perinucleolar heterochromatin (Akhmanova et al., 2000) or nuclear matrix mediate by TIP5 (Zillner et al., 2013).

Therefore, the association of tau and TIP5 at the perinucleolar and perinuclear heterochromatin observed here potentially indicate that tau may contribute to the

rDNA segregation mediated by TIP5. Given the importance of the nucleolus and heterochromatin in nuclear stability and architecture, our finding also suggests that tau may play a role in the maintenance of nuclear architecture (Fig. 6.3). This can be by linking and stabilising the heterochromatin and the nucleolus either independently or cooperatively with TIP5 and other chromatin remodellers that localise to both compartments. This is supported by the finding which revealed that tau KO mouse harbour pericentromeric heterochromatin instability, which can be rescued by tau overexpression to the nucleus (Mansuroglu et al., 2016). This also suggests that tau may be essential for the stability of the satellite repeats at the pericentromeric heterochromatin (Mansuroglu et al., 2016). Consistent with this, experiments with tau knockdown in the SHSY5Y cells led to an increase in rDNA transcription, concomitant with a reduction in H3K9me3 and HP1α foci and nuclear levels of H3K9me2, indicative of the destabilisation of the heterochromatin. A destabilised heterochromatin would be associated with altered interactions between chromatin remodellers that promote heterochromatin formation, such as an interaction between TIP5 and DNMTS and HDACs, needed for silencing of the rDNA transcription (Fig. 6.4).

It is known that DNA methylation plays a role in the preservation of chromatin stability (Phillips, 2008). Consistent with this, in addition to the loss of H3K9 methylation observed here following the depletion of tau, we also observed a global decrease in 5-methylcytosine methylation. Methylation-sensitive restriction digest using MspI and HpaII revealed a reduction in the methylation level of the T0 element of the rDNA following the tau knockdown. The T0 element lies upstream of the rDNA promoter, serving as the binding site for TTF-I to facilitate transcriptional initiation of the rDNA (Längst et al., 1998). Whether the decrease in methylation of this element

following the tau knockdown has an implication on the binding of TTF-I and its interaction with TIP5 at T0 element needs to be investigated by future studies. Hypothetically, the reduction in methylation of the T0 element may indicate that TIP5 was either unable to bind to the T0 element or fails to recruit Dnmt and HDAC1 to methylate and deacetylate the region (Fig. 6.4). Nevertheless, all these provides a strong evidence of the involvement of tau in the nuclear and nucleolar heterochromatin stability, involving a regulation of DNA methylation. The compaction of the chromatin is known to prevent spurious transcription and illicit recombination and enhances genome stability. TIP5-mediated heterochromatin formation has been shown to protect cells from rDNA illicit recombination (Guetg et al., 2010). Consistent with this, splenocytes from tau KO mice were shown to harbour genome instability, characterised by aneuploidy, indicating that tau, similar to TIP5, promotes genome stability (Granic et al., 2010).

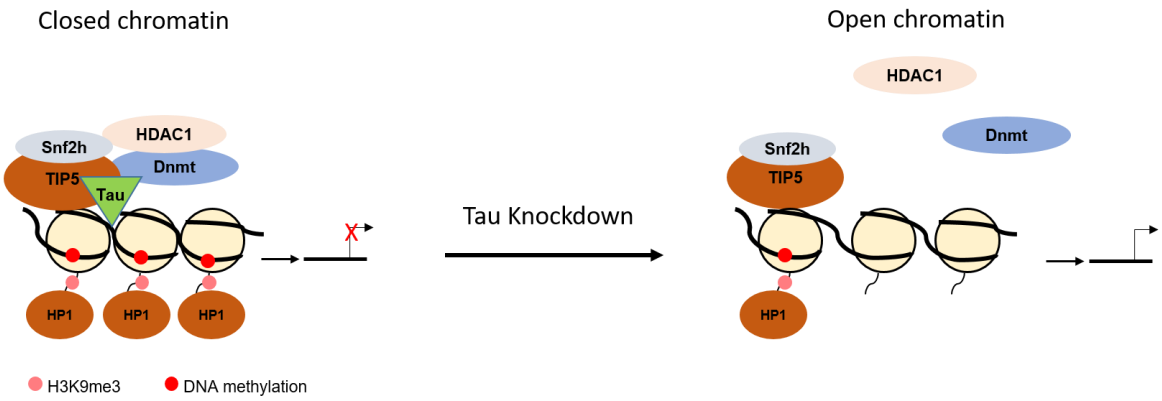

Figure 6. 4 Hypothetical model of tau-induced heterochromatin formation.
Tau may provide stability to the complex formed by TIP5 and HDAC1 and DNMT, leading to heterochromatin formation, silencing of the rDNA and stability of heterochromatin domains. This may be facilitated by tau's ability to bind the minor groove of the DNA. In such case, tau knockdown may destabilise the interaction of TIP5 and its partners, preventing the recruitment of HDAC1 and Dnmt, without which, heterochromatin formation and gene repression may not occur, resulting in loss of H3K9 methylation, DNA methylation and an increase in transcription of the rDNA. Although this may not mean the knockdown would cause TIP5 to lose its entire roles in the chromatin because TIP5 has several chromatin binding motifs (Zillner et al., 2013, McStay and Grummt, 2008).

Tau is only just emerging as a heterochromatin stabiliser, thus at this stage, it is not clear whether tau plays its heterochromatin role via the recruitment of chromatin remodelers to induce heterochromatin formation or whether it serves as an important component of the heterochromatin complex crucial for the maintenance of the heterochromatin (Fig. 6.4). Although the localisation of tau in the SHSY5Y cells and the human brain within the condensed heterochromatin by electron microscopy and its association with H3K9me3 (Mansuroglu et al., 2016), TIP5, Hp1 and CENP-B shown here establishes it as a heterochromatin protein. Interestingly, TIP5 mRNA, not protein, increased following the tau knockdown, suggesting a possible compensatory response at the mRNA level. The fact that no similar increase in the mRNA of fibrillarin or UBF was observed indicates that tau may also play a role in the transcriptional silencing of a defined set of genes. Overall, more research is required to address the mechanism by which tau contributes to chromatin stability and transcriptional silencing.

6.5 Tau as a transcriptional repressor: fitting the jigsaw puzzle

Many heterochromatin stabilising proteins play a role in transcriptional repression. Such proteins exert their function via a protein:protein interaction around the DNA or direct interaction with the DNA that often involves DNA bending and restructuring. Often, the types of domains contained in a protein and its nature of interaction with the DNA highlights its possible function. Thus far, tau has been demonstrated to interact with the DNA via the minor groove (Sultan et al., 2011, Wei et al., 2008). Minor groove binding proteins are known for their DNA bending properties, as a result of which they regulate the transcriptional state of the chromatin (Bewley et al., 1998). For example, the high mobility group A proteins (HMGA) can bind the DNA minor groove or transcription factors, this way, they can form enhanceosomes around enhancers or promoters that lead to transcriptional activation or repression (Sgarra et al., 2006). The H-NS-like proteins, MvaT-like proteins and Lsr2-like proteins which drive Xenogeneic silencing in bacteria target the AT-rich sequences of the minor groove (Will et al., 2015). Interestingly, both HMGA, H-NS and Lsr2 possess an AT-hook, containing a conserved core sequence of arginine-glycine-arginine (R-G-R) that mediate binding to the minor groove (Will et al., 2015, Sgarra et al., 2006). The AT-hook sequences are found in many nuclear proteins playing a role in DNA binding and chromatin remodelling (Aravind and Landsman, 1998). Interestingly, TIP5 also has four AT-hook motifs (Zillner et al., 2013, McStay and Grummt, 2008). On the contrary, similar to MvaT-like proteins which do not have AT-hook motif (Will et al., 2015), tau protein also does not appear to have an AT-hook motif. However, *in vitro* studies demonstrate that tau can independently bind to the minor groove of the DNA (Wei et al., 2008, Qi et al., 2015). Nonetheless, above evidence indicate that TIP5 which has AT-hooks may facilitate the interaction of tau with the DNA *in vivo*. Apart from

interacting with the DNA through arginine residues, some proteins interact with the DNA via the insertion of lysine residues to the minor grooves (Rohs et al., 2010, Rohs et al., 2009). Consistent with this, it was demonstrated that tau interacts with the DNA minor groove via the lysine residues on the second half of the PRD (R209 to A246) and MBD (K267 to S289), thereby twisting as well as changing the conformation of the DNA (Wei et al., 2008, Qi et al., 2015). Its interaction with the DNA that results in DNA bending may lead to the recruitment of transcriptional repressors to cause gene repression; such a mechanism occurs in the regulation of the β-globin gene (Drew et al., 2000). Interestingly, in addition to an increase in rDNA transcription and TIP5 transcripts observed here following the depletion of tau, evidence from tau KO mice showed that its absence enhances the transcription of several genes (Oyama et al., 2004), including the pericentromeric chromatin (Mansuroglu et al., 2016) and smarce1 gene (Gómez de Barreda et al., 2010). Loss of transcriptional repressors only results in a modest increase in the transcription of some selected genes that are normally not silent in wild-type cells (Reynolds et al., 2013). Tau behaves similarly by selectively enhancing TIP5 gene expression not genes for fibrillarin and UBF. Consistent with the heterochromatin stabilising role of tau, these findings support a role for tau in transcriptional repression (Ke et al., 2012).

Decades of research show that transcriptional repression is a complex event driven by many mechanisms and DNA configurations, including DNA methylation (Ogbourne and Antalis, 1998). Generally, the accessibility of transcription machinery to the chromatin depends on the state of the nucleosome, comprised of histone octamer, having each two H2A, H2B, H3 and H4, with an associated 146 base pairs of DNA and associated regulatory proteins (Kornberg, 1974). Condensed chromatin is mostly impermissive to the transcriptional machinery, and the condensation can be

influenced by many mechanisms, especially DNA methylation, which is responsible for heterochromatin formation across the genome (Cedar and Bergman, 2009). Typically, DNA methylation triggered by DNA methyltransferases at CpG islands create a configuration that allows the recruitment of methyl-binding proteins, which initiate repressive cascade through the recruitment of enzymes that lead to deacetylation of histones, such as H3K9me3 and its methylation, subsequently, allowing for the binding of HP1 and chromatin compaction (Phillips and Shaw, 2008). The control of transcription can thus be modulated by a combination of mechanisms, including DNA methylation and heterochromatin formation (Ogbourne and Antalis, 1998). The depletion of tau decreased the levels of histone methylation revealed by the decrease in H3K9me2 and H3K9me3, as well as, DNA methylation, shown by a global reduction in the levels of 5-methylcytosine. Histone modifications such as H3K9me3 can contribute to the formation of local heterochromatin, while DNA methylation provides a long-lasting transcriptional silencing of the chromatin (Cedar and Bergman, 2009). The impact of tau on these modifications suggests that it may mediate its transcriptional repression by impacting on DNA methylation patterns. Tau could achieve this independently or through its association with repressive factors, such as TIP5, which is known to associate with DNMTs and HDACs to mediate silencing of the rDNA (Fig. 6.4) (Santoro et al., 2002). Inhibition of DNMTs or HDACs specifically showed that TIP5 inhibits rDNA transcription via the induction of DNA methylation and histone acetylation (McStay and Grummt, 2008). Thus, the interaction between tau and TIP5 is something particularly exciting, because our data suggests that the tau knockdown replicates some of the heterochromatin destabilising effects observed following the depletion of TIP5 (Santoro et al., 2009, Postepska-Igielska et al., 2013).

TIP5 has different domains that facilitate interaction with chromatin remodellers and the DNA, such as AT hooks, a C-terminal PHD and a bromodomain (McStay and Grummt, 2008). Indeed, TIP5 forms a macromolecular complex that drives gene repression (McStay and Grummt, 2008). Tau does not seem to have some of the key chromatin interacting domains, such as AT-hook (Aravind and Landsman, 1998), bromodomain (Zeng and Zhou, 2002, Sanchez and Zhou, 2009), and PHD domain (Sanchez and Zhou, 2011), found in chromatin remodellers. The association between tau and TIP5 demonstrated here indicates that the heterochromatin and rDNA transcriptional silencing roles of tau may be mediated or facilitated by TIP5 or other chromatin remodellers. It would therefore be important for future research to dissect the interaction between tau and TIP5, provide a detailed map of the regions bound by tau in the genome and how this is affected in health and diseases.

6.6 Implications of the heterochromatin association of Tau in neurodegeneration

The association of tau with the heterochromatin highlights its role in transcriptional silencing and genome stability. The compaction of the chromatin by epigenetic mechanisms like DNA methylation plays a role in the preservation of chromatin stability (Phillips, 2008). The depletion of tau or its complete absence in knockout cells revealed that it stabilises the chromatin and protect against chromosomal aberrations (Mansuroglu et al., 2016, Granic et al., 2010). In tauopathies, tau becomes modified leading to an alteration in its function. In such a scenario, the chromatin stabilising role could become altered leaving cells susceptible to genome instability (Fig. 6.5). Indeed, it has been previously shown by Rossi and colleagues that non-neuronal cells carrying tau mutations show increased chromosomal instability (Rossi et al., 2008, Rossi et al., 2013). This instability may result from reduced heterochromatin

stabilising function of the aberrant tau. Posttranslational modifications, such as phosphorylation reduce DNA binding potential of tau (Qi et al., 2015). Accordingly, a recent study revealed that tau overexpression in *Drosophila melanogaster* leads to mitotic defects (Malmanche et al., 2017). Given the localisation of tau to the chromosomes (Loomis et al., 1990, Rossi et al., 2008, Wang et al., 1993) and its capacity to bind and protect the DNA (Wei et al., 2008, Sjoberg et al., 2006, Sultan et al., 2011), the presence of different chromosomal aberrations and susceptibility to genotoxic stress in cells carrying tau mutation may result from the reduced association of this tau with the chromosome (Fig. 6.5). The absence of normal functioning tau due to mutations can affect tau's role in genome protection and render cells susceptible to chromosomal instability.

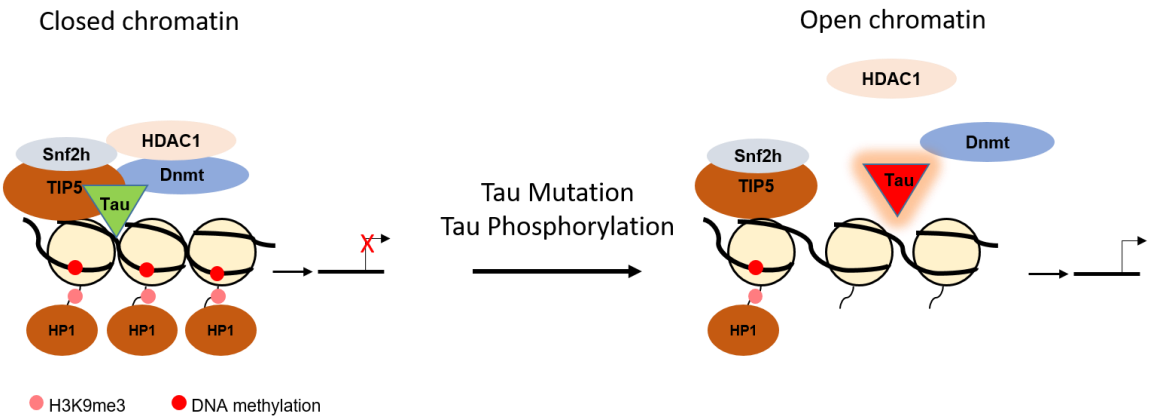

Figure 6. 5 Hypothetical model of tau-induced heterochromatin decompaction.
The heterochromatin decompaction that occurs in AD and tauopathies (Frost et al., 2014) may arise to the inability of phosphorylated tau to interact with the DNA and stabilise the repressive complex formed by TIP5. In this model, this modified tau species would not be available to stabilise the interaction between TIP5 and other repressive epigenetic marks such as Dnmt and HDAC1, leading to instability of the heterochromatin and chromatin relaxation, allowing aberrant gene expression. Since DNA methylation and heterochromatin formation are essential for chromatin stability, the increase in chromosomal aberrations observed in patients carrying tau mutations (Rossi et al., 2013) may arise due to the inability of mutant tau to interact with the DNA and thus fail to stabilise TIP5 and other repressors like Dnmt and HDAC1, leading to instability of the chromosomes.

In the context of neurodegeneration, tau toxicity in *Drosophila* and mouse model of tauopathy revealed that tau induces widespread chromatin relaxation and enhancement in the transcription of initially heterochromatic genes. Recent findings

from Philip De Jager's Lab presented at the AD/PD conference 2017 in Vienna, showed that tau pathology is associated with large-scale epigenetic changes in the genome based on DNA extracted from 600 postmortem prefrontal cortex samples from the Religious Orders Study and the Memory and Aging Project, comprised of subjects with AD and ageing related cognitive problems (Bennett et al., 2013). This revealed that about 6000 genes that become euchromatic in the brains of people are correlated with tau pathology, indicating that the aberration of tau, allows an open chromatin. Philip De Jager confirmed such a chromatin relaxing role of tau in mouse and human iPSC-derived neurons overexpressing tau. Given that hyperphosphorylation (Qi et al., 2015, Camero et al., 2014b) or aggregation (Hua and He, 2002) of tau reduces its binding capacity with the DNA, in AD, nuclear tau binding and protecting the DNA (Sultan et al., 2011) or stabilising the heterochromatin (Sjoberg et al., 2006, Mansuroglu et al., 2016); could be altered due to the change in the tau molecule configuration, such as phosphorylation, leading to its detachment from the chromatin (Lu et al., 2013b, Qi et al., 2015), resulting in the chromatin destabilisation and relaxation, culminating to aberrant gene expression (Fig. 6.5). This hypothesis supports the findings described above which indicate increased transcription of selected genes following the depletion of tau and mass heterochromatin relaxation following tau toxicity.

6.7 Nuclear and nucleolar localisation of Tau

The nuclear import of nucleotides and protein components required for the syntheses of DNA and RNA is done via a passive process. However, the transport of proteins above 40 kDa is an active process, mostly facilitated diffusion that occurs via the interaction of the protein cargo with soluble nuclear transport receptors, mostly members of the β-karyopherin (Kaps) family (Cautain et al., 2015). Depending on the protein cargo, sometimes Kaps interact with their cargo via an adaptor protein, leading

to complex interactions at the nuclear pore complex (NPC), resulting in the transport of proteins across the complex and their release in the nucleus (Mosammaparast and Pemberton, 2004). Many pathways exist for the import of proteins into the nucleus, but this usually occurs through small peptide motifs called nuclear localisation signal (NLS) located on the surface of the protein cargos (Stewart, 2007). It appears that there is no strict consensus sequence for NLS, in most cases, they are comprised of short positively charged basic amino acids residues located anywhere within the protein cargo (Cokol et al., 2000, Kosugi et al., 2009). Although tau has been shown to translocate to the nucleus following cellular stress (Sultan et al., 2011, Violet et al., 2014), here, we revealed that tau localises to the nucleus in unperturbed neuroblastoma cells and in human brain. This suggests that it commonly localises to the nucleus, playing a role even in the non-stress situation. Tau protein weighs above 40 kDa. Thus its nuclear localisation would require an active process, and thus it might require an NLS. The longest isoform of tau contains over 60 basic amino acids from its N-terminal through its C-terminal (Kolarova et al., 2012). Thus its nuclear translocation might involve the interaction of a nuclear import carrier protein with these basic residues. Interestingly, it has been reported that NLS motifs can also be used in binding DNA (Cokol et al., 2000). The PRD and MBD of tau both contain basic amino acid residues and these domains have been shown to mediate its interaction with the DNA (Qi et al., 2015, Wei et al., 2008). The transport of tau could thus occur via these domains.

Over 17% of all eukaryotic proteins are thought to be imported into the nucleus (Cokol et al., 2000). However, the imports of many such proteins are obscure. Such is the case of β-Catenin, whose transport is thought to involve direct interaction with nucleoporins at the NPC, while other mechanism may involve piggy-backing or Ran

and requiring a yet to be identified cytoplasmic binding partner (Wagstaff and Jans, 2009). Piggy-backing is particularly interesting, as it may participate in the transport of some proteins lacking NLS. Such a mechanism has been reported for the transport of a defective mutant of the hepatitis D virus antigen (Xia et al., 1992). Tau could also be transported to the nucleus by "piggybacking" (Wang et al., 1993). Interestingly, here we found that tau associates with TIP5 at the nuclear border in both SHSY5Y cells and the human brain, suggesting that tau could be piggy-backed by TIP5 into the nucleus. This may only apply to the pool of tau normally localised in the nucleus. The stress-dependent influx/efflux of tau may be via an entirely different mechanism.

In this work, we also revealed that tau localises to the nucleolus and cellular stress causes its redistribution in a similar manner to the redistribution of fibrillarin. This indicates that some pool of tau is retained in the nucleolus, where it may play a role in rDNA transcriptional silencing. Proper localisation of proteins to the nucleolus has also been shown to occur via a nucleolar localisation/retention signal (NoRS), which is not well defined but can be comprised of basic amino acid residues, such as Arg or Lys (Emmott and Hiscox, 2009). For instance, the ^{143}-K-K-R-K-K-K^{149} amino acid residues on the N-terminal part of NF-κB-inducing kinase NIK is necessary and sufficient for its nucleolar localisation (Birbach et al., 2004). However, some NoRS can be part of NLS contained in protein (Musinova et al., 2011). From this angle, it may be possible that the basic amino acid residues present on the tau molecule may mediate its nucleolar localisation/retention. However, the nucleolar proteome contains over 4500 proteins and some of these proteins do not have a NoRS, but accumulate in the nucleolus through interaction with nucleolar building blocks, such as the rDNA, its transcripts or protein components (Carmo-Fonseca et al., 2000). For instance, nucleolin, though a nucleolar protein, it does not seem to have NoRS, its transport to

the nucleolus has been suggested to occur via its interaction with nucleophosmin (Emmott and Hiscox, 2009). Consistent with this, the association of tau and TIP5 uncovered in this work, may facilitate tau's localisation to the nucleolus. TIP5 has a TAM domain that has been shown to mediate its nucleolar localisation and association with the nuclear matrix (Zillner et al., 2013). Altogether, our findings reveal that tau's nuclear and nucleolar localisation may be facilitated by the much larger, multidomain protein - TIP5. Future studies are required to fully dissect the relationship between tau and the heterochromatin complex, especially TIP5, and how it is transported to the nucleus and retain in the nucleolus.

6.8 Future directions

This work has uncovered many exciting findings regarding the toxic pathways altered by Aβ and glutamate and a new functional role for tau. Both Aβ and glutamate could ultimately inhibit protein synthesis, suggesting protein synthesis restoration might be a good therapeutic target for AD. The inhibition of the eIF2α pathway is currently considered as one approach to restoring protein synthesis in neurodegeneration since its inhibition using Trazodone hydrochloride and dibenzoylmethane prevented neurodegeneration (Halliday et al., 2017). Here we show that an early effect of Aβ in AD may involve an alteration in rRNA production, which would lead to downstream protein synthesis reduction. Our findings therefore infer that targeting eIF2α pathway may not provide a long-term solution for the protein synthesis defect in neurodegeneration if deficits in rRNA persist, as this would lead to subsequent ribosome deficits and protein synthesis defects in the long run. Importantly, recent evidence shows that nucleolar integrity is essential for synaptic plasticity and memory formation (Kiryk et al., 2013, Allen et al., 2014). For instance, Kim et al. showed that availability of the nascent rRNA transcripts and stability of the nucleolus is essential

for late-phase long-term potentiation in mouse hippocampal neurons, (Allen et al., 2014). Capitano et al. recently also showed that that spatial learning increases the nucleolar organiser region, while RNA pol I inhibition results in memory impairment in mice (Capitano et al., 2015). All these indicate the importance of the nucleolus and rRNA production in learning and memory. This has led to the hypothesis that new ribosomes may be required for new memories (Hernández et al., 2015). Although a series of ground-breaking work from the Mallucci's Lab has demonstrated the potential of targeting the eIF2α pathway to restore protein synthesis as a mechanism to slow neurodegeneration in diverse neurodegenerative diseases (Halliday et al., 2017), future studies are required to assess whether targeting this pathway also normalises the rRNA production pathway that is affected in AD (Ding et al., 2005, Ding et al., 2006), which this work reveals to result from Aβ42 toxicity.

This work also revealed that glutamate and Aβ42 toxicity both lead to the delocalisation of tau in the nucleolus. This is an entirely novel finding that needs further investigation, especially, since tau seems to play a role in heterochromatin stability and rDNA transcriptional silencing. It would be interesting to find out the ultimate fate of the redistributed tau. For instance, does the redistributed tau become degraded eventually or does it recover back to the nucleolus following the restoration of the cellular stress? Importantly, this work revealed that tau is essential for heterochromatin stability and associates with TIP5, the larger subunit of the NoRC. However, the mechanism behind the recruitment of tau to NoRC and its main role in the heterochromatin complex remains unclear. Therefore, future studies are required to investigate this process and dissect the full role of tau in the centromeric heterochromatin and NoRC. For instance, studying the recruitment and stability of different members of these complexes following tau knockout and vice versa would

provide evidence of its role in these complexes. TIP5 knockout would also show whether it is required for the nuclear and nucleolar localisation of tau or its localisation to the heterochromatin or NoRC.

In this work, we also showed that glutamate stress causes the accumulation of phosphorylated tau in the nucleus, which coincides with DNA damage and heterochromatin loss. Frost and colleagues revealed that hyperphosphorylated could cause DNA damage and heterochromatin instability (Frost et al., 2014). Although they did not investigate whether the phosphorylated tau accumulates in the nucleus. It is also not clear whether the species of nuclear phosphor-tau observed here and elsewhere (Noel et al., 2016, Lu et al., 2013a) interact with the chromatin and whether this has any consequence. One hypothesis is that nuclear-hyperphosphorylated tau, among other things, can induce heterochromatin relaxation by preventing the association of tau with the heterochromatin or altering the stability of the protein complexes stabilised by tau in the heterochromatin domains. Therefore, future studies are required to dissect whether this tau species associates with the heterochromatin and NoRC, similar to non-phosphorylated tau.

Given that tau becomes aberrant in AD, a critical question for future studies would be to provide a detailed map of the changes in the tau species that occur from MCI through stage 6 AD and correlate this to changes in NoRC and heterochromatin stability and the association of tau to this complexes. This would provide an answer to the direct involvement of tau in the heterochromatin alteration observed in the disease and whether tau-induced changes in the nucleus precede or occur after tangle accumulation in AD.

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