INVESTIGATING THE DNA DAMAGE RESPONSE IN NEURODEGENERATION

by

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Abstract

Elevated levels of DNA damage are found in neurodegenerative diseases, including Parkinson's disease, Amyotrophic lateral sclerosis, and Alzheimer's Disease. It is not known whether the DNA damage is a cause or a consequence of the pathology. DNA damage in the nervous system has the potential to cause aberrant cell cycle re-entry, trigger apoptosis or induce Alzheimer's disease-like pathological changes, including Tau phosphorylation and amyloid beta (Aβ) plaque deposition. Previous work has shown that knocking down key players involved in the response to DNA double-strand breaks, including ataxia-telangiectasia-mutated kinase (ATM) and checkpoint kinase 2 (Chk2), elicits a neuroprotective in a *Drosophila* model of Alzheimer's disease. Here, I investigated potential mechanisms downstream of the ATM-Chk2 signalling pathway to identify those that may contribute to the neurotoxicity in our *Drosophila* Alzheimer's disease model. I demonstrate the involvement of p53, a key downstream target of ATM and Chk2 in the DNA damage response and show that blocking caspase activity protects against amyloid induced toxicity. Surprisingly, I was not able to find evidence of aberrant cell cycle re-entry or apoptosis in the brains of *Drosophila* expressing toxic Aβ, which indicates a potential non-apoptotic role for caspases in amyloid induced neurotoxicity.

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List of abbreviations

Abbreviation	Meaning
AD	Alzheimer's Disease
Aβ	Amyloid beta
NFTs	Neurofibrillary tangles
MCI	Mild cognitive impairment
LOAD	Late onset Alzheimer's Disease
EOAD	Early onset Alzheimer's Disease
FAD	Familial Alzheimer's Disease
APP	Amyloid precursor protein
PSEN1	Presenilin 1
PSEN2	Presenilin 2
GWAS	Genome wide association studies
ACEi	Acetylcholinesterase inhibitor
APOE	Apolipoprotein E
CNS	Central nervous system
PET	Positron emission tomography
CSF	Cerebrospinal fluid

MRI	Magnetic resonance imaging
NIA-AA	National Institute on Aging and Alzheimer's Association
sAPP α	Soluble amyloid precursor protein alpha
AICD	Amyloid precursor protein intracellular domain
sAPP β	Soluble amyloid precursor protein beta
APH -1	Anterior pharynx defective 1
BACE1	Beta-Secretase 1
MAPT	Microtubule associated protein Tau
MBD	Microtubule binding domain
FTDP-17	Frontotemporal dementia linked to parkinsonism
GSK3β	Glycogen synthase kinase beta
CKI	Casein kinase I
PKA	Protein kinase A
Cdk5	Cyclin dependent kinase 5
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
MARK	Microtubule affinity regulating kinase
AMPK	Adenosine monophosphate activated protein kinase

dAPP	*Drosophila* amyloid precursor protein
UAS	Upstream activating sequence
SSB	Single strand break
DSB	Double strand break
ROS	Reactive oxygen species
DDR	DNA damage response
BER	Base excision repair
NER	Nucleotide excision repair
NHEJ	Non homologous end joining
HR	Homologous recombination
ATM	Ataxia-telangiectasia-mutated
ATR	Ataxia telangiectasia and Rad3-related
PI3K	Phosphoinositide-3-kinase
MRN	Mre11, Rad50 and Nbs1
BRCA1	Breast cancer gene 1
H2AX	Histone family member X
p53	Tumour protein p53
KAP1	KRAB-associated protein-1

DNA-PK	DNA-dependent protein kinase
RPA	Replication protein A
ssDNA	Single stranded DNA
SCD	SQ/TQ cluster domain
FHA	Forkhead association
Cdk2	Cyclin dependent kinase 2
NLS	Nuclear localisation signal
C-NHEJ	Canonical non-homologous end joining
Alt-NHEJ	Alternative non-homologous end joining
ALS	Amyotrophic lateral sclerosis
Cdk	Cyclin dependent kinase
PCNA	Proliferating cell nuclear antigen
BrdU	Bromodeoxyuridine
EdU	5'Ethynyl-2'-deoxyuridine
TBI	Traumatic brain injury
SCI	Spinal cord injury
SV40	Simian virus 40
Rb	Retinoblastoma

APAF1	Apoptotic peptidase activating factor 1
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1
FADD	Fas-associated death domain
DISC	Death-inducing signaling complex
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
PI	Performance index
PCR	Polymerase chain reaction
RT	Room temperature
FBS	Foetal bovine serum
PBS	Phosphate buffered saline
TBS	Tris-buffered saline
TBST	TBS-Tween 20
TBE	Tris-borate-EDTA
IHC	Immunohistochemistry
ICC	Immunocytochemistry
BL	Bloomington
GFP	Green fluorescent protein

RFP	Red fluorescent protein
HRP	Horseradish peroxidase
Cdk11	Cyclin dependent kinase 11
WT	Wildtype
DART	*Drosophila* ARousal Tracking
Dcp-1	*Drosophila* caspase protein 1
FUCCI	Fluorescent ubiquitination-based cell cycle indicator
IR	Irradiation
ATMi	ATM inhibitor
Chk2i	Chk2 inhibitor
AMPKi	AMPK inhibitor
ACC1	Acetyl-CoA carboxylase 1
A-T	Ataxia Telangiectasia
Dronc	Drosophila Nedd2-like caspase
DrICE	Death related ICE-like caspase
Dcp-1	Death caspase 1
TE	Transposable element

1.0 Introduction

1.1. Alzheimer's Disease

1.1.1 Epidemiology of Alzheimer's Disease

Dementia is a group of progressive neurodegenerative diseases that impair cognitive function. Alzheimer's Disease (AD) is the most prevalent form of dementia, contributing to around 60-70% of all cases (Alzheimer's Association, 2021). It is estimated there are 50 million people living with it worldwide which is predicted to increase to 82 million by 2030 (World Health Organisation, 2019).

The two dominant pathological hallmarks of AD are extracellular plaques composed of the amyloid beta (Aβ) peptide, and intraneuronal neurofibrillary tangles (NFT) composed of hyperphosphorylated Tau protein (Glenner and Wong, 1984a; Masters *et al.*, 1985; Grundke-Iqbal *et al.*, 1986; Nukina and Ihara, 1986). Research suggests both proteins contribute to the neurotoxicity seen in AD (*this will be covered in detail in sections 1.2 and 1.3*). These neuropathological changes are accompanied by gliosis, synapse impairment, DNA damage and neuronal loss (Itagaki *et al.*, 1989; Masliah *et al.*, 2001; Ribe *et al.*, 2008; Lin *et al.*, 2020). AD can initially present without any symptoms, leading into mild cognitive impairment (MCI) and eventually progressing to an AD diagnosis (Scheltens *et al.*, 2021). Symptoms of AD include memory loss, decline in cognition, alongside behavioural and speech problems, eventually leading to functional loss of daily activities (Scheltens *et al.*, 2021). In late-stage severe AD, individuals will require full care due to impaired movement. The individual is likely to be bed-bound, increasing the risk of blood clots, infections, sepsis and organ failure (Alzheimer's Association, 2021).

AD can result in severe complications such as inability to swallow and immobility which can result in respiratory and circulatory system disorders, leading to death.

One common cause of death in patients with AD is aspiration pneumonia where food or liquid enters the lungs. Survival time after an AD diagnosis depends on the age of diagnosis and sex, with the average survival time being 4-8 years if diagnosed at age 65 or older (Alzheimer's Association, 2021). In a cohort study taking data from general practises in the UK between 1990-2007 they discovered the median survival of people diagnosed at age 60-69 was 6.7 years, compared to 1.9 years for those diagnosed in there 90 and above (Rait *et al.*, 2010).

There are many genetic and environmental risk factors for developing AD. Age is the most significant risk factor for AD, with 90% of patients being diagnosed over the age of 65, known as late onset AD (LOAD) or sporadic (Hoogmartens, Cacace and Van Broeckhoven, 2021). A diagnosis before 65 is known as early onset AD (EOAD) or familial AD (FAD) and can be dominantly inherited by mutations in one of three genes involved in processing of the Aβ peptide : APP (amyloid precursor protein), PSEN1 or PSEN2 (presenilin 1 & 2) (Cacace, Sleegers and Van Broeckhoven, 2016). Other environmental risk factors for AD include smoking, blood pressure and diet (Livingston *et al.*, 2020).

1.1.2 Genetics of Alzheimer's Disease

AD is a complex disease with varying influencing factors such as age, environment, and genetics. LOAD is a polygenic disease with a heritability of around 56-79%, in contrast to EOAD which is almost entirely genetically determined with a heritability of approximately 92-100% (Gatz *et al.*, 2006; Wingo *et al.*, 2012).

Many different GWAS studies have identified the strongest risk factor for LOAD is carrying at least one ε4 allele of APOE gene, which encodes a transport protein for cholesterol (Bertram *et al.*, 2007; Bertram and Tanzi, 2009). The ε4 allele is suggested to decrease Aβ clearance (Bertram and Tanzi, 2009; Castellano *et al.*, 2011). There is a dose dependent effect of carrying an ε4 allele, with each allele present increasing the risk of developing AD, and decreasing the mean age of onset

(Corder *et al.*, 1993; Farrer *et al.*, 1997; Bullido *et al.*, 1998; Bertram *et al.*, 2007; Coon *et al.*, 2007). Compared to a non-carrier of any ε4 alleles, carrying one allele increases the risk by ~2-3 times, and carrying two alleles has an 8 to 12 fold risk (Corder *et al.*, 1993; Kim, Basak and Holtzman, 2009; Bertram, Lill and Tanzi, 2010). Carrying the ε4 allele is also a risk factor for EOAD (López-Riquelme *et al.*, 2016). The ε2 allele has opposite effect, decreasing the risk of AD and is associated with a later onset of disease (Corder *et al.*, 1994).

EOAD is rare, accounting for ~ 5-10% of total AD cases (Cacace, Sleegers and Van Broeckhoven, 2016). Studies of families showing autosomal dominant inheritance of AD lead to the discovery of three genes for EOAD: APP, PSEN1 and PSEN2 (Kang *et al.*, 1987; Levy-Lahad *et al.*, 1995; Sherrington *et al.*, 1995). It is widely accepted that FAD mutations in these genes either increase the total level of Aβ peptides or increases the ratio of Aβ42/Aβ40 (Hoogmartens, Cacace and Van Broeckhoven, 2021) (*FAD mutations are covered in more detail in section 1.2.2*). Aβ42 is the more aggregative species and forms the amyloid plaques whilst Aβ40 is suggested to be protective against plaque deposition (*see section 1.2 for more on amyloid beta*) (Kim *et al.*, 2007). Mutations in these genes are dominantly inherited and explain around 5-10% of EOAD cases, leaving the majority of EOAD cases genetically unexplained (Wingo *et al.*, 2012; Dai *et al.*, 2018). Mutations in PSEN1 are the most common cause of EOAD, accounting for 6% of total EOAD cases and has the earliest onset of disease (~8.4 and ~14.2 years on average earlier compared to APP and PSEN2, respectively) (Cruts, Theuns and Van Broeckhoven, 2012; Cacace, Sleegers and Van Broeckhoven, 2016). The estimated frequency of mutations in APP and PSEN1 in EOAD cases are <1% and 1%, respectively (Brouwers, Sleegers and Van Broeckhoven, 2008).

1.1.3 Treatment of Alzheimer's Disease

The only drugs available for AD today are targeted at alleviating symptoms to improve cognition function (Yiannopoulou and Papageorgiou, 2020). Most of the drugs available are acetylcholinesterase inhibitors (ACEi's) which work by increasing the amount of acetylcholine neurotransmitter in the CNS (Hampel *et al.*, 2018). In AD there is degeneration of cholinergic neurons, which are responsible for cognitive functions such as memory, learning, attention and behaviour, and therefore contributes to the symptoms seen in AD (Mesulam, 1976; Bowen *et al.*, 1982; Whitehouse *et al.*, 1982). Writing this in June 2022, there are currently 3 FDA approved ACEi's which all improve the cognition of AD patients; donepezil, rivastigmine and galantamine (Hansen *et al.*, 2008; Rountree *et al.*, 2013). Donepezil and rivastigmine are approved for mild, moderate or severe AD whereas galantamine is best effective with mild or moderate AD (Rountree *et al.*, 2013). ACEi's do improve cognition but do not halt the progression of the disease and they come with various side effects. Another FDA approved drug is the NMDA antagonist, memantine, which protects neurons against the toxic effects of glutamate excitotoxicity (Yiannopoulou and Papageorgiou, 2020). Memantine is effective against moderate to severe AD and can be prescribed alone or in combination with an ACEi. Combination therapy is suggested to have a greater effect on cognition, but it is not clear its effects on behaviour and daily living activities (Matsunaga, Kishi and Iwata, 2015; Dominik *et al.*, 2019). Anti-psychotic and anti-depressant drugs are also used to control the behavioural and psychological symptoms of AD (Bessey and Walaszek, 2019).

As of writing this in June 2022, there are 143 agents being investigated in clinical trials to help treat AD (Cummings *et al.*, 2022). Over the years, therapies have predominantly attempted to target the production, aggregation, or clearance of Aβ. However, until now, the development of anti-amyloid therapies have repeatedly failed, raising questions if Aβ is the right target (Jeremic, Jiménez-Díaz and Navarro-López, 2021). The most promising avenue in clinical trials has focused on reducing the aggregation or increasing the clearance of Aβ using immunotherapy (Huang, Chao and Hu, 2020). Most trialled immunotherapies have been vaccines and anti-

amyloid antibodies, known as active and passive immunotherapy, however none have been successful to date due to toxicity or no improvement in cognition, despite reducing Aβ levels (Jeremic, Jiménez-Díaz and Navarro-López, 2021). Currently, there are 4 monoclonal antibody therapies in phase III clinical trials that work by clearing Aβ; Gantenerumab, Lecanemab, Solanezumab, Donanemab (Cummings *et al.*, 2022). Despite repeated failures using anti-amyloid antibodies, recently a monoclonal antibody Aducanumab has been approved by the FDA using the accelerated approval pathway (Sevigny *et al.*, 2016). Controversy around this approval stems from the two identical phase III twin studies EMERGE and ENGAGE showing conflicting clinical efficacy of the drug (Morant, Jagalski and Vestergaard, 2019). The accelerated approval is based upon the assumption that a reduction in Aβ plaques in both trials is likely to produce a clinical benefit. The company Biogen now must conduct a new follow up trial verifying the clinical benefit of the drug.

1.1.4 Neuropathology of Alzheimer's Disease

1.1.4.1 The discovery and diagnosis of Alzheimer's Disease

AD is named after Dr. Alois Alzheimer who first identified 'a characteristic disease of the cerebral cortex'. Dr. Alzheimer found thinning and shrinkage of the brain alongside abnormal deposits of plaques and tangles around the brain. It was later discovered in the 1980's that the unique plaques found in this disease were composed of ' a novel cerebrovascular amyloid protein', now known as the Aβ peptide (Glenner and Wong, 1984a; Masters *et al.*, 1985). Not long after this, the Tau protein was discovered to be a key component of the tangles (Grundke-Iqbal *et al.*, 1986; Kosik, Joachim and Selkoe, 1986; Nukina and Ihara, 1986).

Initially, the diagnosis of 'possible AD' was only based on clinical symptoms which could then only be confirmed upon autopsy after death (McKhann *et al.*, 1984). However, this missed out pre-symptomatic people or those in the early stages. In

2011, the National Institute on Aging and Alzheimer's Association (NIA-AA) updated the guidelines so that AD was now recognised as a progressive disease and classified into 3 stages; preclinical stage, MCI and eventually AD (Albert *et al.*, 2011; McKhann *et al.*, 2011; Sperling *et al.*, 2011). The 2011 guidelines still based the primary diagnosis on clinical symptoms, but biomarkers could now be used to help support the diagnosis. The preclinical stage looks at changes in biomarkers without any clinical symptoms. MCI is defined as subtle changes in memory and thinking alongside biomarker evidence. Not everyone who develops MCI will progress to AD, however approximately one third of patients with MCI will develop AD within 5 years (Alzheimer's Association, 2021). AD is the final stage characterised by noticeable changes in memory, behaviour and eventually impairing the individual's ability to live independently. In 2018 the NIA-AA updated the diagnosis criteria once again, shifting the diagnosis of AD exclusively to the use of biomarkers measuring Aβ and Tau. The biomarkers used are Aβ deposition, Tau and neurodegeneration, known as the ATN system (Jack *et al.*, 2018). Biomarkers for Aβ deposition are a positive positron emission tomography (PET) or low Aβ42 in cerebrospinal fluid (CSF). Tau biomarkers include a positive Tau PET scan or high CSF levels of phosphorylated Tau. Markers of neurodegeneration include reduced ^{18}F – fluorodeoxyglucose PET (as a marker of reduced brain metabolism), MRI for brain atrophy, or high CSF total Tau (Jack *et al.*, 2018).

1.1.4.2 The spread of Aβ and Tau

Aβ plaque and Tau tangle deposition tend to spread in a predictable manner, leading to the identification of stages (Figure 1.1) (Braak and Braak, 1991, 1997; Thal *et al.*, 2002). Aβ deposition starts in the neocortical areas, predominantly the temporal lobe (Phase 1). Phase 2/3 involves further spread throughout the neocortex and affecting subcortical areas including the hippocampus, amygdala, and the basal ganglia. The final stages 4/5 are characterised by Aβ deposition in the brainstem and cerebellum. The stereotypical spread of NFT's has been divided into 6 stages (Braak and Braak, 1991). NFT's start in the trans entorhinal or entorhinal cortex (I/II), which progresses to the hippocampus and some neocortical association areas (III/IV). Stage V-VI

involves spread to the neocortex, with later involvement of the striatum and substantia niagra (Braak and Braak, 1991; Serrano-Pozo *et al.*, 2011; Chen and Mobley, 2019). Recent evidence supports that the spread of Tau pathology could be via a prion-like seeding mechanism meaning abnormal Tau can induce aggregation of normal Tau in a neighbouring cell (Goedert and Spillantini, 2017).

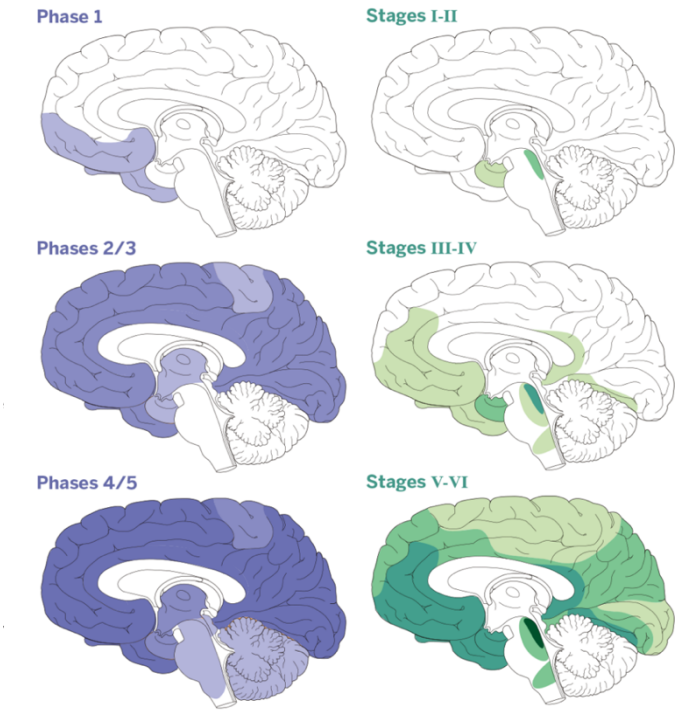


Figure 1.1 Stages of Aβ (purple) and Tau (green) deposition and spread, taken from (Goedert, 2015).
Aβ spread (purple) begins in the neocortical areas including the basal temporal and orbitofrontal neocortex (phase 1). Phase 2/3 includes further Aβ deposition throughout the neocortex affecting structures including the hippocampus and amygdala. The final phases 4 and 5 involve Aβ spread into the brainstem and cerebellum. Tau pathology (green) starts in the trans entorhinal and entorhinal cortex (stage I/II) which spreads to the hippocampus and starts to involve some parts of the neocortex (stage III/IV). Stage V-VI involves most of the neocortex.

1.2 Amyloid Beta

1.2.1 Amyloid beta processing

Aβ peptides arise from the normal processing of APP (Zhang *et al.*, 2011). APP is a type 1 membrane protein which is expressed throughout various tissues but predominantly in the CNS. Three major isoforms of APP exist (APP695, APP751, APP770) with APP 695 being predominantly expressed in neurons. APP has physiological roles in the CNS including neurotropic and neuroprotective effects (Müller and Zheng, 2012). *APP* knock out mice exhibit abnormal phenotypes including reduced brain and body size, impaired learning and long-term potentiation and a hypersensitivity to seizures (Müller and Zheng, 2012).

APP can be processed via two main pathways: the amyloidogenic (Figure 1.2) and the non-amyloidogenic pathway (Zhang *et al.*, 2011). Under normal conditions, the non-amyloidogenic pathway processes the majority of APP, which involves cleavage by α-secretase to give rise to an N terminal fragment soluble APP alpha (sAPPα) fragment, and a C terminal fragment of 83 amino acids known as α-CTF or C83. The enzyme α-secretase belongs to a member of the ADAM (a disintegrin and metalloprotease) family, with ADAM 9, 10, 17 and 19 all likely to be involved (Zhang *et al.*, 2011). *In vitro* and *in vivo* evidence suggests sAPPα is neuroprotective and enhances learning and memory function (Furukawa *et al.*, 1996; Mucke, Abraham and Masliah, 1996; Mattson, 1997; Meziane *et al.*, 1998; Thornton *et al.*, 2006; Gakhar-Koppole *et al.*, 2008; Taylor *et al.*, 2008). sAPPα can also rescue most of the abnormal phenotypes seen in *APP* knockout animal models (Ring *et al.*, 2007). Further cleavage of C83 by the y-secretase complex - a protein complex composed of the presenilin's (PSEN1/2), nicastrin and anterior pharynx defective 1 (APH -1), produces a 3kDa product (P3) and an APP intracellular domain (AICD). The AICD is released in to the cytoplasm and is suggested to play a role in transcriptional regulation (Cao and Sudhof, 2001; Leissring *et al.*, 2002).

The alternative pathway (amyloidogenic) gives rise to Aβ species, varying in length from 38 to 43 amino acids (Figure 1.2). First, APP is cleaved by β-secretase to give rise to soluble APP beta (sAPPβ) and a C terminal fragment of 99 amino acids known as β-CTF or C99. BACE1 and 2 are the β-secretase enzymes, with BACE1 being the primary β-secretase in the brain (Vassar *et al.*, 1999). β-CTF is then processed by the y-secretase complex to give rise to another AICD and Aβ peptides of varying amino acid lengths.

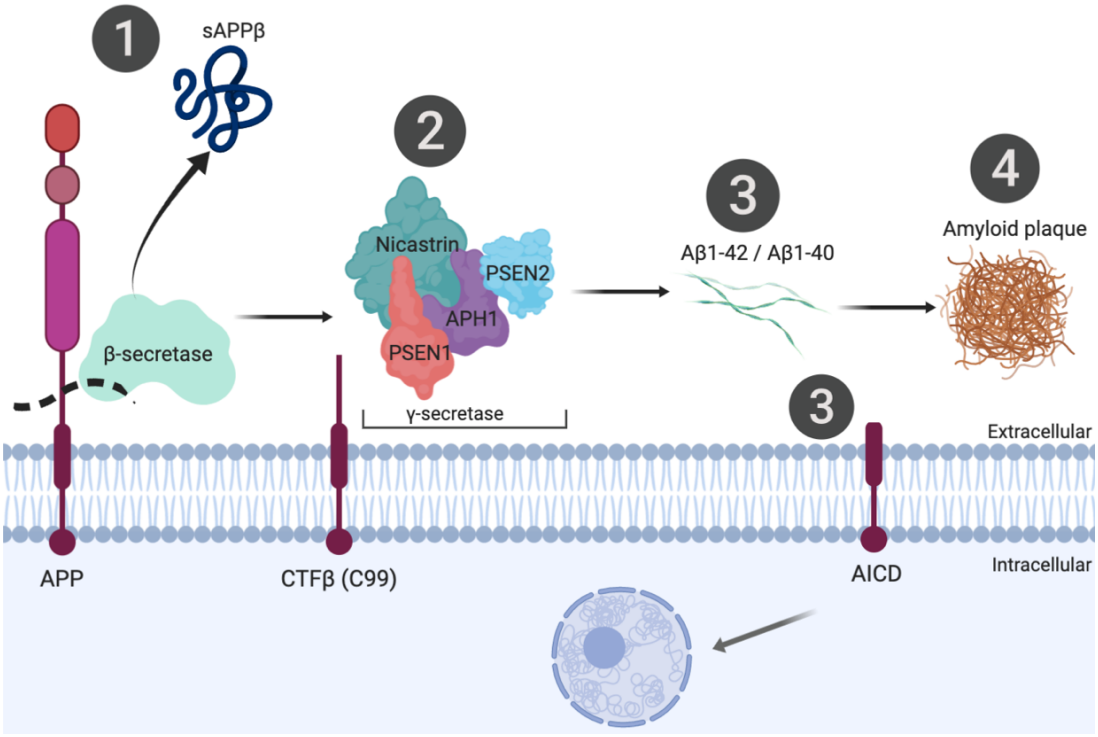


Figure 1.2 Processing of the amyloid precursor protein in the amyloidogenic pathway.
1. The amyloid precursor protein (APP) is made up of a large extracellular domain, a single pass transmembrane region and a cytoplasmic domain. APP is first cleaved by β-secretase to generate a soluble N terminal fragment (sAPPβ) and a membrane bound C terminal (CTFβ) composed of 99 amino acids. **2.** CTFβ is then cleaved by the γ-secretase complex composed of presenilin 1 and 2, nicastrin and APH1. **3.** This generates Aβ peptides of varying lengths and an APP intracellular domain (AICD) which can translocate to the nucleus to modulate transcription. **4.** Under normal conditions, Aβ1-40 is the major species produced, however in Alzheimer's Disease, Aβ1-42 production is increased which forms the Aβ plaques due to its hydrophobic nature.

The Aβ peptides produced via APP processing are composed of various amino acid lengths. Under normal conditions Aβ40 is the most abundant species produced from APP processing (~90%) and is protective against amyloid plaque deposition (Kim *et al.*, 2007; Zhang *et al.*, 2011). The second major species found is Aβ42 (~10%) which is more hydrophobic and aggregative than Aβ40 (Burdick *et al.*, 1992). Aβ peptides are found in the CSF of normally cognitive people at levels of 2-3ng/ml for the major species Aβ40 and 0.75 ng/ml for the minor species Aβ42, indicating Aβ has physiological roles in the CNS (Ida *et al.*, 1996; Pearson and Peers, 2006; Mo *et al.*, 2015). *In vitro* and *in vivo* evidence suggests endogenous Aβ has physiological roles including maintaining the blood brain barrier and regulating synaptic function (Pearson and Peers, 2006; Brothers, Gosztyla and Robinson, 2018). Aβ in its monomeric form is also suggested to be neuroprotective (Tamaoka *et al.*, 1997; Giuffrida *et al.*, 2009). Under normal conditions, the production of soluble Aβ is regulated via enzyme degradation or clearance from the brain via transport across the blood brain barrier into the circulatory system (O'Brien and Wong, 2011).

In AD it is proposed that there is an increase in total Aβ levels or an increase in the ratio of Aβ42/40 (*this is covered in more detail in section 1.2.2*). Aβ42 is the predominant species found in Aβ plaques, probably due to its more hydrophobic nature and therefore more prone to self-aggregate than Aβ40 (Burdick *et al.*, 1992; Jarrett, Berger and Lansbury, 1993; Iwatsubo *et al.*, 1994; Mak *et al.*, 1994; Gravina *et al.*, 1995; Lee *et al.*, 2019).

1.2.2 Evidence for the amyloid hypothesis

The amyloid hypothesis was first proposed in 1991 by John Hardy and David Allsop, suggesting that Aβ is the initiating factor that causes the neurodegeneration seen in AD (Hardy and Allsop, 1991; Selkoe and Hardy, 2016). The first pieces of evidence that supported the amyloid hypothesis was the discovery of the first gene associated with EOAD- APP (Brouwers, Sleegers and Van Broeckhoven, 2008). The identification of the human APP gene came from the observation that patients with down syndrome, caused by complete trisomy chromosome 21, develop AD like pathology with significant Aβ plaques and NFT's by the age of 40 (Glenner and Wong, 1984a, 1984b; Masters *et al.*, 1985; Head *et al.*, 2012). Isolation and sequencing of the Aβ peptide identified it's cDNA and mapped it's gene (APP) to chromosome 21, explaining the increased Aβ production in down syndrome (Kang *et al.*, 1987; St. George-Hyslop *et al.*, 1987). To date, around 70 mutations have been identified in the APP gene in AD, including missense mutations and whole gene duplications (https://www.alzforum.org/mutations). The majority of missense mutations cluster in the Aβ region or at the secretase cleavage sites. Mutations here affect the processing by the secretases, leading to an increase in total Aβ production or an increase in the relative ratio of Aβ42/Aβ40 (Weggen and Beher, 2012; Tcw and Goate, 2017).

Later, further investigation of familial AD led to the discovery of mutations in the presenilin genes, PSEN1 and PSEN2 (Brouwers, Sleegers and Van Broeckhoven, 2008). PSEN1/2 genes encode for the catalytic subunits of the y-secretase that generates the Aβ species in the amyloidogenic pathway (Dorszewska *et al.*, 2016; Li *et al.*, 2016). Discovery of these mutations further strengthened the hypothesis that Aβ is involved in the pathogenesis of AD. The most common cause of FAD is mutations in PSEN1 and are generally missense mutations which alter the way in which the y-secretase complex cleaves APP (https://www.alzforum.org/mutations). In earlier years it was suggested PSEN1 mutations increases the production of Aβ or the relative ratio of Aβ42/40 (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Scheuner *et al.*, 1996). However, since the discovery of more than 300 mutations, an increased Aβ

42/40 ratio is not always observed, contradicting the hypothesis that increased ratio of Aβ42/40 is essential for AD pathogenesis (Sun *et al.*, 2017). PSEN2 is less studied but some of the well-known mutations that have been investigated also increase the Aβ42/40 ratio (Walker *et al.*, 2005).

Further evidence supporting the amyloid hypothesis is from the accumulative research showing that Aβ oligomers are directly neurotoxic in *vivo* and *in vitro* (Selkoe and Hardy, 2016). Aβ oligomers have been found to induce cell senescence, inhibit long term potentiation, decrease synapse number and impair memory learning (Walsh *et al.*, 2002; Townsend *et al.*, 2006; Shankar *et al.*, 2007, 2008; He *et al.*, 2013).

1.2.3 Problems with the amyloid hypothesis

Despite there being a clear link between Aβ plaques and AD pathology, Aβ weakly correlates with the severity and duration of the disease and some plaque deposition is also seen in cognitively normal individuals, suggesting Aβ accumulation could be a normal process of ageing (Arriagada *et al.*, 1992; Davis *et al.*, 1999; Li *et al.*, 2008; Fagan *et al.*, 2009; Nelson *et al.*, 2012; Chételat *et al.*, 2013). Further doubt regarding the amyloid hypothesis has arisen from the study of transgenic AD mouse models. Many APP and PSEN1/2 transgenic mouse models where Aβ accumulates exhibit the pathological hallmarks of AD, including cognitive impairment and synaptic loss, however they fail to show a link between Aβ plaques and neuronal loss (Wirths and Bayer, 2010; Kitazawa, Medeiros and M. LaFerla, 2012) (*AD animal models is covered in more detail in section 1.4*). Additionally, as previously discussed, although Aβ plaques are found in AD, targeting Aβ therapeutically has been largely unsuccessful in the past. Several immunisation therapies have reached human clinical trials, but still present little success in preventing cognitive decline, despite reducing Aβ levels (Jeremic, Jiménez-Díaz and Navarro-López, 2021).

1.3. Tau pathology

1.3.1 The function of Tau

Tau is a microtubule binding protein, predominantly localised in neurons, that promotes microtubule assembly and stability, ensuring normal cytoskeletal organisation and function (Barbier *et al.*, 2019). The cytoskeleton not only provides structure to cells but determines cellular polarity by organising intracellular transport. The neuron is a highly polarised cell with functionally distinct axons and dendrites, which relies on selective transport of cargo to different compartments (Stiess and Bradke, 2011). Tau is predominantly found in axons in neurons but is also found in the nucleus, dendrites and synapses, suggesting it may have multiple roles (Brunello *et al.*, 2020). Synaptic Tau is suggested to be involved in synapse development of newly formed neurons (Pallas-Bazarra *et al.*, 2016) and could potentially implicate neuronal activity with *Tau* knockout mice showing impaired long term depression (Kimura *et al.*, 2014). Nuclear Tau is suggested to play a role in DNA protection against insults (Sultan *et al.*, 2011).

1.3.2 Tau protein structure

Tau is encoded by the MAPT (microtubule associated protein Tau) gene located on chromosome 17 (17q21) which is made up of 16 exons that can be alternatively spliced to give rise to different isoforms (Figure 1.3)(Kolarova *et al.*, 2012). In the human CNS, there at 6 Tau isoforms which arise as a result of alternative splicing of exons 2,3 and 10 (Figure 1.3). Tau protein comprises of 3 domains; a projection domain which interacts with plasma membrane proteins; a proline rich domain which enhances microtubule binding activity; and a microtubule binding domain (MBD). The MBD is made up of repeat regions, each consisting of 18 highly conserved amino acids and is the essential domain allowing microtubule binding (Hanger, Anderton and Noble, 2009). Exons 2 and 3 each encode an acidic 29 amino acid sequence at the N terminus and exon 10 encodes for the second repeat motif in the MBD (Fig.2) (Hanger, Anderton and Noble, 2009). The different isoforms depend on the presence of absence of one

(1N) or two (2N) 29 amino acid inserts at the N terminus, and either three (3R) or four (4R) repeats in the MBD. Phosphorylation and expression of different isoforms is developmentally regulated. All Tau isoforms are present in the mature adult brain compared to foetal Tau which is comprised of only the shortest isoform 0N3R (Hanger, Anderton and Noble, 2009). Foetal Tau has lower microtubule binding affinity due to two factors - increased phosphorylation and the lower number of repeat regions in the MBD (Goode *et al.*, 2000; Panda *et al.*, 2003). In the adult CNS 3R and 4R Tau isoforms are present in an equal ratio (Goedert *et al.*, 1989). Increased 4R:3R ratios are observed in multiple neurodegenerative diseases, including frontotemporal dementia linked to parkinsonism (FTDP-17), Pick's disease, progressive supranuclear palsy and AD, suggesting alterations in Tau splicing contributes to neurodegeneration (D'Souza and Schellenberg, 2005; Ginsberg *et al.*, 2006; Conrad *et al.*, 2007).

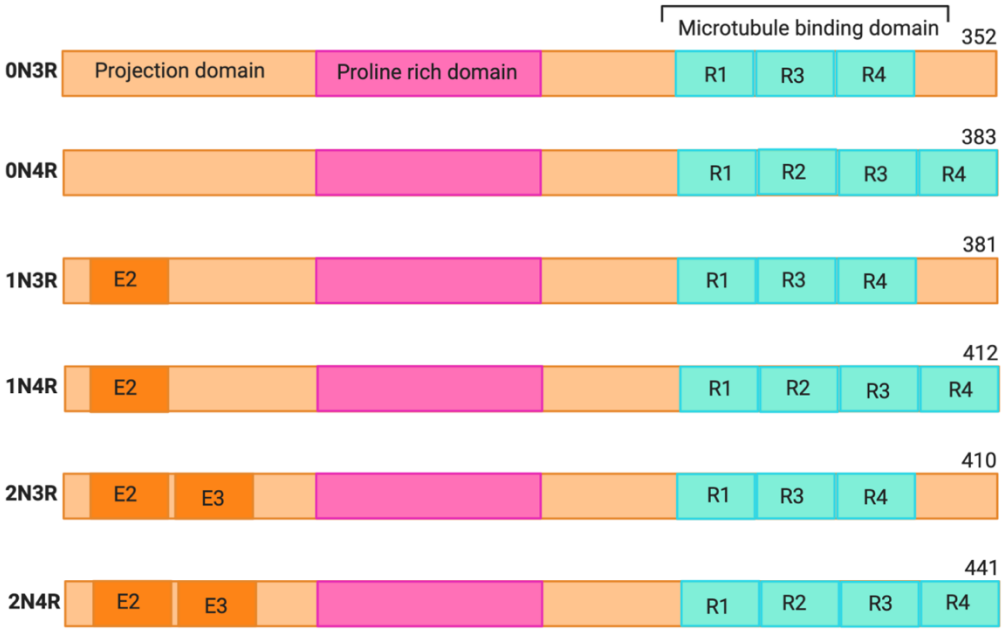


Figure 1.3 The alternative splicing of Tau in the central nervous system. 6 isoforms of Tau exist in the adult CNS that are generated by alternative splicing of exons 2, 3 and 10. Exons 2 and 3 (E2 & E3) encode for a 29 amino acid insert in the N terminus. Exon 10 encodes for the second repeat region (R2) in the microtubule binding domain. The isoforms differ due to the absence or presence of one or two 29 amino acid sequences at the N terminal (0N, 1N, 2N), alongside three (R1, R3, R4) or four (R1-R4) repeat regions in the microtubule binding domain.

1.3.3 Tau phosphorylation in neurodegeneration

Many post translational modifications can occur on Tau in both normal states and in disease, including ubiquitylation, glycosylation, nitration and phosphorylation (Martin, Latypova and Terro, 2011). Phosphorylation of Tau is by far the most discussed because of its role in neurodegeneration (Lee and Leugers, 2013). Formation of insoluble, hyperphosphorylated Tau inclusions are observed in multiple neurodegenerative diseases, which collectively are termed the tauopathies, including frontotemporal dementia with parkinsonism-17 (FTDP-17), Picks disease, progressive supranuclear palsy and AD (Arendt, Stieler and Holzer, 2016). Tau in AD is hyperphosphorylated containing 9-10 moles of phosphate per molecule compared to normal Tau made up of 2-3 phosphates per molecule (Gong and Iqbal, 2008). In AD, hyperphosphorylation of Tau reduces it's binding to microtubules, leaving Tau free to aggregate into insoluble inclusions known as neurofibrillary tangles (NFTs). Phosphorylation at specific sites including Ser262 and Ser356, Ser396,Thr231 and Ser235 have been shown to be particularly important in reducing Tau binding to microtubules (Biernat *et al.*, 1993; Bramblett *et al.*, 1993; Drewes *et al.*, 1995; Sengupta *et al.*, 1998; Buée *et al.*, 2000). Hyperphosphorylated Tau induces microtubule instability, defective axon transport, synaptic and mitochondrial dysfunction and calcium dysregulation (Gómez-Ramos *et al.*, 2008; Morfini *et al.*, 2009; Di *et al.*, 2016; Cheng and Bai, 2018; Wu *et al.*, 2021).

There are 85 potential phosphorylation sites on human Tau. Many Tau phosphorylation sites have been identified from AD brain material via mass spectrometry (Figure 1.4) (Hasegawa *et al.*, 1992; Kawashima *et al.*, 1995; Hanger *et al.*, 2002, 2007). Mass spectrometry studies have allowed the discovery of an estimated 57 phosphorylation sites on Tau from AD brains, compared to 19 sites from control Tau (Figure 1.4) (Hasegawa *et al.*, 1992; Hanger *et al.*, 2002, 2007; Wesseling *et al.*, 2020; Xia, Prokop and Giasson, 2021). A large number of kinases have been shown to be capable of phosphorylating Tau (Hanger, Anderton and Noble, 2009). These include glycogen synthase kinase 3 beta (GSK3β), casein kinase I (CKI), protein kinase (PKA), cyclin dependent kinase 5 (Cdk5), checkpoint

kinase 1 & 2 (Chk1/2) (Mendoza *et al.*, 2013), microtubule affinity regulating kinase (MARK) and adenosine monophosphate-activated protein kinase (AMPK) (Hanger, Anderton and Noble, 2009). The use of kinase inhibitors has already shown the potential beneficial effects of targeting Tau phosphorylation in neurodegeneration, showing reduced neuronal apoptosis *in vitro* and delayed motor deficits in a Tau transgenic mouse (Zheng *et al.*, 2005; Le Corre *et al.*, 2006). Tau phosphorylation could underpin the synergistic interaction between Aβ and Tau in AD (Zheng *et al.*, 2002). Aβ can induce Tau phosphorylation via activation of Tau kinases, including Cdk5, GSK3β, AMPK, MARK and subsequently leads to Tau phosphorylation (Town *et al.*, 2002; Song *et al.*, 2008; Terwel *et al.*, 2008; Hernandez *et al.*, 2009; Zempel *et al.*, 2010; Thornton *et al.*, 2011).

mAEPRQEFEV	MEDHAGTYGL	GDRKDQGGYT	MHQDQEGDTD	AGLKESPLQT	50
PTEDGSEEPG	SETSDAKSTP	TAEDVTAPLV	DEGAPGKQAA	AQPHTEIPEG	100
TTAEEAGIGD	TPSLEDEAAG	HVTQARMVSK	SKDGTGSDDK	KAKGADGKTK	150
IATPRGAAPP	GQKGQANATR	IPAKTPPAPK	TPPSSGEPPK	SGDRSGYSSP	200
GSPGTPGSRS	RTPSLPTPPT	REPKKVAVVR	TPPKSPSSAK	SRLQTAPVPM	250
PDLKNVKSKI	GSTENLKHQP	GGGKVQIINK	KLDLSNVQSK	CGSKDNIKHV	300
PGGGSVQIVY	KPVDLSKVTS	KCGSLGNIHH	KPGGGQVEVK	SEKLDFKDRV	350
QSKIGSLDNI	THVPGGGNKK	IETHKLTFRE	NAKAKTDHGA	EIVYKSPVVS	400
GDTSPRHLSN	VSSTGSIDMV	DSPQLATLAD	EVSASLAKQG L	441	

Figure 1.4. Phosphorylation sites of Tau identified from Alzheimer's disease and control brains from mass spectrometry studies.
Amino acid sequence of the full-length version of Tau (2N4R- 441 amino acids). Tau in neurofibrillary tangles found in Alzheimer's Disease is abnormally hyperphosphorylated. Phosphorylation sites identified from control brain Tau are noted with a black star. Phosphorylation sites identified from pathological AD Tau are in purple bold. The microtubule binding domain is underlined from amino acid 243 – 376.

1.3.4 The link between Tau and neurodegeneration

The shared phenomenon of Tau hyperphosphorylation amongst the tauopathies suggests that Tau pathology could underlie the mechanisms of toxicity in neurodegeneration (Arendt, Stieler and Holzer, 2016). There is evidence to suggest Tau pathology has a stronger link to neurodegeneration compared to Aβ. The first mutations discovered associated with FTDP-17 were found in the MAPT gene, indicating Tau abnormalities can cause neurodegeneration in the absence of Aβ (Lee and Leugers, 2013). In contrast to Aβ, the number of NFTs correlates strongly with cognitive defects and neuronal loss in AD (Bondareff *et al.*, 1989; Arriagada *et al.*, 1992; Gomez-isla *et al.*, 1997; Giannacopoulos, 2003). However, some studies have shown NFT pathology alone cannot induce neuronal dysfunction; inhibiting Tau hyperphosphorylation in a human Tau mutant mouse prevented motor defects without changing NFT density (Le Corre *et al.*, 2006), and turning off mutant Tau expression improves memory and attenuates neuronal loss, whilst NFTs continue to accumulate (SantaCruz *et al.*, 2005).

1.3.5 The synergistic interaction of Tau and amyloid beta

Accumulating evidence indicates that both Aβ and Tau work together (Bloom, 2014). Expression of mutant Tau in mouse models induces NFT formation, which is accelerated by mutant APP co-expression, suggesting Aβ lies upstream of Tau (Lewis *et al.*, 2001; Hurtado *et al.*, 2010). The same phenotype was observed by injecting synthetic Aβ into a Tau mutant mouse model (Götz *et al.*, 2001). As previously discussed, Aβ can also induce Tau phosphorylation via activation of kinases (Terwel *et al.*, 2008; Hernandez *et al.*, 2009; Zempel *et al.*, 2010). Tau pathology does not just appear as a downstream consequence of Aβ but is in fact needed for Aβ neurotoxicity *in vivo* and *in vitro*. Exposure of primary neurons to synthetic Aβ induces neurite degeneration and cell death, which is prevented with a *Tau* knockout (Rapoport *et al.*, 2002). Knocking *Tau* back into neurons restored the toxic effects of Aβ, indicating Tau is essential for Aβ induced cytotoxicity. Other *in vitro* Tau dependent effects of Aβ include neurite degeneration (Jin *et al.*, 2011),

abnormal cell cycle re-entry (Seward *et al.*, 2013), excitotoxicity (Ittner *et al.*, 2010), inhibition of long term potentiation (Shipton *et al.*, 2011), defective axonal transport (Vossel *et al.*, 2010) and microtubule disassembly (King *et al.*, 2006; Zempel *et al.*, 2013). Additionally, *in vivo* evidence shows loss or reduction of Tau rescues memory and learning abnormalities induced by Aβ (Rapoport *et al.*, 2002; Roberson *et al.*, 2007; Leroy *et al.*, 2012). The exact interaction between Aβ and Tau is not fully understood yet, however it is thought there may be a feedback loop involved with both proteins being able to accelerate and induce formation of the other (Götz *et al.*, 2001; Lewis *et al.*, 2001; Ferrari *et al.*, 2003; Oddo *et al.*, 2004; Hurtado *et al.*, 2010; Ittner *et al.*, 2010; Israel *et al.*, 2012; Leroy *et al.*, 2012; Choi *et al.*, 2014; Peters *et al.*, 2019).

1.3.6 Pathological Tau and amyloid beta at the synapse

Of all the neuropathological hallmarks seen in AD, synapse loss has the strongest correlation with the cognitive decline, suggesting it is a critical component of the pathology (Davies *et al.*, 1987; Terry *et al.*, 1991; DeKosky, Scheff and Styren, 1996; De Wilde *et al.*, 2016). Synapse loss appears to be an early event seen in AD and is even observed in MCI before the onset of neurodegeneration (Masliah *et al.*, 2001; Selkoe, 2002; Scheff *et al.*, 2006). The cause of synaptic dysfunction in AD is still poorly understood, but evidence suggests both Aβ and Tau contribute to this pathology (Spires-Jones and Hyman, 2014).

Physiological Aβ has an important role at the synapse, facilitating synaptic plasticity and memory, promoting neurogenesis in development and acting as a feedback loop to maintain normal neuronal activity (Dawson *et al.*, 1999; Saura *et al.*, 2004; Laird *et al.*, 2005; Pearson and Peers, 2006)(López-Toledano and Shelanski, 2004). (Dawson *et al.*, 1999; Kamenetz *et al.*, 2003; Laird *et al.*, 2005; Cirrito *et al.*, 2008). However, it is also known that Aβ can induce synaptic loss and alter synapse function (Palop, 2010). It is suggested that the synaptotoxic effect of Aβ comes from the soluble Aβ oligomers held within the plaques, and not the insoluble Aβ fibrils that comprise the plaques (Selkoe, 2002; Shankar *et al.*, 2008; Mucke and Selkoe, 2012)

A range of studies have demonstrated the effect of Aβ on the synapse, with different forms of Aβ species at various concentrations showing different effects on synapse function.

There is a magnitude of *in vivo* and *in vitro* evidence showing Aβ oligomers, but not monomers, derived from AD brains or in their naturally secreted form impair synaptic plasticity and memory and enhance synaptic depression (Lambert *et al.*, 1998; Walsh *et al.*, 2002; Billings *et al.*, 2005; Cleary *et al.*, 2005; Townsend *et al.*, 2006; Shankar *et al.*, 2007, 2008; Selkoe, 2008; Li *et al.*, 2009). High levels of Aβ oligomers have also been shown to play a role in preventing excessive glutamate release and thus reducing excitatory synaptic transmission (Chapman *et al.*, 1999; Hsia *et al.*, 1999; Mucke *et al.*, 2000; Walsh *et al.*, 2002). The specific mechanism in which Aβ induces synaptic dysfunction is poorly understood however it is known that Aβ can bind to synaptic receptors to alter signalling pathways, neurotransmitter release and gene expression (Li *et al.*, 2009; Xia *et al.*, 2016; Rice *et al.*, 2019). Additionally, downstream increased calcium signalling appears to be a phenomenon observed when oligomeric Aβ is applied to cultured neurons *in vitro* which is associated with a loss of dendritic spines and impaired synapse plasticity (Mattson *et al.*, 1992; Demuro *et al.*, 2005; Wu *et al.*, 2010; Hudry *et al.*, 2012). There may be a balancing act of Aβ levels, with high pathological concentrations of Aβ depressing synapse activity, but lower, physiological Aβ levels positively modulating synaptic activity. One study demonstrated that a slight elevation of endogenous Aβ (1.5 fold) is associated with enhanced synaptic vesicle release and consequently increased neuronal activity *in vitro* (Abramov *et al.*, 2009). Consistent with this, low levels of synthetic Aβ42 in the physiological range facilitate long term potentiation and enhance synaptic plasticity and memory but higher pathological levels in the nanomolar range induce long term depression (Puzzo *et al.*, 2008).

Although a lot of research has focused on the role of Aβ oligomers and synaptic loss, NFTs are the pathological hallmark that correlate strongly with synaptic loss and the severity of cognitive decline seen in AD. Indeed, it is known that pathological Tau also contributes to synaptic loss and dysfunction (Wu *et al.*, 2021). Pathological Tau

detaches from microtubules and mis-localises to synapses where it promotes synapse impairment and synaptic loss via various mechanisms including disturbing synaptic vesicle release, decreased traffic of post synaptic receptors and mitochondrial dysfunction (Wu *et al.*, 2021).

1.4 Alzheimer's Disease animal models

1.4.1 Alzheimer's Disease mouse models

Rodent models are an essential tool for drug discovery and further research into neurodegenerative diseases. The perfect rodent model for AD would be one that develops Aβ plaque deposition, Tau tangles, synaptic and cognitive impairments, and neuronal loss. However, to date there is not one that fully recapitulates the whole AD pathology seen in human disease (https://www.alzforum.org/research-models/alzheimers-disease).

Most rodent used to model AD are mouse models which rely on extensive overexpression of APP with FAD mutations or mutations in the human MAPT gene (Jankowsky and Zheng, 2017). One of the most commonly used FAD mutations in APP is the Swedish mutation which increases Aβ processing and consequently increases production (Citront *et al.*, 1992). The majority of APP transgenic mice show plaque deposition, gliosis, cognitive defects and synapse impairment, however replicating neuronal loss and Tau tangle formation consistently has proven difficult (Drummond and Wisnlewski, 2017). Some of the key mouse models generated include PDAPP and Tg2576 which overexpress a single FAD mutation in APP (Games *et al.*, 1995; Hsiao *et al.*, 1996). These mice develop widespread extracellular Aβ deposition, gliosis, synapse loss and cognitive defects. However, no NFT formation is observed alongside a lack of neurodegeneration. The 5XFAD model overexpresses human APP and PSEN1 transgenes with a total of 5 FAD mutations (Oakley *et al.*, 2006). These mice exhibit amyloid plaque pathology, gliosis, cognitive impairments, and even neuronal loss but still no Tau pathology is observed.

Most mouse models capturing Tau pathology rely on the overexpression of FTDP-17 associated mutant variants of MAPT (Jankowsky and Zheng, 2017). Although these models provide evidence for Tau toxicity, they are probably better suited to modelling FTDP-17 as no MAPT mutation has been liked to AD. Tau mice display age dependent neurodegeneration and the severity and age of onset of the disease is dependent on the form of Tau used (Jankowsky and Zheng, 2017). One of the most popular models used over the years to study Tau toxicity is the rTg4510 model which expresses the P301L mutation in human Tau, one of the most common FTDP-17 mutations (Ramsden *et al.*, 2005; SantaCruz *et al.*, 2005). However, more recently it was show that the random insertion of the MAPT transgene (P310L) significantly affects the phenotype seen in this mouse (Gamache *et al.*, 2019). Another commonly used Tau model is the JNPL3 which also overexpresses the P310L mutation and exhibits Tau tangles, neuronal loss, gliosis, but no evidence of cognitive impairment (Lewis *et al.*, 2000). The triple transgenic model (3xTg) is one of the most complete models available which exhibits both Tau tangle formation and plaque deposition, however it relies on overexpression of mutations in APP, MAPT and PSEN1 (Oddo *et al.*, 2003). These mice develop plaque formation before Tau pathology, in agreement with the amyloid cascade hypothesis.

Although mouse models can generally capture some parts of the neuropathology seen in AD quite well, to replicate the whole pathology multiple mutations and expression of multiple transgenes are needed. This differs to human disease where one single FAD mutation is sufficient to develop the disease. Due to recent light about the effect of transgene insertions on the phenotypes (Gamache *et al.*, 2019), it is questionable how reliable the current models are. Additionally, rodents are expensive to handle and have a long-life cycle to study an ageing disease.

1.4.2 *Drosophila melanogaster* as a model for neurodegenerative diseases

Drosophila melanogaster have emerged as one of the most widely used invertebrate models to study neurodegenerative diseases (Bouleau and Tricoire, 2015). *Drosophila* are cheap and easy to maintain, with a short generation time (~10 days at 25 °C) and short life span (2-3 months), making them a useful model to study age related disease (Lenz *et al.*, 2013). They also exhibit complex behaviours that are seen in humans such as circadian rhythms, memory and learning which can be observed to study diseases. *Drosophila* and humans share a high degree of conservation of fundamental signalling pathways e.g Wnt and Notch. 70% of human disease causing genes having homologs in *Drosophila,* including APP and Tau (Fortini *et al.*, 2000; Rubin *et al.*, 2000; Reiter *et al.*, 2001). *Drosophila* APP (dAPP) shares approximately 25% identify and 39% similarity with human APP, but lacks conservation in the Aβ region meaning no endogenous Aβ species are produced (Luo, Martin-Morris and White, 1990; Luo, Tully and White, 1992). Additionally, *Drosophila* possess a β-secretase like enzyme (dBACE) which exhibits low activity (Yagi *et al.*, 2000). Overexpressing dBACE results in cleavage of dAPP and produces Aβ peptides which aggregate and induce neurodegeneration, suggesting Aβ induced neurotoxicity is a conserved function (Carmine-simmen *et al.*, 2015).

1.4.2.1 UAS-Gal4 system

Another advantage of using *Drosophila* to model human diseases is the genetic tools available to manipulate the genome. The most direct, widely used system to study transgene expression in *Drosophila* is the UAS-Gal4 system which allows human proteins to be expressed in a time and tissue specific manner (Figure 1.5) (Brandt and Lee, 1993). This system involves inserting a transgene downstream of an upstream activator sequence (UAS) which expression is driven by the yeast transcription factor Gal4. Gal4 expression is under the control of a tissue specific

enhancer or promotor, meaning the transgene is only expressed where Gal4 is expressed, giving rise to tissue specific expression of the chosen gene (Duffy, 2002). Thousands of Gal4 driver lines have been created including the pan-neuronal promoter *elav* and the eye-specific promotor *GMR.* This system can not only be used to overexpress transgenes of interest but also to express RNAi lines to silence genes. Gal4 activity is temperature dependent, with low activity at lower temperatures (16/18 °C) and higher activity at higher temperatures (29 °C)(Duffy, 2002). This is something that must be considered when choosing a temperature as Gal4 itself is toxic when expressed at high levels, inducing developmental defects and apoptosis (Kramer and Staveley, 2003). Different modifications of the UAS-GAL4 system exist to allow controlled expression of genes such as including the temperature sensitive Gal4 repressor, known as $Gal80^{ts}$ (Figure 1.5) (McGuire *et al.*, 2003). At low temperatures (18 °C) $Gal80^{ts}$ inhibits the transcriptional activity of Gal4, and at high temperatures $Gal80^{ts}$ is no longer functional meaning the transgene can be expressed. Another modified UAS-Gal4 system is the gene switch system where the transcriptional activation activity of Gal4 is only active when RU486 (mifepristone) is active (Osterwalder *et al.*, 2001).

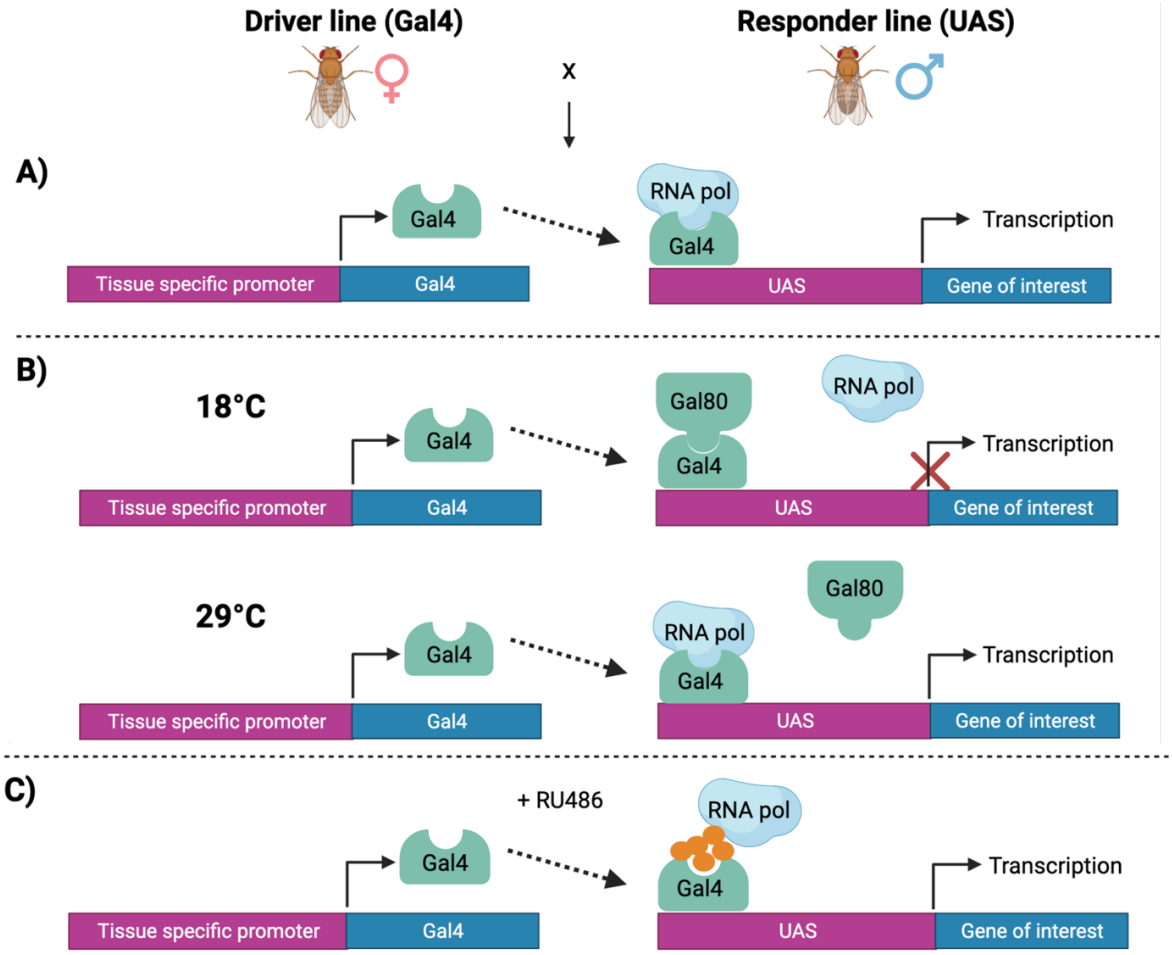


Figure 1.5 The UAS-Gal4 system for targeted gene expression in *Drosophila melanogaster.*

A) For flies to express a gene in a tissue specific manner they must possess both the yeast Gal4 transcription factor, which is expressed under the control of a tissue specific promoter, and a construct containing the gene of interest placed downstream of a Gal4 binding site known as the upstream activating sequence (UAS). Gal4 will be expressed in specific tissues and bind to UAS, recruiting transcription machinery and driving transcription of gene 'X'. **B)** Different modifications of the UAS-Gal4 system exist such as the GAL4- Gal80^{ts}. Gal80 is a temperature sensitive repressor of Gal4. At low temperatures Gal80 inhibits activity of Gal4, whereas at high temperatures Gal80 is inactive allowing Gal4 binding to the UAS. **C)** Another modification of the UAS-Gal4 system is the Gene Switch system which relies on the presence of mifepristone (RU486) for Gal4 to be active.

1.4.2.2 Modelling Aβ and Tau toxicity

The most direct way to model Tau and Aβ42 toxicity in *Drosophila* is using the UAS-Gal4 system to express the proteins in the CNS (Bonner and Boulianne, 2011; Lenz *et al.*, 2013). Overexpression of Tau or Aβ42 in the *Drosophila* nervous system results in many different phenotypes including reduced lifespan, locomotor defects and neurodegeneration, making it easy to investigate different aspects of the pathology involved in AD (Funez, Mena and Limas, 2015).

Modelling amyloid toxicity in *Drosophila* involves overexpressing a modified Aβ42 peptide containing a secretory signal peptide to mimic the extracellular Aβ found in AD (Funez, Mena and Limas, 2015). Overexpression of Aβ42 in the *Drosophila* nervous system results in formation of insoluble aggregates, locomotor defects, reduced lifespan, learning defects and age dependent neurodegeneration seen by neuronal vacuolisation and cell loss (Finelli *et al.*, 2004; Greeve *et al.*, 2004; Iijima *et al.*, 2004; Crowther *et al.*, 2005; Iijima *et al.*, 2008; Speretta, Thomas R Jahn, *et al.*, 2012). Expression of Aβ42 in the retina using the GMR-Gal4 driver also produces a rough eye phenotype, another indicator of neurodegeneration due to loss of retinal cells (Finelli *et al.*, 2004; Crowther *et al.*, 2005). This is contrasting to mouse models of Aβ toxicity where neuronal loss can only be seen in the presence of a Tau transgene (Hall and Roberson, 2012). The severity of these phenotypes in *Drosophila* depends on the form of Aβ42 being expressed. Expression of the Arctic mutation [E693G] within the Aβ region of APP, which increases Aβ42 aggregation (Nilsberth *et al.*, 2001), enhances the severity of phenotypes seen compared to Aβ42 alone, such as earlier memory defects, more severe locomotion defects and earlier death (Crowther *et al.*, 2005; Iijima *et al.*, 2008). Other variant models of Aβ42 include the 12L version in which tandem dimers of Aβ42 were created using a 12 amino acid sequence in between (Speretta, Thomas R Jahn, *et al.*, 2012). Expression of tandem Aβ42 in the nervous system results in an increased of insoluble aggregates and increased neurotoxicity, compared to its monomeric form (Speretta, Thomas R Jahn, *et al.*, 2012).

Modelling Tau induced neurodegeneration in *Drosophila* involves overexpressing human wildtype Tau or FTDP-17 linked Tau mutants R406W or V337M (Wittmann *et al.*, 2001; Jackson *et al.*, 2002; Nishimura, Yang and Lu, 2004). *Drosophila* Tau shares 46% identify and 66% similarity to human Tau, containing 5 microtubule binding domains and zero N terminal repeat insertions (Heidary and Fortini, 2001). Overexpression of wildtype Tau or mutant Tau in *Drosophila* neurons results in neurodegeneration, observed by neuronal vacuolisation (Wittmann *et al.*, 2001; Jackson *et al.*, 2002; Nishimura, Yang and Lu, 2004; Iijima-Ando, 2010). Neurodegeneration in *Drosophila* has been observed in the absence of NFT formation, although abnormal Tau phosphorylation is detected (Wittmann *et al.*, 2001; Jackson *et al.*, 2002; Nishimura, Yang and Lu, 2004). This idea that pathological Tau alone cannot induce neurodegeneration is also seen in Tauopathy mouse models of Tauopathies where NFT formation does not correlate with the severity and progression of neurodegeneration (Le Corre *et al.*, 2006; Kimura *et al.*, 2007). However, abnormal Tau phosphorylation does associate with areas of degeneration in *Drosophila* (Wittmann *et al.*, 2001). NFT pathology has been observed in *Drosophila* expressing wildtype human Tau alongside the GSK3β homologue – shaggy, indicating phosphorylation is key to NFT formation in *Drosophila* (Roberson *et al.*, 2007). Expression of Tau or mutant Tau in *Drosophila* also results in learning and memory defects, shortened life span and a rough eye phenotype (Wittmann *et al.*, 2001; Jackson *et al.*, 2002; Mershin *et al.*, 2004; Nishimura, Yang and Lu, 2004). Most phenotypes induced by Tau overexpression are more toxic in the mutant Tau flies compared to wildtype human Tau such as extent of neurodegeneration and a much shortened life span (Wittmann *et al.*, 2001; Prüßing, Voigt and Schulz, 2013).

1.5 The link between DNA damage and neurodegeneration

1.5.1 Introduction to DNA damage

DNA carries all the information for normal cellular survival and functioning, thus maintaining genome integrity is essential. Cells are continuously exposed to DNA damage from both endogenous and exogenous sources which can give rise to a variety of lesions including single-strand breaks (SSBs), double-strand breaks (DSBs) and oxidative damage (Chatterjee and Walker, 2017). On average per cell per day, 10-50 DSBs occur, 10,000 SSBs and 10,000-100,000 oxidative lesions occur (Madabhushi, Pan and Tsai, 2014). Endogenous sources of DNA damage include spontaneous deamination and depurination, misincorporation of nucleotides by DNA polymerase and reactive oxygen species (ROS) produced by normal cellular metabolism including respiration (Chatterjee and Walker, 2017). Exogenous sources of DNA damage include chemical mutagens such as tobacco smoke and radiation, especially ultraviolet light (UV) and ionizing radiation. Unrepaired lesions can block transcription or replication leading to genome instability, or directly induce apoptosis (Jackson and Bartek, 2009). Mis-repaired lesions can induce mutagenesis and cellular dysfunction, potentially leading to cancer (Jackson and Bartek, 2009).

To protect the genome, cells have evolved a highly conserved system called the DNA damage response (DDR) which senses damaged DNA and initiates a variety of cellular responses to maintain genome integrity. Each distinct lesion formed is repaired by a specific pathway, for example, oxidative damage is repaired by base excision repair (BER), bulky lesions are repaired via nucleotide excision repair (NER) and DSBs are repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR) (Chatterjee and Walker, 2017). In replicating cells, activation of the DDR pathway leads to cell cycle arrest to allow time for repair, or if the damage is too great or is unrepairable then cell senescence and apoptosis can be triggered (Schmitt *et al.*, 2007). Defects in the DDR in replicating cells can result in cancer or

apoptosis whereas defective repair systems in post-mitotic cells such as terminally differentiated neurons are suggested to underly accelerated aging and neurological diseases (Kulkarni and Wilson, 2008).

1.5.2 Types of DNA damage in neurons

Oxidative damage is the major source of DNA damage in the brain due to the high metabolic demands of neurons, consuming 20% of the body's total oxygen uptake (Deitmer *et al.*, 2019). Neurons are particularly vulnerable to oxidative damage due to a combination of low levels of anti-oxidant defences and their long-lived, post-mitotic nature meaning they are highly reliant on repair systems (Deitmer *et al.*, 2019). The attack on DNA by reactive oxygen species (ROS) attack on the DNA can result in more than 100 different types of base modifications, with 8-oxoG being the most common (Ba and Boldogh, 2018). Oxidised bases are repaired via BER which gives rise to SSBs as intermediates during the repair process (Narciso *et al.*, 2016). ROS can also directly induce SSB by direct attack on the DNA backbone (Narciso *et al.*, 2016). DSBs can arise indirectly from SSBs when either transcription or replication machinery collides with the SSB, causing collapse into a DSB, or if SSBs form close together (Cannan and Pederson, 2017). DSBs also arise in neurons in response to normal activity due to activation of gene transcription (Suberbielle *et al.*, 2013; Madabhushi *et al.*, 2015). DSBs form in early response gene promoters to facilitate gene expression associated with learning and memory (West and Greenberg, 2011; Madabhushi *et al.*, 2015; Cholewa-Waclaw *et al.*, 2016). These activity-induced DSBs are mediated by Topoisomerase IIβ which releases torsional stress so RNA polymerase can elongate (Ju *et al.*, 2011; Bunch *et al.*, 2015). The DSB occurs less frequently than the SSB but is the most lethal form of DNA damage. DSBs involve loss of integrity in both DNA strands, meaning the repair mechanisms are more prone to mis-repair, leading to chromosomal translocations (Rodgers and McVey, 2016). Additionally unrepaired DSBs are highly mutagenic and prone to recombination with other genomic areas. More importantly, as few as one DSB may

be sufficient to trigger apoptosis which could be catastrophic in the CNS as neurons can-not replace themselves (Jackson and Bartek, 2009).

1.5.3 The DNA damage response pathway

The DNA damage response pathway is a highly complex, interconnected set of signalling pathways (Figure 1.6). In response to DSBs, the DDR is initiated largely by activation of one of two serine/threonine kinases; ATM (ataxia telangiectasia mutated) or ATR (ataxia telangiectasia and Rad3- related protein), both members of the phosphoinositide-3-kinases (PI3K) family (Awasthi, Foiani and Kumar, 2015). These master regulatory kinases are responsible for phosphorylating numerous downstream targets on S/TQ amino acid motifs to elicit cellular responses such as DNA repair, cell cycle arrest or apoptosis (Kim *et al.*, 1999; Matsuoka *et al.*, 2007). ATM and ATR respond to different lesions with ATM primarily involved in responding to DSBs (Paull, 2015) and ATR responding to a wider variety of lesions including those generated by replication stress (Saldivar, Cortez and Cimprich, 2017).

1.5.3.1 ATM and ATR activation and downstream signalling

In its inactive form, ATM normally exists as a homodimer. Upon DNA damage, ATM undergoes at least four autophosphorylation events on serine residues 367,1893, 1981 and 2996, which induces dissociation in to active monomers (Kastan and Bakkenist, 2003; Kozlov *et al.*, 2006, 2011). The trimeric MRN complex (Mre11, Rad50, Nbs1) is one of the first complexes of proteins to be recruited to the site of the DSB and is needed for full activation and recruitment of ATM (Paull, 2015). Activated ATM then phosphorylates many downstream targets directly such as BRCA1 (Cortez *et al.*, 1999; Gatei *et al.*, 2000), H2AX (Burma *et al.*, 2001), p53 (Banin *et al.*, 1998) and KAP1 (Ziv *et al.*, 2006) (Figure 1.6). One of the first responses in DSB repair is phosphorylation of the histone variant H2AX at Ser139 (γH2AX) predominantly by ATM, but also can be phosphorylated by ATR or DNA-dependent protein kinase (DNA-PK) (Kinner *et al.*, 2008). Phosphorylated H2AX is essential for the initial signal amplification of the DDR, recruiting repair proteins at the site of break and also promoting chromatin remodelling

to enhance DSB repair (Podhorecka, Skladanowski and Bozko, 2010). ATM facilitates DNA repair via phosphorylation of various other substrates such as BRCA1 which promotes DSB repair, XRCC1 which is involved in base excision repair or KAP1 which facilitates loosening of heterochromatin to allow repair proteins to access the break. ATM can also trigger DNA end resection by stimulating the activity of key nucleases; Mre11 and CtIP, an essential step needed for DSB repair by homologous recombination (see section 1.5.3.3 for repair of double-strand breaks) (Lamarche, Orazio and Weitzman, 2010). ATM contributes to p53 phosphorylation which induces gene transcription to regulate cell cycle checkpoints for repair or apoptosis if the damage is unrepairable.

ATR responds to a variety of lesions but mainly in response to ssDNA created as a result of replication stress (Cimprich and Cortez, 2008). ATM can also activate ATR through DNA end resection which generates ssDNA (Jazayeri *et al.*, 2006; Myers and Cortez, 2006; Shiotani and Zou, 2009). The ssDNA is coated by the replication protein A (RPA) which then recruits ATR, via its binding partner ATRIP, and the Rad9-Hus1-Rad1 clamp (9-1-1) (Zou and Elledge, 2003). The role of the 9-1-1 clamp is to recruit TopBP1 which then activates ATR. ATR plays an essential role in the replication stress response by stabilising stalled forks, preventing origin firing and promoting DNA repair and fork restart (Figure 1.6) (Paulsen and Cimprich, 2007). ATR phosphorylates downstream targets such as FANCI to inhibit dormant origin firing (Chen *et al.*, 2015) and SMARCAL1 to prevent fork reversal and collapse (Couch *et al.*, 2013). ATR signalling also promotes various repair pathways via phosphorylation of BRCA1 for DSB repair (Tibbetts *et al.*, 2000) and FANCD2 for inter-strand crosslink repair (Andreassen, D'Andrea and Taniguchi, 2004). ATR phosphorylates many targets shared by ATM including p53 and H2AX (Figure 1.6).

While ATM and ATR phosphorylate a large number of downstream targets (~700 protein targets together) to amplify the response (Matsuoka *et al.*, 2007), two important effectors are the transducer kinases Chk1 and Chk2 which are downstream of ATR and ATM, respectively (Shiloh and Ziv, 2013).

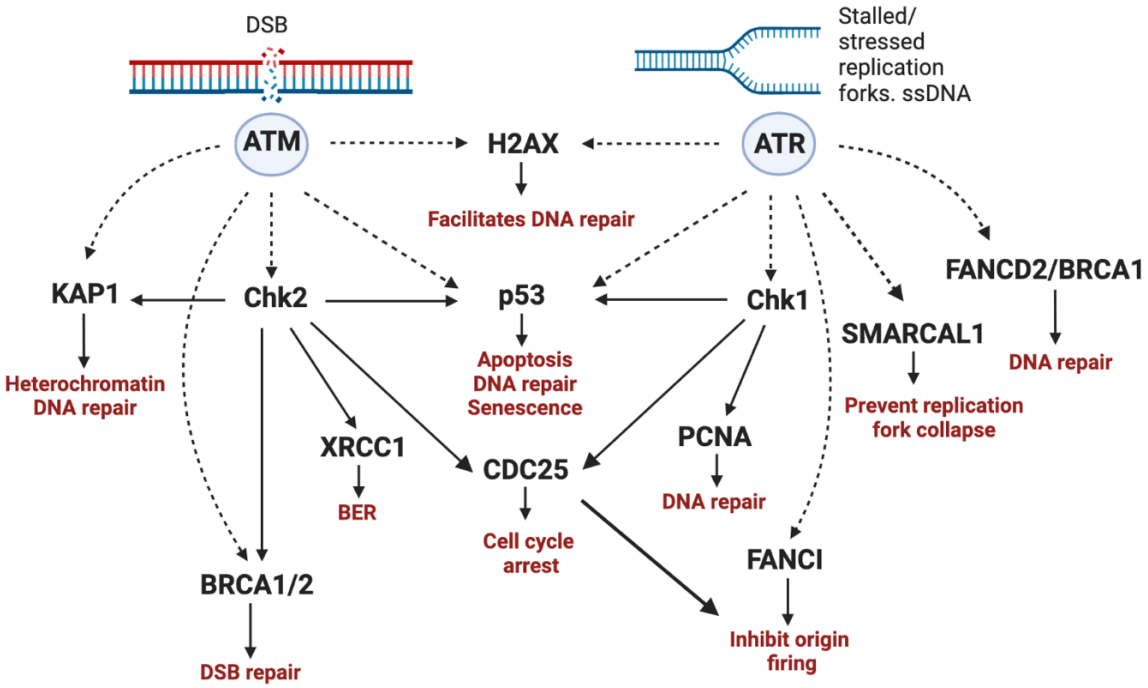


Figure 1.6 Overview of the DNA damage response pathway.
The DDR is initiated by recruitment and activation of ATM and ATR. ATM is primarily activated in response to DSBs, whereas ATR is mainly activated in response to replication stress. ATM and ATR phosphorylate many downstream substrates, including the transducer kinases Chk2 and Chk1, respectively, to amplify the response. Phosphorylation of the CDC25 phosphatases inhibits the activity of cyclin dependent kinases to induce cell cycle arrest. Phosphorylation and stabilisation of p53 initiates transcription of genes involved in cell cycle arrest, DNA repair, senescence, or apoptosis. Additional ATM/Chk2 targets include BRCA1/2 to promote homologous recombination and XRCC1 for break excision repair. ATM/Chk1 pathway mainly responds to stalled replication forks to maintain genome integrity via stabilising forks and inhibiting further origin firing.

1.5.3.2 Checkpoint kinases 1 and 2

The transducer kinases Chk1 and Chk2 lie downstream of ATR and ATM, respectively, and phosphorylate many downstream substrates to amplify the response and enhance ATM/ATR signalling. Here I will focus mainly on Chk2 activation due to its importance in the DSB repair pathway.

Chk2 is a 543 amino acid protein which has three functional domains: the N terminus consisting of SQ/TQ cluster domain (SCD), a central forkhead association (FHA) domain and a C terminal kinase domain (Figure 1.7). The SCD contains regions rich in serine-glutamine or threonine-glutamine clusters (SQ/TQ clusters) which are targets for phosphorylation by ATM and ATR (Buscemi *et al.*, 2006; Matsuoka *et al.*, 2007). The FHA domain interacts with other phosphorylated proteins, including the SCD of other Chk2 monomers, an important step involved in Chk2 activation (Li *et al.*, 2002). At the C terminus is the kinase domain and a nuclear localisation signal (NLS). Inside the kinase domain is the activation loop (T-loop) which contains residues that must undergo autophosphorylation for efficient kinase activation (Guo *et al.*, 2010).

Chk2 activation is mediated by various phosphorylation events (Figure 1.7). Chk2 is normally inactive in a monomeric form until ATM phosphorylates at threonine 68 (T68) in the SCD which triggers dimerization with another Chk2 monomer through the FHA region (Matsuoka *et al.*, 2000; Ahn *et al.*, 2002). Chk2 dimerization induces autophosphorylation events within the kinase domain and activation loop at serine 260, threonine 432, threonine 383, threonine 387 and serine 516 which induces an additional conformational change, causing dimer dissociation into active monomers (Schwarz, Lovly and Piwnica-Worms, 2003; Wu and Chen, 2003). Active Chk2 phosphorylates at least 24 identified substrates, most of which are involved in the DDR (Zannini, Delia and Buscemi, 2014). The checkpoint kinases are essential for induction of cell cycle checkpoints through phosphorylation of the CDC25 phosphatases, which inhibits Cdk/Cyclin activity. Chk2 phosphorylates many substrates shared with ATM

including BRCA1, KAP1 and p53, reinforcing ATM signalling (Figure 1.6) (Zannini, Delia and Buscemi, 2014).

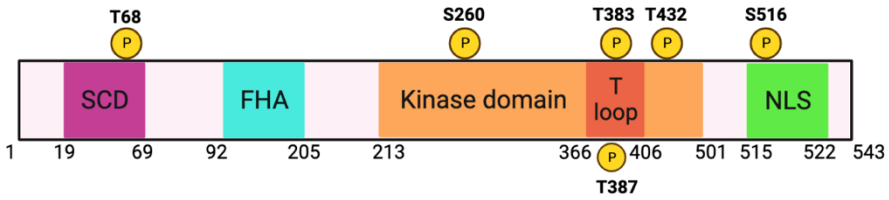


Figure 1.7. Schematic diagram showing the structure of Chk2.
The SCD domain (residues 19-69) contains SQ/TQ amino acid rich clusters which can be phosphorylated by ATM/ATR. Phosphorylation by ATM at T68 induces SCD dimerization with another phosphorylated Chk2 monomer through the forkhead association (FHA) domain (residues 92-205). At the C terminus the kinase domain (residues 213-501) contains an activation loop (T-loop) which undergoes autophosphorylation events that are essential for full Chk2 activation including S260, T383, T387, T432 and S516. Chk2 contains a nuclear localisation signal (NLS) at residues 515-522 to ensure correct localisation.

More briefly, Chk1 is phosphorylated by ATR at serine 317 and serine 345 for activation (Liu *et al.*, 2000; Zhao and Piwnica-Worms, 2001). ATR-Chk1 signalling is essential for genome stability during replication via many mechanisms such as regulating origin firing and maintaining fork progression (Buisson *et al.*, 2015). (Petermann, Woodcock and Helleday, 2010; Couch *et al.*, 2013; Eykelenboom *et al.*, 2013). During replication stress Chk1 acts to further suppress origin firing by phosphorylation of CDC25 phosphatases which in turn inactivates Cdk2 (cyclin dependent kinase 2) activity, which is essential for needed for firing (Zhao, Watkins and Piwnica-Worms, 2002). Chk1 phosphorylation of the CDC25 phosphatases also induces cell cycle arrest.

1.5.3.3 Repair of double-strand breaks

DSBs are largely repaired by either one of two pathways– homologous recombination (HR) or canonical non-homologous end joining (C-NHEJ) (Ceccaldi, Rondinelli and D 'andrea, 2016). Evidence suggests that NHEJ is the dominant pathway in which DSBs are first attempted to be repaired, probably due to faster kinetics (Mao *et al.*, 2008; Karanam *et al.*, 2012; Shahar *et al.*, 2012; Ceccaldi, Rondinelli and D 'andrea, 2016). C-NHEJ can occur in all phases of the cell cycle, but predominantly occurs in G1 (Brandsma and Gent, 2012). C-NHEJ is the more error prone DSB repair method because no homologous template is used during the repair process. In contrast, HR is mainly error-free as it uses a sister chromatid as a template for repair. However, the reliance on a sister chromatid means HR can only occur in proliferating cells after S phase (Brandsma and Gent, 2012). Since neurons are post mitotic, it is likely they rely exclusively on NHEJ for repair with the potential for mutations to accumulate (Jeppesen, Bohr and Stevnsner, 2011; Iyama and Wilson, 2013).

During C-NHEJ, the break ends are recognised by the Ku70/80 complex which recruits the catalytic subunit of the DNA dependent protein kinase (Lieber, 2010). DNA ends are processed by nucleases and filled in by DNA polymerases to generate compatible ends to be sealed by DNA ligase IV. When C-NHEJ is impaired, an alternative NHEJ pathway (alt-NHEJ) can occur independent of Ku70/80 binding and relies on homologous sequences at the ends of DNA to seal broken ends together (Sfeir and Symington, 2015). Alt-NHEJ involves extensive deletions and insertions at junctions, making it more error-prone than C-NHEJ.

In HR the DSB is recognised by the MRN complex (Mre11, Rad50 and Nbs1) which initiates DNA end resection, alongside the cofactor CtIP (Wright, Shah and Heyer, 2018). End resection is an important early step in HR which produces ssDNA, followed by RPA loading. This allows Rad1 to initiate strand invasion of the sister chromatid, which in turn allows correct repair using the sister template. ATM plays a

major role in DNA repair by triggering end resection, an essential step in HR, by phosphorylating and stimulating the activity of key nucleases; Mre11 and CtIP (Lamarche, Orazio and Weitzman, 2010). Alt-NHEJ also requires the initial resection mediated by Mre11 (Huang and Dynan, 2002; Dinkelmann *et al.*, 2009; Xie, Kwok and Scully, 2009; Zhang and Jasin, 2011; Truong *et al.*, 2013). It is proposed that long extended resection by the MRN complex favours HR compared to short resection which favours alt-NHEJ (Rass *et al.*, 2009).

1.5.4 DNA damage and neurodegeneration

DNA damage is implicated in normal ageing and is suggested to contribute to ageing phenotypes such as declining cellular function and dysregulation of signalling systems (Schumacher *et al.*, 2021). This highlights the impact of DNA damage and how essential DNA repair is in the CNS.

Elevated levels of DNA damage alongside reduced DNA repair activities are observed in multiple neurodegenerative diseases including Parkinson's disease, Amyotrophic lateral sclerosis (ALS) and AD (Madabhushi, Pan and Tsai, 2014; Coppedè and Migliore, 2015). It remains unclear if the accumulating DNA damage observed is a cause or a consequence of the pathology. A variety of forms of DNA damage are associated with neurodegeneration, including oxidative damage, abasic sites, bulky adducts, SSBs and the lethal DSB (Jeppesen, Bohr and Stevnsner, 2011; Madabhushi, Pan and Tsai, 2014; Merlo *et al.*, 2016). As previously discussed, the DSB presents a significant threat to the genome stability of neurons due to their long-lived nature and their high reliance on error prone NHEJ for repair. The DSB is not only found in neurodegenerative diseases but also are a feature of acute neuropathological conditions such as spinal cord injury (SCI) and following ischemia, suggesting they may be a universal feature of neurological disorders (Chen *et al.*, 1997; Hayashi *et al.*, 1998; Didenko *et al.*, 2002; Kotipatruni *et al.*, 2011).

1.5.4.1 DNA damage in Alzheimer's Disease

A plethora of evidence exists documenting DNA damage such as SSBs, DSBs and oxidative damage in multiple neurodegenerative disease, including Huntington's disease, Parkinson's disease, ALS and AD (Madabhushi, Pan and Tsai, 2014; Coppedè and Migliore, 2015).

Accumulating DSBs have been documented in AD, even at the very early stages of the disease, before NFT formation is apparent (Mullaart *et al.*, 1990; Sheng, Mrak and Griffin, 1998; Adamec, Vonsattel and Nixon, 1999; Myung *et al.*, 2008; Madabhushi, Pan and Tsai, 2014; Merlo *et al.*, 2016; Shanbhag *et al.*, 2019; Thadathil *et al.*, 2020). Evidence suggests that increasing numbers of DSBs in post-mortem AD brain material (marked by yH2AX accumulation) correlates with reduced cognitive function in patients before death (Simpson *et al.*, 2015). Studies in mice have shown that DSBs form naturally in neurons through increased neurological activity, such as during learning and memory tasks, but that the presence of Aβ exacerbates this effect (Suberbielle *et al.*, 2013; Merlo *et al.*, 2016). Addition of soluble Aβ to cultured neurons has also been shown to generate DSBs (Tuxworth *et al.*, 2019).

In addition to strand breaks accumulating in AD, oxidative DNA damage is detectable early during the MCI phases and later in AD (Gabbita, Lovell and Markesbery, 1998; Lovell, Gabbita and Markesbery, 1999; Wang, Markesbery and Lovell, 2006; Lovell, Soman and Bradley, 2011). The source (or sources) of the oxidative damage in AD are not yet fully established, but one possibility is the Aβ peptide which directly induces ROS production in neuronal cell culture (Behl *et al.*, 1994; Harris *et al.*, 1995), potentially through redox metal ions binding to Aβ and catalysing the production of ROS (Cheignon *et al.*, 2018). Additionally BER activity is also compromised in AD with essential proteins being reduced such as DNA glycosylase and Polβ, allowing oxidative damage to accumulate (Lovell, Xie and Markesbery, 2000; Weissman *et al.*, 2007).

DSB DNA repair pathways are also impaired in AD (Jeppesen, Bohr and Stevnsner, 2011). Key NHEJ repair proteins, including the Ku70/80 subunit and the DNA-PK catalytic subunit show lowered expression levels in AD, which likely contributes to poor repair and the accumulation of DSBs (Kanungo, 2013). Moreover, Aβ can directly inhibit one of the principal kinases regulating NHEJ, DNA-PK (Cardinale *et al.*, 2012). Although neurons are post-mitotic and therefore reliant on NHEJ for DSB repair, there is also evidence of a reduction in HR which could also contribute to DSB accumulation. Key proteins involved in HR are also reduced in AD patients such as the MRN complex, ATM and BRCA1, with BRCA1 depletion associated with memory and learning impairments in mice and reduced ATM levels correlating with neuronal loss (Jacobsen *et al.*, 2004; Suberbielle *et al.*, 2015; Shen *et al.*, 2016).

1.5.4.2 DNA damage induces cell cycle re-entry

Neurons are post-mitotic, terminally differentiated cells that reside in G0 and are defined as out of the cell cycle. Although neurons are traditionally classed as “out” of the cell cycle, an abundance of evidence exists to show abnormal neuronal cell cycle re-entry in CNS pathological insults (Wang *et al.*, 2009). Ectopic cell cycle re-entry is observed in chronic neurodegenerative diseases include ALS, Parkinson’s disease and AD and also after acute CNS insults such as cerebral ischemia after stroke (Love, 2003; Rashidian, Iyirhiaro and Park, 2007), traumatic brain injury (TBI) (Di Giovanni *et al.*, 2005) and spinal cord injury (SCI) (Velardo *et al.*, 2004; Wang *et al.*, 2009). It is more likely that neurons are actively held in a G0 state and in disease that regulation is disrupted.

A wealth of evidence suggests that cell cycle dysregulation in CNS pathologies induces cell death, reviewed in (Wang *et al.*, 2009). The first evidence that aberrant cell cycle re-entry could induce neuronal cell death came from studies forcing neurons into the cell-cycle which resulted in neurodegeneration (Al-Ubaidi *et al.*, 1992; Feddersen *et al.*, 1992). This was achieved via expression of the Simian virus 40 (SV40) large T antigen which inhibits the retinoblastoma (Rb) and p53 tumour

suppressor proteins (Ahuja, Sáenz-Robles and Pipas, 2005). Other studies have found cell cycle markers often associated with neuronal death induced by Tau or Aβ, further supporting this hypothesis (Copani *et al.*, 1999; Giovanni *et al.*, 1999; Andorfer *et al.*, 2005; Khurana *et al.*, 2006). Moreover, targeting cell cycle re-entry by using compounds to induce G0-G1 arrest has proven to be neuroprotective in rodent models of stroke and traumatic brain injury (Woods, Snape and Smith, 2007; Bonda *et al.*, 2010).

In AD, expression of cell cycle markers representing evidence of G1, S, G2 and transition into mitosis have been documented in post-mortem brain tissue, but no completion of mitosis has been observed (McShea *et al.*, 1997, 2007; Nagy *et al.*, 1997; Nagy, Esiri and Smith, 1997; Busser, Geldmacher and Herrup, 1998; Yang, Geldmacher and Herrup, 2001; Bonda *et al.*, 2010). Cell cycle markers have been shown to colocalise with NFTs and Aβ plaques (van Leeuwen and Hoozemans, 2015) and are also observed in the early stages of the disease, including MCI, suggesting that cell cycle re-entry could be involved as an initiating trigger for AD pathology (Nagy *et al.*, 1997; Nagy, Esiri and Smith, 1997; Vincent *et al.*, 1998; Yang, Mufson and Herrup, 2003; Varvel *et al.*, 2008). Cell cycle markers are also associated with formation of AD-like neuronal alterations such as Tau phosphorylation and Aβ plaque deposition (McShea *et al.*, 2007; Park *et al.*, 2007). Cell cycle markers are found in degenerating neurons and correlate with regions of neuronal loss and degeneration (Busser, Geldmacher and Herrup, 1998; Ding *et al.*, 2000; Yang, Geldmacher and Herrup, 2001; Yang, Mufson and Herrup, 2003) . Both Tau and Aβ, are connected to cell cycle events. Cell cycle-related kinases involved in checkpoint signalling and the DDR are able to phosphorylate Tau, including Cdk2, Cdk5, Chk2 and Chk1 (Baumann *et al.*, 1993; Liu *et al.*, 1995; Noble *et al.*, 2003; Mendoza *et al.*, 2013; Kimura, Ishiguro and Hisanaga, 2014). Aβ itself can act as a mitogen *in vitro* and induce neuronal cell cycle re-entry (Copani *et al.*, 1999; Giovanni *et al.*, 1999; Wu *et al.*, 2000; Varvel *et al.*, 2008).

DNA damage, which accumulates in neurodegeneration, has been shown to be a stimulus to trigger neurons to re-enter the cell cycle. This ultimately ends in neuronal

cell death and, importantly, it requires ATM activation (Park *et al.*, 1997; Kruman *et al.*, 2004; Fielder, Von Zglinicki and Jurk, 2017). There is now a body of evidence indicating that cell cycle activation can induce neuronal cell death, yet some cell cycle markers are expressed at low levels in neurons in the brains of healthy individuals (van Leeuwen and Hoozemans, 2015). One suggestion is that neuronal cell cycle re-entry is needed for NHEJ to repair DSBs and therefore possibly essential for their survival (Kruman *et al.*, 2004; Tomashevski *et al.*, 2010). Potentially in neurodegeneration where repair systems are compromised and DNA damage is elevated, this pathway is excessively activated or over-ridden, thus inducing neuronal apoptosis.

1.6 Mechanisms of cell death in neurodegeneration

1.6.1 Introduction to death in the central nervous system

Neuronal cell death occurs physiologically during development but also in pathology. During CNS development, neuronal cell death is crucial during neurogenesis but as neurons mature after development, they become highly resistant to cytotoxic stimuli, which is essential due to their long-lived, post-mitotic state (Kole, Annis and Deshmukh, 2013; Yamaguchi and Miura, 2015). During normal ageing, little neuronal loss occurs (Yankner, Lu and Loerch, 2008). This contrasts with neurodegenerative diseases where extensive neuronal cell death is observed, contributing to the progressive decline in cognition (Gorman, 2008; Serrano-Pozo *et al.*, 2011). The mechanism through which neurons die during neurodegeneration is controversial and not fully understood. Potential mechanisms of neuronal cell death include necrosis, apoptosis and autophagy (Fricker *et al.*, 2018). There are several complicating factors for detecting cell death in neurodegeneration (see section 4.1 for more detail). AD is a slow progressive disease, thus the number of neurons undergoing cell death at one point are low. Additionally, some cell death mechanisms are quick meaning the timing

of cell death assays is crucial: if carried out too early or too late, false results may occur.

1.6.2 Apoptosis and necrosis

The two principal types of cell death thought to occur in neurodegeneration are apoptosis and necrosis (Chi, Chang and Sang, 2018). Apoptosis is a tightly regulated cell death mechanism that is triggered by stimuli such as DNA damage, oxidative stress, viruses and toxins (D'Arcy, 2019). Apoptosis is essential for tissue homeostasis, nervous and immune system development and for clearing infected or damaged cells (Singh, Letai and Sarosiek, 2019). In contrast, necrosis is a passive pathological cell death mechanism initiated by direct trauma to the cell (D'Arcy, 2019). Apoptosis and necrosis have distinct morphological characteristics. Apoptosis includes chromosome condensation, DNA fragmentation, cell membrane blebbing and formation of apoptotic bodies to minimise an immune response (Elmore, 2007). Necrosis is identified by features such as cellular and organelle swelling and cell membrane lysis (D'Arcy, 2019).

Apoptosis is executed by the caspases, a family of proteases which cleave cellular proteins to induce cell death, including nuclear lamins and cytoskeletal proteins (D'Arcy, 2019). Caspases can be divided into initiator caspases (caspases 2,8,9 and 10), which initiate the caspase dependent mechanism and executioner caspases (caspases 3,6 and 7) which directly cleave cellular proteins to induce death (Elmore, 2007). Apoptosis can be activated by two main pathways: the intrinsic pathway (mitochondrial) and the extrinsic pathway (death receptor) (Figure 1.8) (Elmore, 2007).

The intrinsic pathway relies on an internal stress stimuli which induces transcription of pro-apoptotic genes via p53 dependent transcription (Figure 1.8)(Singh, Letai and Sarosiek, 2019). Induction of pro-apoptotic genes induces permeabilisation of the mitochondria and consequently mitochondrial proteins are released, including cytochrome C. The Bcl2 protein family are responsible for regulating membrane permeability of the mitochondria, which can be pro-apoptotic (Bax, Bak and Bad) or

anti-apoptotic (Bcl-2, Bcl-x) (Singh, Letai and Sarosiek, 2019). Anti-apoptotic proteins such as Bcl-2 can inhibit the activity of pro-apoptotic proteins, suggesting that the ratio of anti-apoptotic to pro-apoptotic is more important in dictating cell death (Billen *et al.*, 2008; Singh, Letai and Sarosiek, 2019). Cytochrome C forms a complex with apoptotic protease activating factor 1 (APAF-1) and pro-caspase 9, known as the apoptosome, which leads to activation of pro-caspase 9 (Li *et al.*, 2017).

The extrinsic pathway is activated when death ligands such as Fas ligand (FasL) and tumor necrosis factor (TNF) bind to death receptors (Figure 1.8) (Kumar, Herbert and Warrens, 2005). Death receptors have an extracellular domain for ligand binding and an intracellular domain known as the death domain which transmits the signal internally. Cytoplasmic adaptor proteins are recruited to the death domain upon ligand binding such as FasL binding to the FAS receptor recruits the adaptor protein FADD (Kumar, Herbert and Warrens, 2005). The adaptor proteins associate with pro-caspase 8 to form a death inducing signalling complex (DISC) which provides a platform for activation of pro-caspase 8 by inducing dimerisation. Both intrinsic and extrinsic pathways converge on to activation of the executioner caspases (3, 6, 7) to carry out apoptosis. Caspase 3 is the major executioner caspase (Slee, Adrain and Martin, 2001; Walsh *et al.*, 2008).

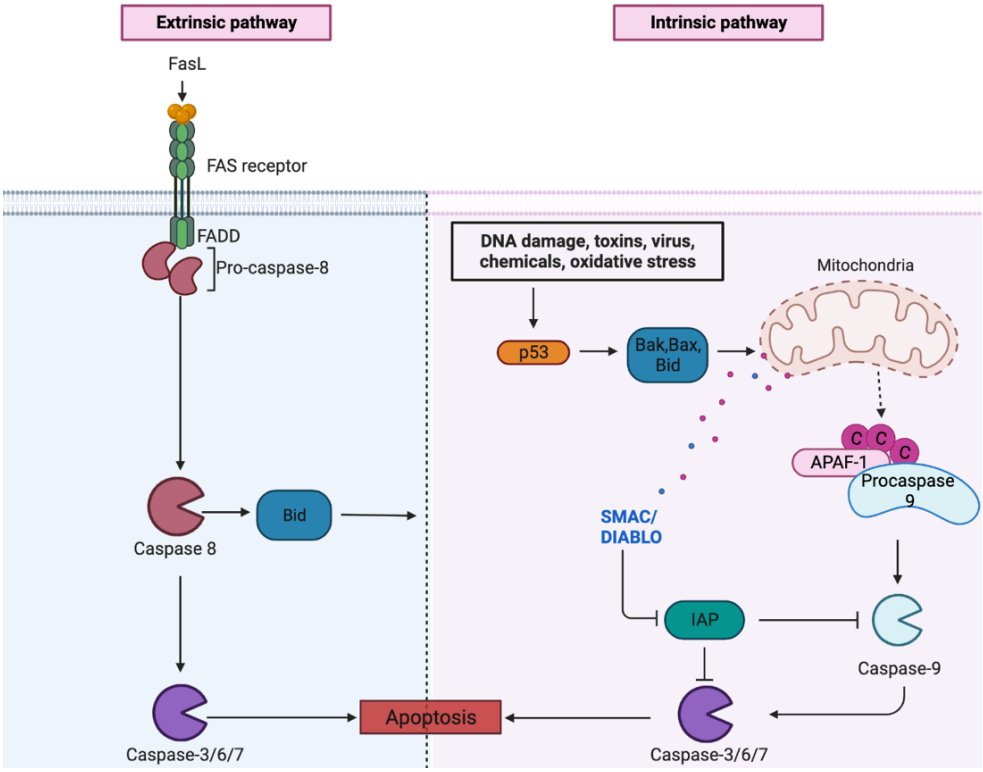


Figure 1.8. The intrinsic and extrinsic pathway of apoptosis.

Apoptosis can be triggered via the intrinsic or extrinsic pathway. The intrinsic pathway is triggered by internal stimuli such as DNA damage or oncogene activation which induces p53 dependent transcription of pro-apoptotic proteins. Pro apoptotic proteins include members of the Bcl2 protein family (Bax, Bak and Bid) which triggers permeabilisation of the mitochondria membrane. Consequently, cytochrome C is released from the mitochondria and associates with APAF1 and procaspase 9 to form the apoptosome, resulting in activation of caspase 9. Other mitochondrial proteins released are SMAC/DIABLO which also promote apoptosis by blocking inhibitors of apoptosis. The extrinsic pathway is activated by binding of death ligands (FasL) to their receptors (FAS). This recruits adaptor proteins (FADD) to the cytoplasmic death domain and pro-caspase 8. Association of pro-caspase 8 results in dimerization and activation of caspase 8. Active caspase 8 can also cleave the pro-apoptotic protein Bid, inducing its translocation into the mitochondria, linking the extrinsic and intrinsic pathways. Both intrinsic and extrinsic pathways converge onto activation of the executioner caspases (3,6 and 7) to cleave cellular proteins to induce cell death.

1.6.3 The role of p53 in apoptosis

p53 is a tumour suppressor protein that is essential to cellular stress responses (Feroz and Sheikh, 2020). p53 regulates gene expression in response to DNA damage to protect the genome via cell cycle arrest, apoptosis, DNA repair or senescence (Feroz and Sheikh, 2020). In an unstressed cell, p53 is present at relatively low levels due to proteasomal degradation by the E3 ubiquitin ligase MDM2 (Aubrey *et al.*, 2018). Upon cellular stress, such as DNA damage, metabolic dysfunction or oncogene activation, p53 undergoes multiple post transcriptional modifications, resulting in its stabilisation and accumulation (Liu, Tavana and Gu, 2019). Many DDR kinases have been implicated in phosphorylation and stabilisation of p53 (Kruse and Gu, 2009). Phosphorylation at serine 15 by ATM, ATR and DNA-PK and phosphorylation at serine 20 by Chk2 and Chk1 is particularly important in p53 stabilisation by impairing MDM2 mediated degradation (Shieh *et al.*, 1997, 2000; Banin *et al.*, 1998; Canman *et al.*, 1998; Tibbetts *et al.*, 1999; Unger *et al.*, 1999; Chehab *et al.*, 2000). Once stabilised, p53 can bind to its target genes to drive expression to carry out various cellular response. p53 drives expression of approximately 500 target genes which induce cell cycle arrest, DNA damage repair pathways, cell senescence or apoptosis (Aubrey *et al.*, 2018). In response to irreparable DNA damage, p53 induces apoptosis by controlling both the intrinsic and extrinsic pathway. p53 activates the intrinsic pathway by upregulation of the expression of pro-apoptotic members of the Bcl-2 protein family to induce mitochondrial membrane permeability, which is the key no return point in the apoptotic signalling cascade (Singh, Letai and Sarosiek, 2019). p53 activates the extrinsic pathway by upregulating gene transcription of death receptors and its death ligands (Kumar, Herbert and Warrens, 2005). p53 activation also upregulates expression of caspases and coactivators of caspases such as APAF1 (Elmore, 2007).

1.6.4 p53 dysregulation in AD

Elevated p53 levels and activity has been reported many neurodegenerative diseases including AD, Huntington's Disease, Parkinson's Disease and ALS (De La Monte, Sohn and Wands, 1997; Chang *et al.*, 2012; Szybinska and Lesniak, 2017). Elevated p53 levels could be due to the accumulation of DNA damage seen in neurodegenerative diseases, resulting in stabilisation (Merlo *et al.*, 2016). Alternatively, p53 activity can be influenced by proteins implicated in AD. The AICD released from APP processing can bind to p53 and stimulate its transcriptional activity, enhancing p53 mediated apoptosis (Alves da Costa *et al.*, 2006; Ozaki *et al.*, 2006). Additionally Aβ can directly activate p53 itself or trigger kinase activity to induce p53 phosphorylation and stabilisation (Ohyagi *et al.*, 2005; Lapresa *et al.*, 2019). p53 activity is not just linked to Aβ, but also Tau. p53 can interact with Tau and indirectly induce Tau phosphorylation, possible by activation of GSK3β (Hooper *et al.*, 2007; Farmer *et al.*, 2020).

Under normal physiological conditions p53 activity controls gene expression to regulate cell cycle events, DNA repair, senescence, or apoptosis to protect the genome (Feroz and Sheikh, 2020). It therefore may not be surprising that expression of apoptotic proteins are altered in AD (MacGibbon *et al.*, 1997; Nagy and Esiri, 1997; Su, Deng and Cotman, 1997; Kitamura *et al.*, 1998; Giannakopoulos *et al.*, 1999). Although elevated p53 are seen in AD, it's been reported that p53 is conformationally different in AD, resulting in oligomerisation, mis localisation and impaired p53 signalling (Uberti *et al.*, 2006; Lanni *et al.*, 2008; Farmer *et al.*, 2020). Mechanisms through which dysfunctional p53 could induce neurodegeneration are summarised in figure 1.9. It is unclear if p53 activity in AD is a cause of cellular pathology or is instead a protective mechanism. p53 mediates neurotoxicity in *in vivo* models of Alzheimer's disease and ALS *and in vitro* neuronal exposure to Aβ1-42, suggesting p53 activity mediates neurotoxicity (Culmsee *et al.*, 2001; Merlo *et al.*, 2014; Taylor and Tuxworth, 2019; Maor-Nof *et al.*, 2021). In contrast, in a *Drosophila* tauopathy model, p53 activity was shown to be neuroprotective possibly via regulating synaptic function (Merlo *et al.*, 2014).

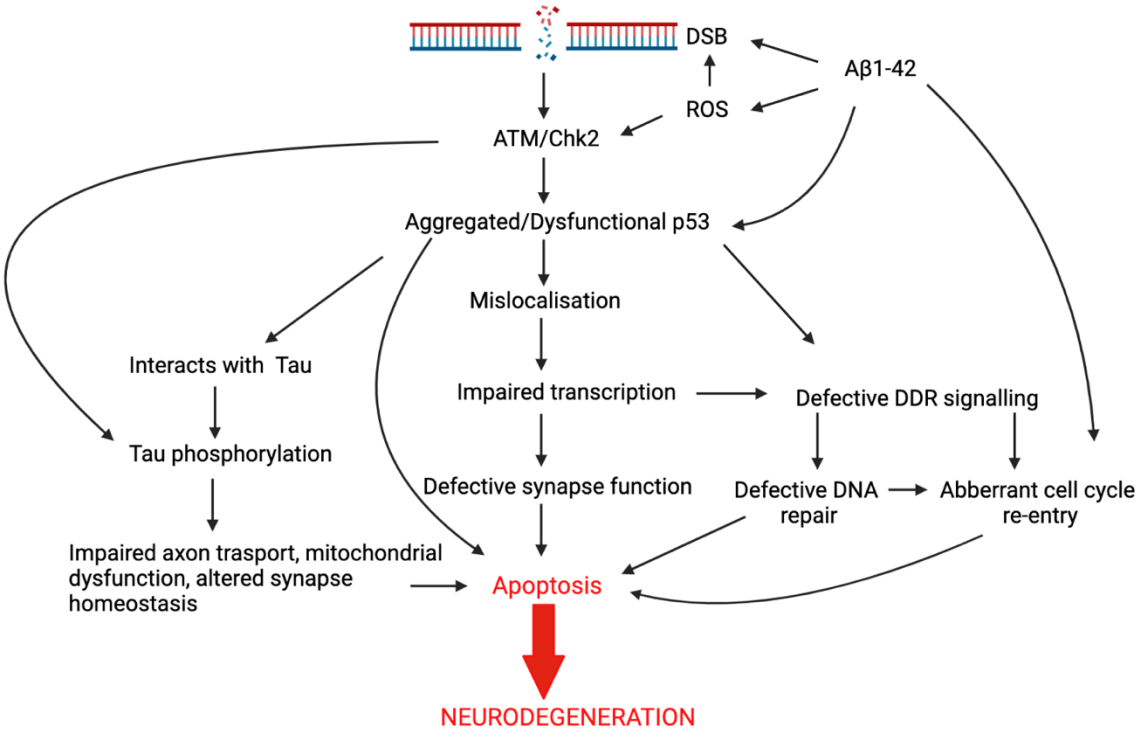


Figure 1.9. Potential mechanisms in which dysfunctional p53 could induce apoptosis in Alzheimer's Disease.

DNA damage is elevated in AD, potentially via Aβ1-42, resulting in activation of the ATM/Chk2 pathway and stabilisation of p53. There is evidence p53 is conformationally changed and forms oligomers in AD, causing mis-localisation and aggregation outside of the nucleus. Mis-localisation leads to defective transcription and consequently impaired DDR repair and apoptosis. A defective DDR signal and consequently more DNA damage could also induce aberrant cell cycle events. Aggregated p53 could interact with the intrinsically disordered Tau protein and induce phosphorylation. Tau phosphorylation could induce cell death via multiple means including disrupted axonal transport and mitochondrial dysfunction.

1.6.5 Evidence of apoptosis in Alzheimer's Disease

AD is characterised by progressive neuronal loss, predominantly in the hippocampus and entorhinal cortex which gives rise to the classical symptoms (West *et al.*, 1994; Gómez-Isla *et al.*, 1996; Troncoso, Martin, *et al.*, 1996; Price *et al.*, 2001; Serrano-Pozo *et al.*, 2011; Andrade-Moraes *et al.*, 2013; Arendt *et al.*, 2015). In AD, regional neuronal loss is more closely correlated to the cognitive decline than deposition of Tau tangles or Aβ plaques (Hof *et al.*, 2003; Arendt *et al.*, 2015; Martínez-Pinilla *et al.*, 2016) and the frequency of apoptosis correlates to the progression of neurodegeneration (Stadelmann *et al.*, 1999; Andrade-Moraes *et al.*, 2013). Several stimuli in AD could be a trigger for neuronal cell death including protein aggregation, DNA damage, oxidative stress and aberrant cell cycle re-entry (Fricker *et al.*, 2018).

Aβ itself is cytotoxic to neurons *in vitro* (Yankner *et al.*, 1989; Yankner, Duffy and Kirschner, 1990; Pike *et al.*, 1991, 1993; Behll *et al.*, 1992; Takadera *et al.*, 1993). Strong evidence exists that Aβ induces cell death via apoptosis. Addition of Aβ *in vitro* results in classical apoptotic morphology, including chromatin condensation, DNA fragmentation and cell membrane blebbing (Loo *et al.*, 1993; Watt *et al.*, 1994; Forloni *et al.*, 1996; Li *et al.*, 1996; Harada and Sugimoto, 1999). Additionally, cell death of neurons expressing Aβ *in vitro* is mediated by p53, alongside microglia apoptosis in AD (Zhang *et al.*, 2002; Davenport *et al.*, 2010). Although there is evidence for apoptosis occurring in AD, not all studies agree with each other. Traditional apoptotic hallmarks have been detected in post-mortem AD brains, including DNA fragmentation by TUNEL staining (Lassmann *et al.*, 1995; Smale *et al.*, 1995; Troncoso, Sukhov, *et al.*, 1996; Lucassen *et al.*, 1997; Sugaya, Reeves and McKinney, 1997; Masliah *et al.*, 1998; Stadelmann *et al.*, 1998; Colurso, Nilson and Vervoort, 2003). However, other studies have failed to observe any morphological features of apoptosis in post-mortem AD brains (Lassmann *et al.*, 1995; Smale *et al.*, 1995; Troncoso, Sukhov, *et al.*, 1996; Lucassen *et al.*, 1997; Stadelmann *et al.*, 1998; Raina *et al.*, 2001; Woodhouse *et al.*, 2006). Upregulation of death ligands and changes in apoptotic protein expression in AD brains have been documented (De La Monte, Sohn and Wands, 1997; Su *et al.*, 2003). Pro-apoptotic proteins such as BAX are upregulated in AD brains and are reported to frequently

localise in tangle and plaque bearing neurons (MacGibbon *et al.*, 1997; Nagy and Esiri, 1997; Su, Deng and Cotman, 1997; Kitamura *et al.*, 1998; Giannakopoulos *et al.*, 1999). Elevated levels of the anti-apoptotic Bcl-2 protein are found in AD brains but decreased in neurons undergoing degeneration, suggesting it has a protective role in AD (Satou, Cummings and Cotman, 1995; O'Barr, Schultz and Rogers, 1996; Kitamura *et al.*, 1998). However, other studies report no or minimal changes in expression of Bcl-2 and BAX between control and AD brains (Braak, Braak and Mandelkow, 1994; Nagy and Esiri, 1997; Kitamura *et al.*, 1998; Stadelmann *et al.*, 1998; Woodhouse *et al.*, 2006). Changes in apoptotic protein expression in AD brains could be due to direct activation of the p53 promoter by Aβ1-42 (Paradis *et al.*, 1996; Zhang *et al.*, 2002; Ohyagi *et al.*, 2005). Further evidence of apoptosis occurring in AD is the presence of activated caspases in post-mortem AD brains including caspase 3,6, 8 and 9 (Selznick *et al.*, 1999; Stadelmann *et al.*, 1999; Rohn, Head, Nesse, *et al.*, 2001; Su *et al.*, 2001; Rohn *et al.*, 2002; Gastard, Troncoso and Koliatsos, 2003; Pompl *et al.*, 2003; Zhao *et al.*, 2003; Guo *et al.*, 2004; Albrecht *et al.*, 2009). Aβ can directly activate caspases, including the dominant executioner caspase 3, and induce apoptosis (Loo *et al.*, 1993; Uetsuki *et al.*, 1999; Marín *et al.*, 2000; Awasthi, Matsunaga and Yamada, 2005; Takada *et al.*, 2020). Although caspase upregulation is associated with AD it is suggested that there is ineffective caspase signalling to the executioner caspases, resulting in a phenomenon known as abortive apoptosis, with neurons surviving although the caspase cascade has been initiated (Raina *et al.*, 2001). It is likely that the heightened caspase activity seen in AD is a consequence of an apoptotic stimuli, however it is also suggested that caspase activity could contribute to the neurodegeneration since APP can be cleaved by caspases to produce a toxic fragment which induces neuronal cell death and synapse loss (Gervais *et al.*, 1999; Lu *et al.*, 2003; Park *et al.*, 2020). Tau is also a substrate of caspases, which when cleaved promotes its assembly into NFT's (Canu *et al.*, 1998; Gamblin *et al.*, 2003; Rissman *et al.*, 2004).

1.7 Preliminary data

In AD, DSBs accumulate and DDR proteins such ATM and p53 are elevated, implicating the DDR is active (Chang *et al.*, 2012; Katsel *et al.*, 2013; Merlo *et al.*, 2016). Previous work in the Tuxworth lab has shown that targeting the DSB repair pathway suppresses neurodegeneration and promotes regeneration (Tuxworth *et al.*, 2019; Taylor *et al.*, 2022). Targeting the MRN complex by reducing or inhibiting Mre11 or Nbs1 activity elicits a neuroprotective effect in a *Drosophila* model of AD (Tuxworth *et al.*, 2019). Similarly, inhibiting the MRN complex promotes axon regeneration after an optic nerve and spinal cord injury model *in vitro* and *in vivo* (Tuxworth *et al.*, 2019). Furthermore, targeting the ATM-Chk2 pathway is neuroprotective. Knocking down *ATM* or *Chk2* suppresses neurodegeneration in *Drosophila* models of amyloid toxicity (Taylor *et al.*, 2022). DSBs are a common feature of most (and potentially all) neurological disorders, including following acute traumatic injury (Merlo *et al.*, 2016). Consistent with this, inhibiting Mre11, ATM or Chk2 with small molecule inhibitors also promotes functional recovery in rats from spinal cord and optic nerve injury (Tuxworth *et al.*, 2019; Taylor *et al.*, 2022). All the above evidence suggests that DSB signalling through the ATM-Chk2 pathway mediates neurotoxicity in our amyloid model in *Drosophila* and after acute neurotrauma. As Chk2 is downstream of ATM, it is likely that a target downstream of Chk2 is involved in the neurotoxic effect when the DDR pathway is activated in neurological disease. The aim of this project is to attempt to uncover the potential mechanisms downstream of Chk2 activation that could mediate neurotoxicity.

1.8 Aims and hypothesis

Hypothesis: Chronic activation of the DNA damage response in neurological disease mediates neurodegeneration via pathways downstream of Chk2.

Aims: To investigate signalling events downstream of Chk2 to identify potential neurotoxic events focusing on the following mechanisms:

- Apoptosis
- Cell cycle re-entry
- Tau phosphorylation

2.0 Materials and methods

2.1 *Drosophila melanogaster*

2.1.1 *Drosophila melanogaster* husbandry

Drosophila melanogaster stocks (listed in Table 2.1) were maintained on a standard food (50 g/L yeast, 50 g/L glucose, 0.8 % agar (w/v), 1 % (w/v) soy flour, 0.1% p-Hydroxy-benzoic acid methyl ester, 0.03% (v/v) propanoic acid) at 18 °C on a 12 h light/dark cycle. Fly crosses were set up and maintained on standard food at 25 °C with relative humidity kept constant at 70 %. Food containing 200 μM mifepristone (Generon) was prepared in the same standard way with the addition of 200uM of mifepristone (added before pouring into vials from a 40 mM stock solution in 100 % ethanol). Mifepristone food was made fresh weekly and flies were changed into fresh food every 3 days.

2.1.2 *Drosophila* transgene expression

For all *Drosophila melanogaster* movement tracking experiments, the driver used was the pan-neuronal driver *Elav-GAL4^{C155}* on the X chromosome combined with a *tubP-GAL80^{ts}* insertion on chromosome II. The temperature sensitive Gal80 repressor inhibits any Gal4 activity at 18 °C, allowing us the restriction of transgene expression to adult neurons only. Virgin females of the driver lines were crossed to males of the correct genotype. Emerging adult flies were separated into vials and shifted to either 27 °C to induce transgene expression.

For apoptosis (UAS-GC3ai) and FLY-FUCCI experiments the Ok107-Gal4 driver was used, which drives expression in the Kenyon cells of the mushroom bodies. Virgin females of Ok107-Gal4 were collected and crossed to males of the appropriate fly line.

The progeny of these crosses were shifted to 27°C 1 day post eclosion and aged for brain dissections. For neuronal counting experiments, the mushroom body Gene Switch driver (MB-GeneSwitch) was used. Virgins of MB-GeneSwitch were collected and crossed to males of the appropriate fly line. 1 day post eclosion adult females were placed on 200 μM Mifepristone food and aged for brain dissections.

2.1.3 Movement tracking using DART

Adult males flies eclosing from crosses were separated into groups of 20 per genotype and each individual fly was placed into vials and shifted to the correct temperature to induce transgene expression. Individual male flies were kept in 65 x 5 mm locomotor vials (Trikinetics). Vials in groups of 20 were secured onto platforms and movement recorded from above via a Logitech C920 HD camera. DART applied a vibrational stimulus (3.5V) in 5 x 0.5s bursts with each stimulus separated by 10 mins for a total of 5 total events. The position of each fly was then tracked at 5Hz. The movement of the fly pre- and post- stimulation was quantified using DART software running in MATLAB 2017a (Faville *et al.*, 2015). The mean speed of the 20 flies for each group over the 120s before the stimulus, and the peak amplitude of response was quantified by DART. The performance index (PI) was calculated by taking the mean amplitude of response (maximum speed post stimulation minus pre stimulus speed) on the days prior to temperature shift. This was used as the baseline movement (day 0, PI=1). All recordings after temperature shift were normalised to this baseline value. Linear trend lines were fitted to this data using linear regression in Prism 9 and the gradients of the lines were compared using a one-way ANOVA with Tukey's multiple comparisons tests. The gradients of the lines were plotted as a bar chart, with error bars representing S.E.M. A full description using the DART system to quantify the stimulus response of flies was published previously (Taylor and Tuxworth, 2019).

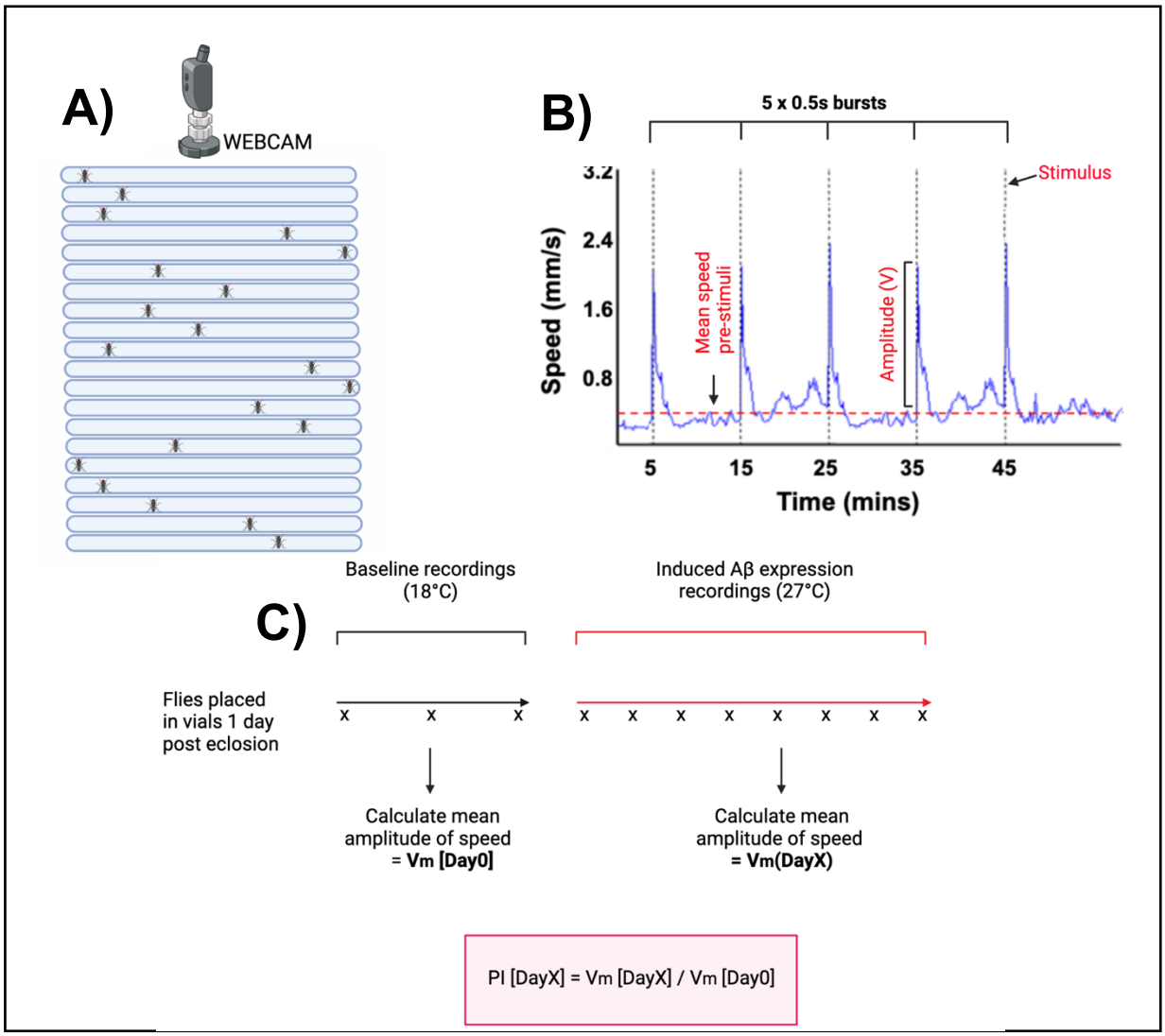


Figure 2.1. Experimental design of the DART tracking software to measure the Aβ induced motility decline in *Drosophila*.
A) A diagram showing the set-up of the DART platforms. Flies in groups of 20 are housed in locomotor vials 1 day post eclosion and a webcam from above tracks their movement over the course of the experiment. **B) A trace diagram showing the mean speeds and amplitude of responses from 20 flies over 1 experiment, adapted from Taylor and Tuxworth,2019.** 5 x 0.5s vibrational stimuli are delivered separated by 10 minutes. The amplitude of each fly is calculated by the peak of the response minus the pre stimulation speed. An average amplitude of response of the 20 flies is taken to give a mean amplitude of response (Vm). **C) Schematic diagram showing the experimental design of the DART tracking system recordings before and after temperature shift.** Flies are developed at 18°C to suppress Aβ expression. 1 day post eclosion flies are placed in vials ready for baseline tracking. Within 1 week, on 3 different days, fly movement is recorded at 18°C to calculate a baseline mean amplitude of speed (Vm[Day0]). Flies are then shifted to a 27°C to allow Aβ expression. The mean amplitude of speed is recorded on different days (Vm [DayX]). The performance index (PI) for each day is calculated by Vm [DayX] / Vm[Day0] for each genotype. The PI for the baseline values represent day 0 and is 1 for all genotypes.

2.1.4 Genomic DNA preparation

2 whole *Drosophila* were squashed with a pipette tip in 50 µl of squishing buffer (see table 2.3 for buffers and reagents) supplemented with 200 µg/ml Proteinase K in a 1.5ml Eppendorf tube followed by incubation in a water bath at 37 °C for 30 min. The Proteinase K was inactivated by incubating for 3 min in hot block at 95 °C. The supernatant was removed and stored at -80 °C until use.

2.1.5 Polymerase chain reaction (PCR)

Standard PCR mixtures contained 1 X Green GoTaq Reaction Buffer (Promega), 200 µM dNTP mix (Thermo), 0.5 µM forward and reverse primers (see table 2.2 for primers), 1-10 ng template DNA, and 0.02 U/µl DreamTaq DNA polymerase (Thermo). Standard PCR reactions were performed with an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, followed by annealing at desired temperature for 30 s, followed by extension at 72 °C for 1 min. PCR reaction then finished with a final extension at 72 °C for 5 min. PCR products were then analysed by agarose gel (in TBE buffer) electrophoresis to verify product size.

2.1.6 Immunohistochemistry

Drosophila brains and imaginal discs from third instar larvae were dissected in cold PBS under a dissecting microscope. Imaginal discs were left attached to the mouth then micro dissected after fixing and staining. *Drosophila* tissues were fixed in 4% paraformaldehyde (Sigma) in PBS on ice for 15 min and then washed with PBS 3 x 5 min. Adult brains and larval imaginal discs were permeabilised in PBS containing 0.3% (v/v) Triton X-100 (Sigma) for 15 min at room temperature (RT). Brains were blocked in IHC block (see table 2.3 for buffers and reagents) for 2 h at RT (or 15 min for imaginal discs). Samples were then stained with primary antibodies (see table 2.4 for primary antibodies) diluted in PBS for 48 h at 4 °C (or 4 h at RT for imaginal

discs). Brains were washed 5 x 30 min at RT (or 3 x 15 min for imaginal discs), and then incubated with secondary antibody (see table 2.5 for secondary antibodies) diluted in PBS for 48 h at 4 °C, kept in the dark (or 24 h at 4 °C for imaginal discs). Brains were washed 5 x 30 min at RT with all PBS being removed in the final stage (3 x 15 min for imaginal discs). For adult brains fluoroshield with DAPI (Sigma) was added to the Eppendorf tube and the brains were left to settle overnight at 4 °C before being mounted onto slides. During the final wash with PBS, imaginal discs were micro disected before being mounted onto slides with Fluoroshield with DAPI (Sigma).

2.2 Cell culture

2.2.1 Neuroblastoma and epithelial cell lines

SK-N-AS human neuroblastoma cells were gifted from Dr. Malgorzata Zatyka. SH-SY5Y cells were obtained from Sigma. Neuroblastoma cell lines were maintained in Dulbecco's Modified Eagle Medium, with high glutamine and without sodium pyruvate, supplemented with 10 % foetal bovine serum (FBS), 1 % non-essential amino acids, 1 % Glutamine and 1 % penicillin-streptomycin (all Gibco) at 37 °C under 5 % CO_2 in air. A549 epithelial cell line were a gift from thes Coleman group. A549 cells were maintained in Ham's F-12 Nutrient Mixture, with 10 % FBS and 1 % penicillin-streptomycin. Cells were passaged at 80 % confluency every 2-3 days.

2.2.2 Drugs applied to neuroblastomas

Kinase inhibitors were added to each dish at a final concentration of 10 μM. Drugs used were: ATM inhibitor (KU60019, Tocris) (Golding *et al.*, 2009), Chk2 inhibitor (CCT241533, Tocris) (Anderson *et al.*, 2011) and an AMPK inhibitor (Cayman Chemical) (Dite *et al.*, 2018). All inhibitors were dissolved in DMSO to a stock concentration of 10mM. DMSO alone was used as a vehicle control. Cells were left 1

h and then irradiated with 8 Gys of X-irradiation using a CellRad irradiator (Faxitron). Cells were then fixed 1 h after irradiation for immunofluorescence.

2.2.3 Inducing oxidative stress

Hydrogen peroxide (H_20_2) (Sigma) was added to the dish at a final concentration of 200 μM in PBS. Cells were left 30 min before fixation for immunofluorescence.

2.2.4 Site directed mutagenesis

Plasmid pLenti-DsRed_IRES_MAPT:EGFP (11.5kb) was a gift from Huda Zoghbi (Addgene plasmid #92196) (Rousseaux *et al.*, 2016). The plasmid was midi prepped using the GenElute™ HP Plasmid Midiprep Kit (Sigma). Tau Serine 262 in the plasmid was mutated to an alanine using the Q5 Site-Directed Mutagenesis Kit (NEB #E0554). PCR cycling conditions were initial denaturation at 98 °C for 30 s, 25 cycles of denaturation at 98 °C for 10 s, followed by annealing at 55 °C for 30 s, followed by extension of 4 min 15 s at 72 °C. The PCR reaction finished with a final extension of 2 min at 72 °C followed by digestion of the template DNA with DpnI for 30 min at 72 °C, as per the kit protocol. Transformation of NEB 5-alpha Competent *E. coli* cells were carried out following the transformation protocol in the Q5 Site-Directed Mutagenesis Kit (NEB #E0554). Single colonies were mini prepped using the GenElute™ HP Plasmid Miniprep Kit (Sigma) and sent for Sanger sequencing to confirm the presence of the Ser262Ala mutation (see table 2.2 for primers).

2.2.5 Transfection

After confirmation of the Ser262Ala mutation, a single correct bacterial colony was midi prepped using the GenElute™ HP Plasmid Midiprep Kit (Sigma) ready for transfection. 24 h before staining, A549 cells were seeded at 50 % confluency in 60 mm culture dishes and allowed to adhere to coverslips coated in 0.1% w/v poly-L-

lysine (Sigma). Cells were transfected using the Invitrogen Lipofectamine™ 3000 Reagent Protocol. 1 μg of DNA was diluted in OptiMEM™ Medium with add P3000™ reagent. The diluted DNA mixture was added to diluted Lipofectamine™ 3000 Reagent (1:3 ratio). A total of 100 μl of DNA-lipid complex was added to cells. After 6 h, the transfection medium was removed from cells and replaced with standard A549 medium. The day after transfection cells were treated with irradiation and then fixed and stained.

2.2.6 Immunocytochemistry

24 h before staining, cells were seeded at 70 % confluency in 60 mm culture dishes and allowed to adhere to coverslips coated in 0.1% w/v poly-L-lysine (Sigma). On the day of fixation, cell media was removed and washed with 2 x PBS. Cells were fixed with 4 % paraformaldehyde (Sigma) in PBS for 20 min at RT. Cells were then permeabilised in PBS 0.1% (v/v) Triton X-100 (Sigma) for 10 min followed by incubation with ICC block (see table 2.3 for buffers and reagents) for 20 min. Cells were incubated with primary antibodies (see table 2.4 for primary antibodies) diluted in ICC block (see table 2.3 for buffers and reagents) for 1 h at RT. Unbound primary antibody was removed with 3 x 5 min washes in PBS 0.1 % (v/v) Triton X-100. Secondary antibodies (see table 2.5 for secondary antibodies), diluted in ICC block, were added for 40 min at RT and kept in the dark. The wash steps were repeated with 2 final additional washes with ddH_20 to remove salt. Coverslips were then mounted onto glass slides using Prolong Gold Antifade with DAPI (Invitrogen) and left at 4 °C overnight before imaging.

2.2.7 Cell lysate preparation

1 h after irradiation, the cell growth media was removed and the cells were washed with 1 ml of ice-cold PBS and scraped from the dish. The cells were centrifuged at 14,000 RPM for 3 min at 4 °C and the supernatant discarded. 200 μl of ice-cold cell lysis buffer (see table 2.3 for buffers and reagents) was added and then incubated on ice for 5 min. 6 x Laemmli SDS Sample buffer (Alfa Aesar) was added to a final

concentration of 1 X. The lysates were heated to 95 °C for 5 min then run on SDS-PAGE gels or stored at -20 °C until needed.

2.2.8 Western blot

10 µl of whole cell lysates were loaded into wells of an 8 % SDS-PAGE gel. Gels were ran at 150V for approximately 1 h in tris-glycine-SDS running buffer. Proteins were transferred to a PDVF membrane in a BioRad wet transfer apparatus in tris-glycine transfer buffer at 0.4 A for 90 min. Membranes were blocked in 5 % milk in TBST for 30 min and then incubated in primary antibodies (see table 2.4 for primary antibodies) diluted in block overnight at 4 °C. Unbound primary antibody was washed off with 3 x 5 min washes in TBST. Membranes were incubated in secondary antibodies (see table 2.5 for secondary antibodies), diluted in TBST, for 40 min at RT. Pierce enhanced chemiluminescence kit (Thermo) were used to detect antibody binding in conjunction with a Vilber Fusion FX scanner.

2.3 Microscopy and image analysis

2.3.1 Confocal microscopy

All immunofluorescence experiments were imaged on a Zeiss LSM880 inverted confocal microscope (Carl Zeiss) using AiryScan super resolution settings. Cells were imaged using a Plan-Apo 100x/1.46 oil immersion objective with an optical slice thickness set at 1.5 µm. For *Drosophila* brains, either a Plan-Apo 25x N.A 0.8 water immersion or a Plan-Apo 40x N.A 1.2 water objective was used with optical slice thickness set at 1.0 µm or 2.55 µm, respectively. All Z-series were taken using a step size of 0.5 µm. Images were processed in Zeiss Zen or in ImageJ/FIJI software.

2.3.2 Image analysis

All image analysis was carried out in Zeiss Zen or ImageJ/FIJI software. Figures were prepared using Microsoft PowerPoint. All statistical tests were performed in GraphPad Prism 9.

2.3.2.1 Nuclear Tau pSer262 and yH2AX quantification

Before any quantification was carried out, Z series were compressed to a maximum intensity Z-projection in ImageJ. To quantify nuclear Tau phosphorylation at Ser262 (pSer262) and yH2AX staining, DAPI positive cells were selected manually and the corresponding mean pixel intensity of Tau pSer262 (green) and yH2AX (red) staining was recorded. Values from independent experiments were then collated together for analysis.

2.3.2.2 Mushroom body cell count

The ImageJ plugin TrackMate was used to count all Kenyon cells present in the mushroom bodies, marked with mCherry. The diameter of dot detected was set at 2.8 μm for all genotypes. Automatic thresholding was not consistent between samples, so thresholding was performed manually for each adult brain to include as many Kenyon cells as possible without overestimating, based on a visual assessment. The settings were as follows: LoG detector, Z/T not swapped, estimated dot diameter 2.8 μm, use sub-pixel localization but not median filter. The threshold was started at 300 and then adjusted in increments of 50, inspecting the image manually to observe is an approximately correct threshold was chosen. The average number of Kenyon cells per mushroom body hemisphere and standard deviation was calculated for each genotype on day 3, day 14 and day 30. Linear trend lines were fitted to this data using linear regression in Prism 9 and the gradients of the lines were compared using a one-way ANOVA with Tukey's multiple comparisons tests. The gradients of the lines were plotted as a bar chart, with error bars representing S.E.M.

2.3.2.3 GC3ai image processing

Before images were analysed, all files were blinded and renamed using a random number system by my supervisor Richard Tuxworth. After analysis was carried out, blinded, the random numbers allocated were matched back up with the original file name. Before cell counting was carried out, Z series were compressed to a maximum Z-projection in ImageJ. To quantify the number of GFP positive cells (active GC3ai) the image was processed to remove background staining by subtracting 10,000 pixels and then a maximum filter with radius 1 was applied. The threshold of 'moments' was applied with a value of 19019 consistently across all images. The manual cell counter in ImageJ was used to count the number of green dots present in each image. Each image was analysed 3 times to produce an average number of 'GFP' positive cells.

Table 2.1 *Drosophila melanogaster* genotypes and sources

Fly stock	Full genotype	Source
w ^{1118}	*w ^{1118}; +; +*	BL5905
Elav - GAL4	*Elav-Gal4 $^{[c155]}$; +; +; +*	BL458
Actin - GAL4	*y^{1} w*; +; Act5C-Gal4 / TM6B, Tb ^{1}*	BL3954
If / Cyo; MKRS / TM6B	*w*; Kr^{If-1} / Cyo; MKRS, Sb^{1} / TM6B, Tb^{1}*	Tuxworth lab
UAS – mCherry NLS	*w*; UAS-mCherry.NLS; MKRS, Sb^{1} / TM6B, Tb^{1}*	BL38425
UAS - Aβ12L	*w*; UAS-Aβ12L*	(Speretta, Thomas R Jahn, *et al.*, 2012)
UAS - hTau0N4R R406W	*w*; +; UAS-hTau0N4R406W / TM6B, Tb^{1}*	(Wittmann et al., 2001)
UAS - FLYFUCCI	*w*; Kr^{If-1} / Cyo; UAS-GFP.E2f1, UAS-RFP1.NLS.CycB.1 / TM6B, Tb^{1}*	BL55111
UAS - p35 (II)	*w*; UAS-P35; +*	BL5072
UAS - p35 (III)	*w*; +; UAS-P35*	BL5073
UAS - GC3Ai	*w*; +; UAS-GC3ai*	Magali Suzanne (Schott et al., 2017)
Ok107 - Gal4	*w*; + ;+ ; ey[Ok107-Gal4]*	BL854
MB - GeneSwitch	*w ^{1118}; +; MB-Switch / TM6C, Sb^{1}*	BL81013
GMR - Gal4	*w*; GAL4-ninaE-GMR*	BL1104
UAS – p53 RNAi	*y^{1} sc* v^{1} sev^{21}; +; TRiP.HMS02286*	BL41720
UAS - ATM RNAi	*y^{1} sc* v^{1} sev^{21}; +; TRiP.GL00138*	BL44417
UAS - Chk2 RNAi	*y^{1} sc* v^{1} sev^{21}; +; TRiP.GL0020 / TM3, Sb^{1}*	BL35152

BL = Bloomington *Drosophila* Stock Center, University of Indiana, USA.

Table 2.2 Primers for *Drosophila* crosses and genetic recombination

Name	Sequence	Annealing temperature (°C)	Purpose
Tau S262A forward Tau S262A reverse	CAAGATCGGCgCCACTGAGAA GACTTGACATTCTTCAGGTC	57	Mutate Tau serine 262 to alanine 262.
Tau seq forward Tau seq reverse	AAAAGCAAAGACGGGACTGG GTGGTCTGTCTTGGCTTTGG	55	Sanger sequencing to confirm successful mutation of Tau serine 262 to alanine 262
UAS-GC3ai forward UAS-GC3ai reverse	GCCACCATGTACCCCTACGACGTG CGCTTACAGGTCCTCCTCGCTGATC	65	To confirm UAS-GC3ai was present in fly lines by PCR.
VALIUM 22 forward VALIUM 22 reverse	GGTGATAGAGCCTGAACCAG TAATCGTGTGTGATGCCTACC	57	To confirm the UAS-RNAi line to Chk2 (TRiP.GL0020) or ATM (TRiP.GL00138) were present in fly line by PCR.
VALIUM20 Forward VALIUM20 reverse	ACCAGCAACCAAGTAAATCAAC TAATCGTGTGTGATGCCTACC	57	To confirm the UAS-RNAi to p53 (TRiP.HMS02286) was present in fly line by PCR.

Table 2.3. Composition of buffers and reagents

Buffers and reagents	Composition
Phosphate buffered saline (PBS)	ddH_2O, 137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, PH 7.4
Tris-buffered saline (TBS)	ddH_2O, 50mM Tris, 150mM NaCl, PH 7.6
TBS – Tween 20 (TBST)	TBS, 0.1% (v/v) Tween 20
Cell lysis buffer	1 X RIPA buffer, 1mM DTT (Thermo) supplemented with phosphatase and protease inhibitor cocktails* (Calbiochem)
RIPA buffer (5X) (Cell Biolabs)	125 mM Tris pH 7.6, 750 mM NaCl, 5% Igepal CA-630, 5% sodium deoxycholate, 0.5% SDS.
SDS – PAGE running buffer	ddH_2O, 25mM Tris, 192mM Glycine, 0.1% (w/v) SDS, pH 8.3
Transfer buffer	ddH_2O, 25mM Tris, 192mM Glycine, 20% (v/v) methanol, pH 8.3
Laemmli SDS sample buffer (6X) (Alfa Aesar)	375mM Tris-HCl, 9% SDS, 50% glycerol, 9% beta-mercaptoethanol, 0.03% bromophenol blue, PH 6.8
Squishing buffer	ddH_2O, 10mM Tris, 25mM NaCl, 0.05% (v/v) Triton X-100, 1mM EDTA-Na_2, 4mg/ml proteinase K*, pH 8.0
Tris-borate-EDTA (TBE) buffer	ddH_2O, 100mM Tris, 100mm boric acid, 20mM EDTA-Na_2
IHC block	PBS, 0.3% (v/v) Triton X-100, 1% (w/v) BSA
ICC block	PBS, 1% (v/v) goat serum (Sigma)
Drosophila lysis buffer	ddH_2O, 1 X RIPA, 1/200 X protease and phosphatase inhibitor cocktails* (Calbiochem), 1/50 DTT (Thermo)

*Proteinase K/protease & phosphatase inhibitors were all added fresh before use

Table 2. 4. Primary antibodies

Antibody (m/p)	Species	Experiment	Dilution	Source (catalogue number)
Phospho- Tau Serine 262 (p)	Rabbit	ICC	1:1000	Invitrogen (44-750G)
Phospho- Tau Serine262 (p)	Rabbit	ICC	1:1000	Signalway (11111)
Total Tau (p)	Rabbit	ICC	1:1000	Dako (A0024)
Phospho-histone H2A.X Serine 139 (m)	Mouse	ICC	1:1000	Millipore (05-636)
Phospho-histone H2A.X Serine 139 (m)	Rabbit	ICC	1:5000	Abcam (ab81299)
Serine 396/Serine 404 (PHF-1) (m)	Mouse	ICC	1:1000	Dr Peter Davies (Greenberg *et al.*, 1992)
Beta actin (m)	Mouse	WB	1:1000	Proteintech (60008-1-IG)
Phospho-ATM Serine 1981	Mouse	WB	1:1000	Abcam (AB36810)
GFP (p)	Rabbit	IHC	1:2000	Abcam (AB290)
RFP (m)	Rat	IHC	1:1000	Chromotek (5F8)

p = Polyclonal m= Monoclonal ICC = Immunocytochemistry
WB = Western blot IHC = Immunohistochemistry

Table 2.5. Secondary antibodies

Antibody (m/p)	Conjugate	Experiment	Concentration & Block	Source (Catalogue number)
Goat anti-rabbit IgG (p)	Alexa Fluor 488	ICC, IHC	1:1000	Invitrogen (A-11034)
Donkey anti-mouse igG (p)	Alexa Fluor 594	ICC, IHC	1:1000	Invitrogen (A-32758)
Goat anti-rabbit IgG (p)	Alexa Fluor 594	IHC	1:1000	Invitrogen (A-11012)
Goat anti-rabbit IgG (p)	HRP	WB	1:5000	Abcam (ab6721)
Goat anti-mouse IgG (p)	HRP	WB	1:5000	Abcam (ab205719)

p = Polyclonal m= Monoclonal ICC = Immunocytochemistry WB = Western blot IHC = Immunohistochemistry HRP = Horseradish peroxidase

3.0 Investigating downstream targets of Chk2 - p53

3.1 Introduction

Previous work leading up to this project has shown that targeting the ATM-Chk2 pathway is neuroprotective in a *Drosophila* model of chronic amyloid toxicity and in two models of acute neurotrauma in rat: spinal cord injury and an optic nerve injury (Taylor *et al.*, 2022). As Chk2 is downstream of ATM in the DNA damage response (DDR), it is likely that the neuroprotective target is due to inhibiting signalling downstream of Chk2. Chk2 has at least 24 known protein targets (Figure 3.1) which are predominantly involved in the DDR including DNA repair, cell cycle regulation, p53 signalling and apoptosis (Zannini, Delia and Buscemi, 2014). Chk2 has other targets that have roles outside of the DDR including Cdk11 and Tau (Zannini, Delia and Buscemi, 2014).

	Chk2 substrate	Phosphorylation sites	Biological function
DNA repair			
	BRCA1	S988	HDR and NHEJ
	BRCA2	T3387	HDR
	XRCC1	T248	BER
	FOX-M1	S361	BER
	KAP-1	S473	Chromatin reorganization
Cell cycle regulation			
	CDC25A	S123	G1/S checkpoint
	LATS2	S408	G1/S checkpoint
	Rb	S612	G1/S checkpoint, apoptosis repr.
	CDC25C	S216	G2/M checkpoint
	TTK/hMPS1	T288, S281	G2/M checkpoint
p53 signaling			
	p53	T18, S20	Apoptosis
	HDMX	S367, S342	p53 accumulation
	CABIN1	NA	p53 activation on chromatin
	pVHL	S111	p53 activation
	STRAP	S221	G2/M checkpoint
	CHE-1	S141, S474, S508	G2/M checkpoint
Apoptosis			
	PML	S117	Apoptosis
	E2F1	S364	Apoptosis
	HuR	S88, S100, T188	Apoptosis or prosurvival
Other or unknown role			
	PP2A	NA	CHK2 inactivation?
	TRF2	S20	NA
	BLM	NA	NA
	TAU	S262	NA
	CDK11	S737	Pre-mRNA splicing

Figure 3.1. List of known Chk2 targets edited from Zannini, Delia and Buscemi, 2014.
Table of known Chk2 targets which are mainly involved in DNA damage repair signalling, including DNA repair, cell cycle regulation, p53 signalling and apoptosis. Other substrates not involved in the DNA damage include Cdk11 and Tau.

One target downstream of ATM-Chk2 activation is p53, which is suggested to be implicated in neurodegeneration (Chang *et al.*, 2012). Elevated p53 levels and activity is observed in multiple neurodegenerative disease, including AD, and there is suggestion p53 signalling is dysregulated in pathology (Chang *et al.*, 2012). This evidence all suggests p53 could play an important role in eliciting the toxicity seen in our amyloid model.

3.2 Results

3.2.1 Using the DART system to model neurodegeneration

Most *Drosophila* models of neurodegeneration result in a progressive decline in motility (Romero *et al.*, 2008; Voigt *et al.*, 2010; Kerr *et al.*, 2011; Beharry, Alaniz and Alonso, 2013). The most common way to measure the locomotor defects is to use the climbing assay which relies on the negative geotaxis response (upward climbing) seen when *Drosophila* are tapped to the bottom of a vial (Gargano *et al.*, 2005). This method requires many flies and is subject to a high degree of variability. Previously in the Tuxworth lab, the *Drosophila* ARousal Tracking (DART) software, developed initially to follow sleep patterns, was re-purposed and optimised as an alternative to the negative geotaxis assay (Taylor and Tuxworth, 2019). The DART system measures the startle response of individual flies housed horizontally in small vials on a platform (Taylor and Tuxworth, 2019)(Figure 3.2A). The system uses a vibrational stimulus to induce the startle response while a camera overhead records the increased movement of the flies. The software then tracks the position of each fly and measures the amplitude of the increase in their movement speed after each stimulus (Figure 3.2B). The behaviour of 20 flies is aggregated to give a population response. The quantifiable output from this data is known as the performance index (mean amplitude of response) which is calculated as the maximum speed post-vibration minus the pre-stimulation speed. The performance index of each genotype of flies after temperature shift to 27 °C was normalised to the performance index of the same flies housed at 18 °C pre-shift to reduce the variability of the data, since individual cohorts of genetically identical flies were seen to start with different levels of startle response. Normalising to the starting response results in highly reproducible data (Taylor and Tuxworth, 2019).

To model AD in *Drosophila*, the UAS-Gal4 system is commonly used to overexpress Tau or Aβ proteins in the CNS, usually in neurons (Bonner and Boulianne, 2011;

Lenz et al., 2013). Overexpression of Tau or Aβ peptides results in neurodegenerative phenotypes such as locomotor deficit, reduced lifespan, and neuronal loss (see section 1.4.2.2 for modelling Aβ and Tau toxicity in *Drosophila*). Here I modelled AD using the DART system by overexpressing Aβ or Tau peptides under the control of the temperature sensitive ElavGal4 ($Gal80^{ts}$) driver, allowing restricted transgene expression to adult post-mitotic neurons only. The flies are kept at 18 °C during development and then shifted to 27 °C as adults to induce expression of Aβ or Tau. Although this system had been used in the group previously to help establish the DART startle response technique (Taylor and Tuxworth, 2019), it was important for me to ensure that I could replicate the motility decline induced by Aβ before further experiments. The peptides used were Aβ 1-42 dimers separated with a 12- amino acid flexible linker. The Aβ 1-42 dimers are more aggregative and consequently more toxic when separated by a flexible 12- or 22-amino acid linker (Speretta, Thomas R. Jahn, *et al.*, 2012). The Tau peptide used was the Tau mutant R406W which is found in FTDP-17 patients and is more toxic when expressed in the *Drosophila* brain than wildtype Tau (Wittmann *et al.*, 2001). It is important to clarify that although Aβ 1-42 dimers were used, this is not a specific model of Aβ42 toxicity but instead a model for Aβ toxicity, as other Aβ forms such as Aβ1-40 were not used as controls. This was suitable for our experiments as we simply wanted to model Aβ toxicity to investigate underlying mechanisms, and we were not looking into the toxicity of different Aβ forms. Expression of Aβ 1-42 using the ElavGal4 system was a well-established model of amyloid toxicity in the lab.

Our expression system used here is adult-onset with expression of Aβ and Tau being restricted to adult neurons only, under the control of the temperature sensitive repressor Gal80. Aβ and Tau have physiological roles in the CNS, including synapse homeostasis and maintenance of normal axon transport, respectively (Kent, Spires-Jones and Durrant, 2020). Overexpression of these proteins during development, instead of restricting to adult neurons only, may interfere and alter these processes resulting in confounding phenotypes, making it less clear the direct effects of the toxic proteins. It is already known that overexpression of Tau in the CNS throughout development results in abnormal phenotypes, including locomotor defects, synaptic

dysfunction and disrupted axon transport (Mudher *et al.*, 2004; Chee *et al.*, 2005; Folwell *et al.*, 2010a). Most importantly, in further experiments we wanted to modulate components of the DNA damage pathway, including *p53*, to investigate downstream mechanisms that elicit the neurotoxicity by Aβ. This would involve expressing an RNAi to knock down *p53* using the same expression system (Elav-Gal4; Gal80^{ts}). Loss of *p53* in the early stages of *Drosophila* is shown to affect development, and again we wanted to prevent these phenotypes from being carried into the adult so the effect of *p53* knock down on Aβ toxicity was clear to analyse (Contreras, Sierralta and Glavic, 2018).

The DART system models Aβ toxicity well, with an initial phase where little decrease in motility is seen followed by a more rapid decline, producing a sigmoidal shape curve for Aβ expressing flies (Figure 3.3A). This bi-phasic response mirrors reasonably well the clinical course of AD: a preclinical, asymptomatic phase, a phase of decline and a final period of severe dementia. It is interesting that a slight increase in motor activity is observed at the start of the experiment between day 0 and day 5 when Aβ is expressed before the decline occurs (Figure 3.3A). As previously discussed (see section 1.3.6) a slight increase of Aβ levels is associated with synaptic vesicle release and consequently increased neuronal activity *in vitro*, whereas high pathological levels are associated with synaptic impairment (Puzzo *et al.*, 2008; Abramov *et al.*, 2009). Potentially here, due to the Elav-Gal4 expression system being turned on, the levels of Aβ are slowly increasing between day 0 and 5, before maximum production is reached at day 5 to induce a decline. There is also a decline in motility observed in the control fly (ElavGal4^{ts} x w^{1118}), which is due to normal ageing, but here the decline is linear and not sigmoidal (Figure 3.3A). Although the decline seen with the Aβ produces data points that fit a sigmoidal curve, linear regression lines were fitted to the performance index to simplify the comparisons between genotypes (Figure 3.3B). Despite the Aβ genotype producing a sigmoidal curve, a good line of best fit is still achieved using linear regression lines (Aβ r^2 value = 0.6772, compared to the control r^2 value = 0.3375 (Figure 3.3B).

In contrast to Aβ, Tau did not appear to induce any detectable decline in motility with the linear regression lines of Tau and the control fly overlapping each other (Figure 3.3B). The gradients of the slopes were calculated and compared using a one-way ANOVA. The rate in the decline of motility is significantly accelerated with Aβ compared to the control fly (Figure 3.3C, **p=0.0014). However, there is no significant effect produced when Tau is expressed compared to the control (Figure 3.3C, p=0.9980). There was a significant difference between the Aβ expressing fly compared to Tau alone, with Aβ proving to be drastically more toxic (Figure 3.3C, ***p=0.0008). Tau is suggested to act downstream of Aβ and enhance neurodegeneration but here co-expression of Tau and Aβ did not enhance Aβ induced neurotoxicity with no significant difference between flies expressing Aβ alone compared to Aβ + Tau (Figure 3.3B and C, p=0.3977). Although the dominant hypothesis is that Tau acts downstream of Aβ (Bloom, 2014), some research does suggest there may be a feedback loop mechanism in which both proteins can induce formation of the other (Götz *et al.*, 2001; Lewis *et al.*, 2001; Ferrari *et al.*, 2003; Oddo *et al.*, 2004; Hurtado *et al.*, 2010; Ittner *et al.*, 2010; Israel *et al.*, 2012; Leroy *et al.*, 2012; Choi *et al.*, 2014; Peters *et al.*, 2019). Although co-expression of Tau and Aβ induce a steeper decline in motility compared to Tau alone, this was not significant (Figure 3.3B and C, p=0.0666).

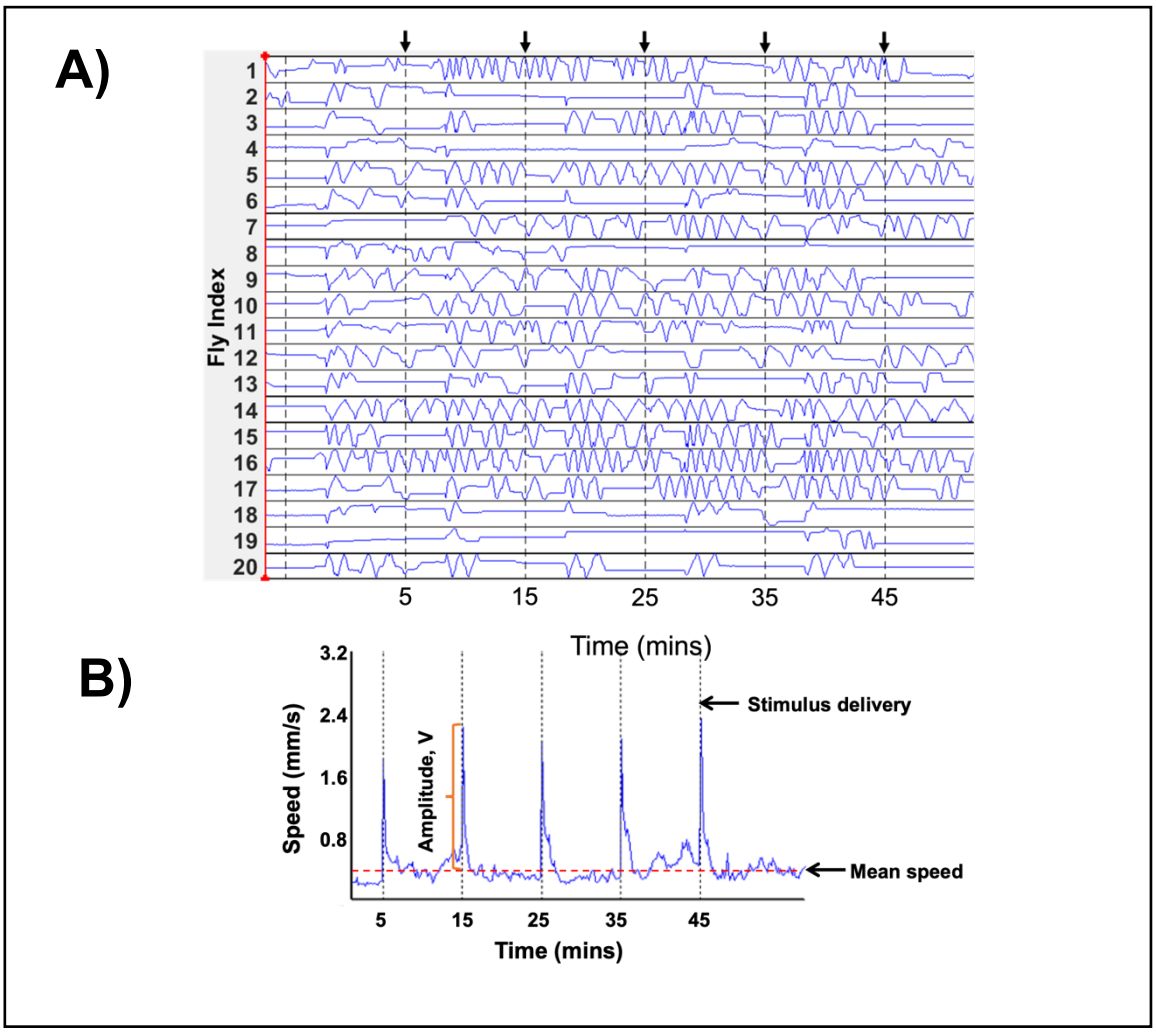


Figure 3.2 The DART tracking system measures the increase in movement of *Drosophila* in response to a vibrational stimulus.
A) An x-y plot showing the location of each fly over the time course of the experiment. The DART system tracks the movement of each fly within its vial. The position of the fly is represented as a vertical trace. The horizontal axis represents the 60 min duration of the experiment with black arrows indicate when the 5 vibrational stimuli was applied at 10 min intervals. **B) Example trace of the mean response to the vibrational stimulus from one group of 20 flies taken from Taylor and Tuxworth,2019**. The dashed lines show when the stimulus was applied, with each stimulus separated by 10 minutes. The amplitude recorded is the increase in movement after the vibration is delivered minus the pre-stimulation speed.

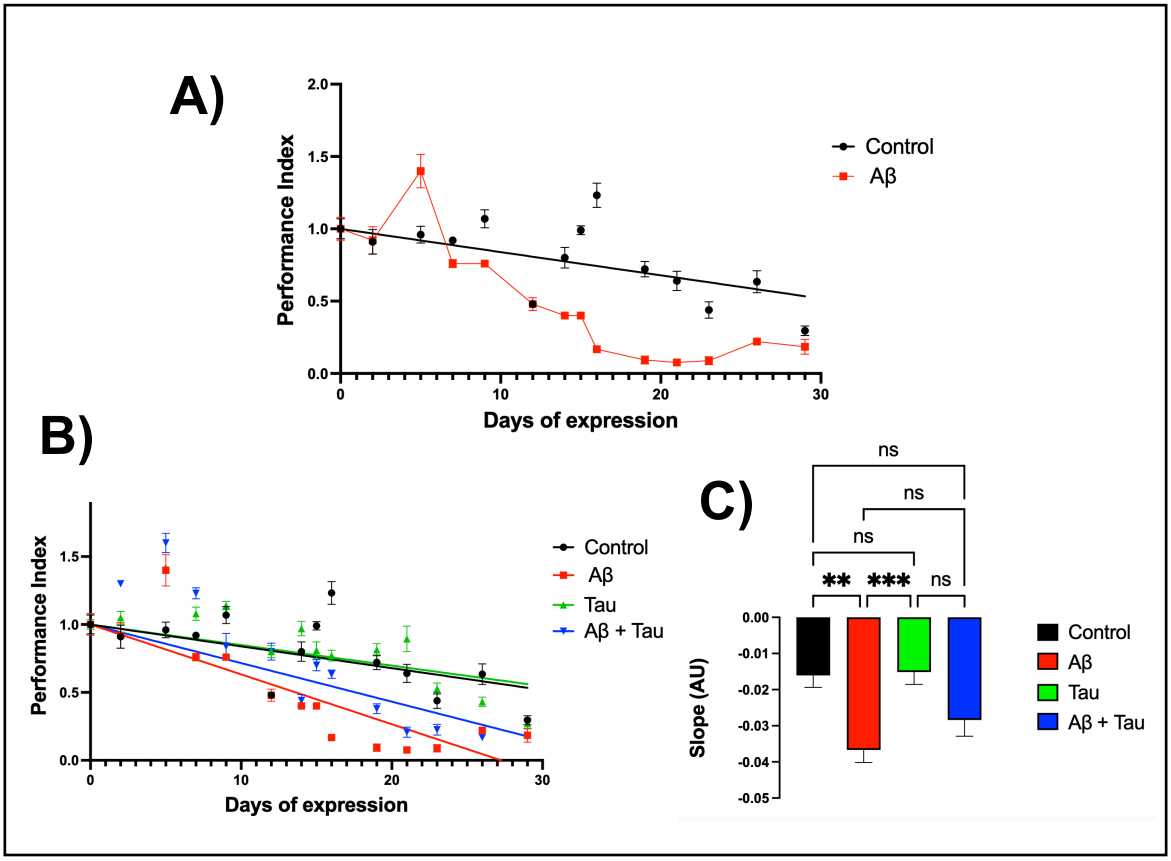


Figure 3.3 Measuring Aβ and Tau induced neurodegeneration using the DART system.

A) Performance index of the flies in response to the 5 vibration stimulations. Each point represents the mean amplitudes (with S.E.M) of the 20 flies for each of the 5 stimulations on one day. Aβ expression produces a decline which fits a sigmoidal shape**.** Control flies decline in a linear manner. **B) Performance index of the flies in response to the 5 vibration stimulations.** Each point represents the mean amplitudes (with S.E.M) of the 20 flies for each of the 5 stimulations on one day. Linear regression lines were fitted to the performance index. r^2 values; Aβ = 0.6772, Control = 0.3375, Tau = 0.5661, Aβ + Tau = 0.6709. **C) Bar chart showing the gradients of the lines (and S.E.M), representing the rate in decline in response to the stimulations**. The mean amplitudes were plotted using linear regression and their gradients were compared using a One-way ANOVA with Tukey's multiple comparison test. Aβ expression significantly enhances the age- dependent motility decline seen in the control (**p=0.0014, n=14). Tau expression did not produce a significant change compared to the control nor did Tau expression enhance Aβ induced toxicity. Aβ was significantly more toxic in this system than Tau (***p=0.0008, n=14). n = the number of days of recordings.

3.2.2 *p53* knockdown is neuroprotective against Aβ-induced neurodegeneration

When the DART system was being optimised, a *p53* knockdown line was used, which proved to elicit a neuroprotective effect in our amyloid toxicity model (Taylor and Tuxworth, 2019). This result was directly relevant to the question I was asking here, but this needed confirmation. I attempted to repeat this finding using a different RNAi line to knockdown *p53* expression (TRiPHMS00286). As before, the driver Elav-Gal4 (Gal80^{ts}) was used to express Aβ in post-mitotic adults using a temperature shift to 27 °C. As seen before, flies expressing Aβ show a significantly higher rate in the decline of motor performance compared to the control fly (Figure 3.4A and B, ****p<0.0001). *p53* knockdown significantly reduced the rate in decline in motor performance induced by Aβ toxicity (Figure 3.4A and B, *p=0.423). There was a difference in performance between the control flies and those expressing the *p53* RNAi, with the RNAi flies performing worse than the control flies, suggesting that p53 could have a protective effect when on its own and not in pathology (Figure 3.4A and B, **p= 0.0100). In conclusion, my results suggest that p53 could be involved in propogating the toxicity involved with Aβ.

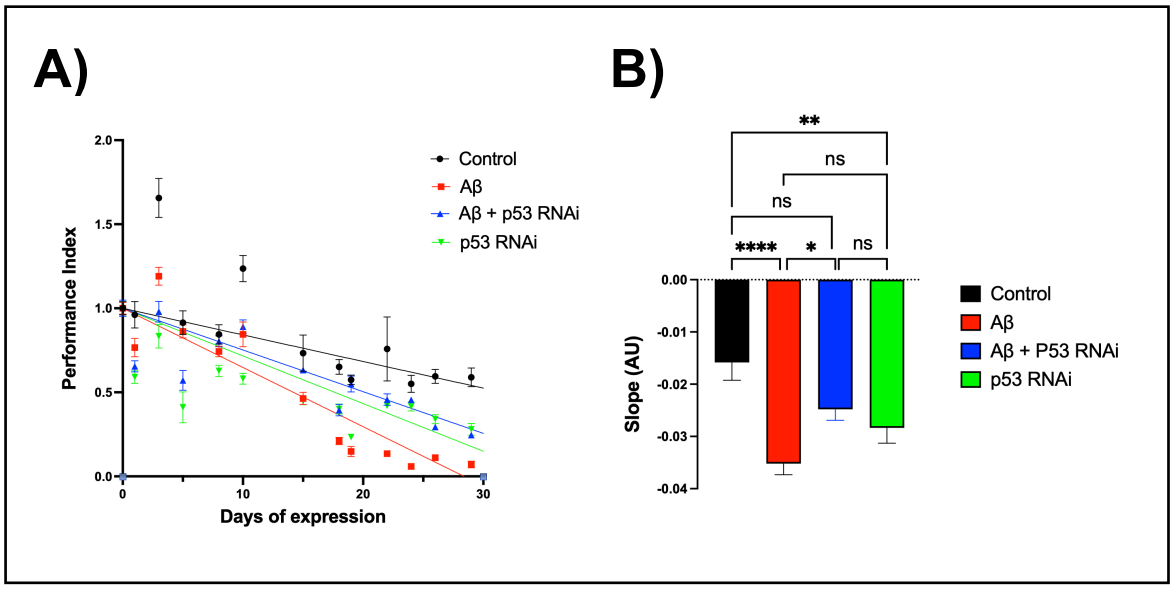


Figure 3.4 *p53* knockdown slows the decline in motility induced by Aβ in *Drosophila*.
A) Performance index of the flies in response to the 5 vibration stimulations. Each point represents the mean amplitudes (with S.E.M) of the 20 flies for each of the 5 stimulations on one day. Linear regression lines were fitted to the performance index. r^2 values; Control = 0.4425, Aβ = 0.8580, Aβ + *p53* RNAi = 0.6722, *p53* RNAi = 0.6191.
B) Bar chart showing the gradients of the lines (and S.E.M) and representing the rate in decline in response to the stimulations. The mean amplitudes were plotted using linear regression their gradients were compared using a one way-ANOVA with Tukey's multiple comparisons test. *p53* knockdown significantly rescues the decline in motility induced by Aβ (*p=0.0423, n=14). n = the number of days of recordings.

3.3 Discussion

3.3.1 Modelling Aβ and Tau toxicity using DART

Expression of Aβ and Tau in the *Drosophila* nervous system results in age-dependent locomotor defects, premature death and neurodegeneration which are normally assessed by vacuolation in the brain or loss of photoreceptors in the eye (Funez, Mena and Limas, 2015). The most common way to measure the age-dependent motility decline seen in *Drosophila* is using a climbing assay, which relies on the negative geotaxis response seen when *Drosophila* are knocked to the bottom of a vial (Gargano *et al.*, 2005). Disadvantages of this method include the large number of flies needed, the binary outcome scored (climb *vs*. not climb) and the degree of high variability, which reduces the sensitivity to detect differences in behaviour. The DART software is an alternative way to measure the startle response in *Drosophila* (Taylor and Tuxworth, 2019). Previously in the Tuxworth lab, the DART sleep tracking software was re-purposed as an alternative to the climbing assay to measure the startle response in *Drosophila* as a continuous variable. This method is more sensitive to differences in behaviour than the climbing assay (Taylor and Tuxworth, 2019). I used the DART system to first, establish a neurodegenerative phenotype induced by Aβ in *Drosophila,* and then to investigate if this could be rescued using an RNAi to p53.

Expression of Aβ in adult neurons induced a significant motility decline compared to the control fly (Figure 3.3B and C, **p=0.0014) (Taylor and Tuxworth, 2019). The control flies do also exhibit a normal, linear, age-dependent decline in motility, but the rate of decline is enhanced by the toxic effects of Gal4 at higher incubation temperatures (Kramer and Staveley, 2003; Taylor and Tuxworth, 2019). When the DART system was being optimised, it became clear that increasing Gal4 activity by rearing the flies at higher temperature had significant impact on their motility (Taylor and Tuxworth, 2019). Therefore, I chose a paradigm where flies were developed at 18 °C to prevent any expression of Aβ in the immature nervous system then switching the flies to 27 °C as adults. This was a compromise to induce sufficient toxicity from Aβ expression without the inherent Gal4 toxicity confounding results. A

second advantage of the reduced temperature was that the toxicity of the Aβ was reduced: I considered it would be unlikely that extreme toxicity could be rescued by knockdown of ATM-Chk2 signalling.

When modelling Tau toxicity in *Drosophila,* the expression of wildtype Tau or mutant Tau (V337M or R406W) are commonly used. These mutations were identified in FTDP-17 patients and are more toxic in the *Drosophila* nervous system than wildtype Tau, resulting in a much shortened lifespan and more severe neurodegeneration (Wittmann *et al.*, 2001; Jackson *et al.*, 2002; Khurana *et al.*, 2006; Prüßing, Voigt and Schulz, 2013). Here I show that Tau R406W does not induce any detectable motility defects when flies are temperature shifted to 27 °C as adults, with no significant difference in the performance of Tau expressing flies and the control flies (Figure 3.2B and C, not significant). Previous studies using climbing assays to measure Tau induced neurodegeneration show mild phenotypes. In one study Tau R406W expression, using a high expression version of the pan-neuronal driver Elav-Gal4, produced a significant decline in motility only at day 35 when compared to the control. However, when Tau R406W was expressed at lower levels using the standard Elav-Gal4 driver, no significant difference was seen at any time point (Beharry, Alaniz and Alonso, 2013), and also as shown here using the DART system. It is possible if I had aged the flies for longer, I might have been able to detect a significant decline induced by Tau. Another study showed that overexpression of wildtype Tau using the Elav-Gal4 driver showed a general reduced negative geotaxis response using the climbing assay, but the time to decline was not significantly different to the control (Kerr *et al.*, 2011). It is hard to compare my results here to these previous studies as they overexpress Tau throughout the development of the nervous system from embryo, through the larval stages and through the remodelling to the adult nervous system that occurs in metamorphosis. In contrast, I restricted expression to adult mature neurons using the temperature sensitive Gal80 repressor specially to avoid potential issues with the developing nervous system. For instance, over expressing Tau in the CNS throughout development is known to cause abnormal phenotypes, including locomotor defects, synaptic dysfunction and disrupted axon transport (Mudher *et al.*, 2004; Chee *et al.*, 2005; Folwell *et al.*, 2010a). More importantly, as discussed above**,** in future experiments I was knocking

down DDR gene expression (p53) using the same Gal4 system used to overexpress Tau (and Aβ). Had I driven expression of Tau (or Aβ) through development p53 would also have been targeted in the developing nervous system.
Additionally, as I have explained later in discussion 7.0, several inherited neurodevelopmental disorders are associated with loss of DDR components, so we decided to restrict expression to adulthood. It is possible that Tau expression is less toxic in adult mature neurons in comparison to neuroblast progenitors or immature neurons. One possibility is that neurons depend more on Tau for specific axon transport roles during the development of the CNS when neurons are establishing their polarised morphology and elaborating and refining neurites. It is also possible that Tau expression in my system does in fact produce mild neurodegeneration which might have been detectable as vacuolation of the brain or resulted in shortened lifespan.

There is a magnitude of evidence to suggest Tau lies downstream of Aβ and enhances Aβ induced neurodegeneration, creating the 'trigger and bullet' model (see section 1.3.4) (Bloom, 2014). Tau is essential for Aβ induced toxicity *in vitro* (Rapoport *et al.*, 2002) and for memory and learning impairments and even cell death in APP transgenic mouse models (Roberson *et al.*, 2007; Leroy *et al.*, 2012). Although the main hypothesis is that Tau acts downstream of Aβ, Aβ can accelerate the formation of NFT's and enhances Tau pathology in mouse models (Götz *et al.*, 2001; Ferrari *et al.*, 2003; Oddo *et al.*, 2003; Terwel *et al.*, 2008; Hurtado *et al.*, 2010) Therefore, I expected to see a synergistic phenotype when both Aβ and Tau were expressed together, enhancing the neurodegeneration observed when Aβ or Tau were expressed alone. Here in this system the addition of Tau with Aβ did not significantly enhance any locomotor defects when compared to Aβ alone (Figure 3.3B and C, $p = 0.3977$). Co-expression of Tau and Aβ did induce a steeper decline in motility compared to Tau alone, however this was not significant (Figure 3.3B and C, $p = 0.0666$). Previous reports in *Drosophila* showing the synergistic interaction of Tau and Aβ have expressed the more toxic Arctic mutation of Aβ under the expression of the very strong GMR-Gal4 driver (Sun *et al.*, 2022). The GMR-Gal4 driver alone causes apoptosis in the developing wing disc and sensitises the

photoreceptors to toxic insults (Kramer and Staveley, 2003). It is, therefore, not a particularly good model of human Tauopathy. The standard Elav-Gal4 driver has also been used to drive co-expression of Aβ and Tau and, in this case, Aβ does enhance Tau-induced phenotypes including axonal transport and locomotor defects. However as with other studies, expression was driven throughout development with the complications that approach brings, as discussed (Folwell *et al.*, 2010b). The synergistic interaction of Tau and Aβ has been consistently observed in mouse models (Lewis *et al.*, 2001; Ribé *et al.*, 2005; Bolmont *et al.*, 2007; Clinton *et al.*, 2010; Chabrier *et al.*, 2014; Pooler *et al.*, 2015; Jackson *et al.*, 2016)**.** These mouse models all use expression of a mutant APP, with the majority using the Swedish APP mutation which is known to significantly enhance Aβ production.

It is possible that Tau is not toxic at 27 °C using the Elav-Gal4 Gal80^{ts} system because Tau is not being expressed at high enough levels to induce toxicity. Potentially using a different, stronger driver or rearing the flies at a higher temperature would have enhanced toxicity. However, as previously discussed, the problem with rearing flies at higher temperatures is that Gal4 shows toxic phenotypes at higher temperatures, as observed here when optimising the DART system and previously (Kramer and Staveley, 2003). In fact, the movement of the control flies (Elav-Gal4 x w^{1118}) flies declines more rapidly at 29 °C that those expressing Aβ at 27 °C (Taylor and Tuxworth, 2019). Later experiments during my PhD revealed that Elav-Gal4 Gal80^{ts} system is likely to be only partially de-repressed at 27 °C resulting in stochastic expression in adult neurons in the brain (Figure 4.6). Therefore, it is a reasonable conclusion to reach that Tau may not have been expressed at high enough levels to induce toxicity in my experiments.

As an alternative system to the Gal80^{ts} repressor for spatial and temporal control of expression, I could have used include the drug-inducible drivers Gene-Switch Gal4 system or the newer Q system, which is independent of Gal4.(Osterwalder *et al.*, 2001; Potter *et al.*, 2010). Both systems remove the need for a temperature shift. The Gene-Switch system has been successfully used to model amyloid toxicity in *Drosophila* including locomotor dysfunction and survival when expressed under the

elav driver (Finelli *et al.*, 2004; Sofola *et al.*, 2010; Burnouf *et al.*, 2015, 2016) but limited studies have used the Gene-Switch system to model Tau toxicity. In the Tuxworth lab now we are using the Elav-GeneSwitch system for motility tracking experiments. Due to the time lost with the COVID-19 lockdown I did not have time to optimise this system and was reliant on using ElavGal4; $Gal80^{ts}$ which had already been optimised for our conditions and calibrated for levels of toxicity to the flies (Taylor and Tuxworth, 2019). Disadvantages of the GeneSwitch system are that it can be leaky and drive expression in the absence of mifepristone and there is also a practical issue: mifepristone is considered potentially dangerous for female researchers (Poirier *et al.*, 2008; Scialo *et al.*, 2016).

The Q system is based on the same bi-partite principals as Gal4-UAS but uses an alternative DNA binding and transactivating protein (QF) with its own binding sequence in DNA. A third component (QS) is a repressor and including this allows expression to be repressed until quinic acid is fed to the flies in the food. This deactivates QS and allows transgene expression (Potter *et al.*, 2010). The original Q system showed toxicity when broadly expressed, but since then less toxic QF2 drivers have been developed. Using an alternative driver may have expressed Tau at higher levels and therefore I may have potentially observed a significant decline in motility.

3.3.2 p53 signalling is implicated in Aβ- induced neurodegeneration

Under normal physiological conditions p53 controls gene expression to regulate cell cycle events, DNA repair, senescence, or apoptosis to protect the genome (Feroz and Sheikh, 2020). In neurodegenerative diseases, p53 levels and activity are elevated, however it is unknown whether elevated levels of p53 in pathology is a cause or consequence of the disease (De La Monte, Sohn and Wands, 1997; Chang *et al.*, 2012; Szybinska and Lesniak, 2017).

Here, I show that knocking down *p53* is neuroprotective in our amyloid toxicity model in *Drosophila* (Figure 3.4A and B, *p=0.0423). This result was initially discovered as

part of a screen for suppressors during establishment of the DART startle response technique (Taylor and Tuxworth, 2019), but was not followed up as the message for that paper was that suppression of toxicity was detectable using the DART system, not what the suppressor was. It was important that I confirmed this result with a different RNAi as different lines can have different levels of knockdown, plus an off-target effect of the RNAi could be responsible for the phenotype. As we have now shown that two different p53 RNAi knockdown lines are neuroprotective in our amyloid model, it is probable that a mechanism downstream of p53 signalling is responsible for at least some of the neurotoxic effects seen when the ATM/Chk2 pathway is activated chronically in AD.

Although unconfirmed here, p53 levels and activity could be elevated in the amyloid fly due to the accumulation of DNA damage or due to Aβ directly activating p53 or stabilising it via activation of kinases (Paradis *et al.*, 1996; Zhang *et al.*, 2002; Ohyagi *et al.*, 2005; Lapresa *et al.*, 2019). Although p53 knockdown is neuroprotective, we have not definitively confirmed that p53 is a target of Chk2 in our amyloid expressing fly. However, it is known that Chk2 phosphorylation of p53 is also conserved in *Drosophila*, suggesting p53 is likely to be a target of Chk2 here in the amyloid expressing fly (Peters *et al.*, 2002; Brodsky *et al.*, 2004; Tuxworth *et al.*, 2019). I did attempt to analyse p53 levels in the amyloid expressing fly using a western blot, however I detected no signal. If I had had more time I would have tried different blocking methods and antibody concentrations and optimised *Drosophila* protein extraction.

Despite evidence of p53 elevation in AD, it is reported that p53 in AD is conformationally different and aggregates to form oligomers (Uberti *et al.*, 2002; Lanni *et al.*, 2008; Farmer *et al.*, 2020). Aggregated p53 results in mis-localisation outside of the nucleus (Moll *et al.*, 1995, 1996; Wolff *et al.*, 2001, 2016; Lasagna-Reeves *et al.*, 2013; Farmer *et al.*, 2020), a common phenomenon observed in disease including cancer (Ano Bom *et al.*, 2012; Wiech *et al.*, 2012; Lasagna-Reeves *et al.*, 2013). p53 is a nuclear protein so mis-localisation results in dysfunction and impaired p53 signalling in response to DNA damage (Uberti *et al.*, 2002, 2006;

Farmer *et al.*, 2020). p53 localisation in the amyloid fly brain could have been investigated here using an antibody against the p53 protein by immunofluorescence.

Our results here agree with previous studies that p53 signalling mediates toxicity in neurodegenerative diseases, including a mouse models of ALS and an *in vitro* system with neuronal exposure to Aβ 1-42 (Culmsee *et al.*, 2001;Maor-Nof *et al.*, 2021). In contrast, one study has shown that *p53* knockdown enhanced neurodegeneration in a *Drosophila* tauopathy model, via alterations in synaptic gene transcription, suggesting p53 plays a protective role in neurodegeneration (Merlo *et al.*, 2014). Potential explanations for the differences in our results could be because Merlo *et al* knocked p53 down throughout development, in contrast to my system where *p53* is knocked down in adults only. In *Drosophila*, loss of p53 produces fertile adults, with no abnormal phenotypes in the adult, suggesting p53 is not needed for development (Sogame, Kim and Abrams, 2003; Xie and Golic, 2004). However more recently this was found to not be entirely true as loss of p53 function elicited defects in larval growth and developmental timing but these defects were not present in the adult fly (Contreras, Sierralta and Glavic, 2018). Here in my experiment, I knocked down p53 in adult neurons as I wanted to prevent any confounding phenotypes from p53 knockdown in the developing nervous system. Other explanations could be due to different models of neurodegeneration or because they used a different RNAi line to the one I used and different RNAi lines elicit different knockdown levels. This is where qPCR could have been used to quantify the extent of knockdown in our lines.

3.4 Conclusions

My results here suggest that activation of the ATM/Chk2 pathway could elicit neurotoxicity in our amyloid *Drosophila* model via p53 signalling. The Tuxworth lab, including my results here, have shown that *p53* knockdown with two different RNAi lines produces a neuroprotective effect.

4.0 Potential targets downstream of p53 - apoptosis

4.1. Introduction

Knocking down *p53* in our amyloid toxicity model is neuroprotective (Figure 3.4A and B). This indicates a target or mechanism downstream of p53 signalling contributes to the deleterious effect seen when the ATM/Chk2 pathway is activated (Taylor *et al.*, 2022). p53 activates target genes that regulate many events in response to DNA damage including cell cycle arrest, DNA repair and apoptosis (Feroz and Sheikh, 2020). The neuroprotective effect seen when knocking down *p53* could therefore be due to any of, or a combination of these events. I decided to investigate if inhibiting apoptosis would replicate the neuroprotective effect seen with *p53* knockdown. If so, this would indicate apoptosis is occurring in the amyloid model and could be contributing to the deleterious effect ATM-Chk2-p53 activation has on neural survival and function (Taylor *et al.*, 2022).

4.2 Problems with detecting apoptosis in Alzheimer's Disease

The mechanism of cell death in neurodegenerative diseases remains controversial. There are a number of problem with attempting to detect apoptosis in a slow degenerative disease such as AD. Definitive detection of apoptosis is complex since well-known assays detect features that are not specific to apoptosis (Behl, 2000). One widely used method of detecting apoptosis is by observing DNA fragmentation using TUNEL staining. TUNEL staining detects DNA breaks generated in apoptosis. However, these breaks are also associated with necrosis (Grasl-Kraupp *et al.*, 1995; Nishiyama *et al.*, 1996). An alternative method to detect DNA fragmentation is using gel electrophoresis to visualise DNA laddering but this is only useful when large

amounts of cells are undergoing apoptosis simultaneously, which is an issue during a slow progressive disease such as AD where only a small subset of neurons are dying at any one time. It is recommended that two or more apoptotic assays are used to detect apoptosis, including visualising the characteristic hallmarks, which captures more late-stage apoptosis. This is also a problem in AD since apoptosis is a quick process and so apoptotic features are likely to be missed (Elmore, 2007). Another problem with trying to observe apoptosis, is that it can be followed by secondary necrosis if apoptotic bodies aren't efficiently removed by phagocytes (Silva, 2010). Due to the issues with visualising apoptosis, the prospect of apoptosis as a mechanism of cell death in AD remains a controversial topic.

4.3 Results

4.3.1 Inhibiting caspase activity is neuroprotective against Aβ-induced neurodegeneration

Initially, I chose to block apoptosis in neurons of the fly by overexpressing the baculovirus caspase inhibitor p35 alongside Aβ (Jabbour *et al.*, 2002). If this could replicate the neuroprotective effect of ATM, Chk2 or p53 knockdown, it would argue that apoptosis is a component of the toxicity. In *Drosophila,* p35 inhibits the activity of the major effector caspases DrICE (Death related ICE-like caspase) and Dcp-1 (Death caspase 1) activity but not the main initiator caspase Dronc (*Drosophila* Nedd2-like caspase). Expressing p35 eliminates most, if not all, cell death (Hay, Wolff and Rubin, 1994; Hawkins *et al.*, 2000; Meier *et al.*, 2000; Lannan, Vandergaast and Friesen, 2007).

As seen previously, flies expressing Aβ significantly enhance the rate in decline of motor performance compared to the control fly, seen as a sigmoidal decline (Figure 4.1A and B, *p=0.0462). As before, linear regression lines were used to compare the rate of decline between the genotypes. Interestingly, the slight increase in motor activity is observed again at the start between day 0 and day 5 when Aβ is expressed before the decline occurs, as previously seen before (compare Figure 3.1 with Figure

4.1). This could again be due to slow increases in Aβ expression which has been shown to increase synaptic vesicle release and increase neuronal activity, in *vitro* (Puzzo *et al.*, 2008; Abramov *et al.*, 2009). Overexpression of the caspase inhibitor p35 with Aβ significantly reduces the decline in motor performance induced by Aβ toxicity (Figure 4.1A and B, ***p=0.0008). There was no difference in performance between the control and those expressing UAS-p35 alone, which suggests inhibiting or reducing apoptosis does not have a general protective effect against age related motor decline and is specifically neuroprotective to the Aβ induced toxicity (Figure 4.1A and B, p=0.3514, not significant).

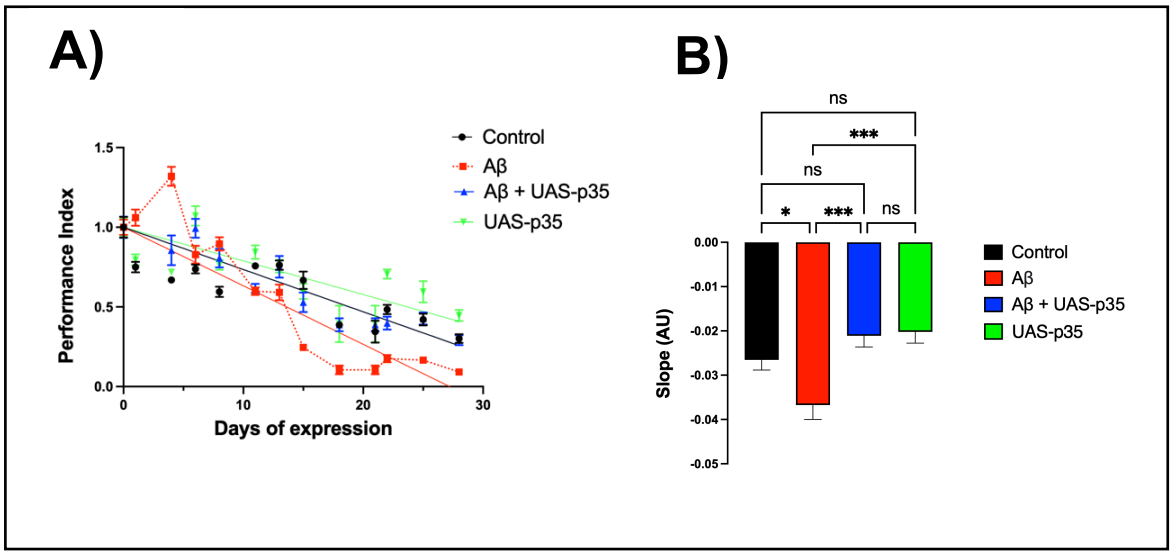


Figure 4.1. Overexpressing the caspase inhibitor p35 slows the decline in motility induced by Aβ in *Drosophila*.
A) Performance index of the flies in response to the 5 vibration stimulations. Each point represents the mean amplitudes (with S.E.M) of the 20 flies for each of the 5 stimulations on one day. Linear regression lines were fitted to the performance index. r^2 values; Control = 0.6406, Aβ = 0.8096, Aβ + UAS-p35 = 0.6138, UAS-p35 = 0.4538. Aβ sigmoidal curve is added to emphasise the rapid decline in Aβ. **B) Bar chart showing the gradients of the lines (and S.E.M) and representing the rate in decline in response to the stimulations**. The mean amplitudes were plotted using linear regression and their gradients were compared using a one way-ANOVA with Tukey's multiple comparisons test. Aβ accelerates the age-dependent motility decline observed in the control (*p=0.0462). Overexpression of p35 with Aβ significantly rescues the decline in motility induced by Aβ (***p=0.0008, n=13).

4.3.2 Reducing apoptosis does not correlate with a rescue of Kenyon cell loss

Given that I had shown that overexpressing the caspase inhibitor p35 is neuroprotective in the amyloid fly model, I next wanted to show that apoptosis was indeed occurring in the brain of the Aβ expressing flies and that it was reduced upon p35 overexpression. Previous members of the group had tried various methods to observe apoptosis, including staining with anti-Dcp1, TUNEL and acridine orange staining: all had failed to detect apoptosis. We considered that one problem may be detecting infrequent events in an entire brain. One alternative might be to express Aβ in only a small subset of neurons in a defined, stereotyped part of the brain. In this way, I would be able to focus attention on these neurons to detect apoptosis.

With this aim, I created a recombinant fly expressing the mifepristone inducible MB-GeneSwitch combined with a UAS-mCherryNLS signal. MB-GeneSwitch drives expression in the Kenyon cells of the mushroom bodies, which regulate learning and memory and therefore a disease-relevant population of neurons (Figure 4.2). They are localised in a small, defined area of the brain, which is relatively easy to image by confocal microscopy, allowing consistent counting in the same region of the brain each time. Including UAS-mCherryNLS signal would mark the neurons with a bright, nuclear-localised signal, allowing me to image and count the Kenyon cell nuclei. Importantly, the MB-GeneSwitch driver is inducible with the steroid mifepristone (Figure 4.3), allowing normal development to occur without expression of Aβ, as before.

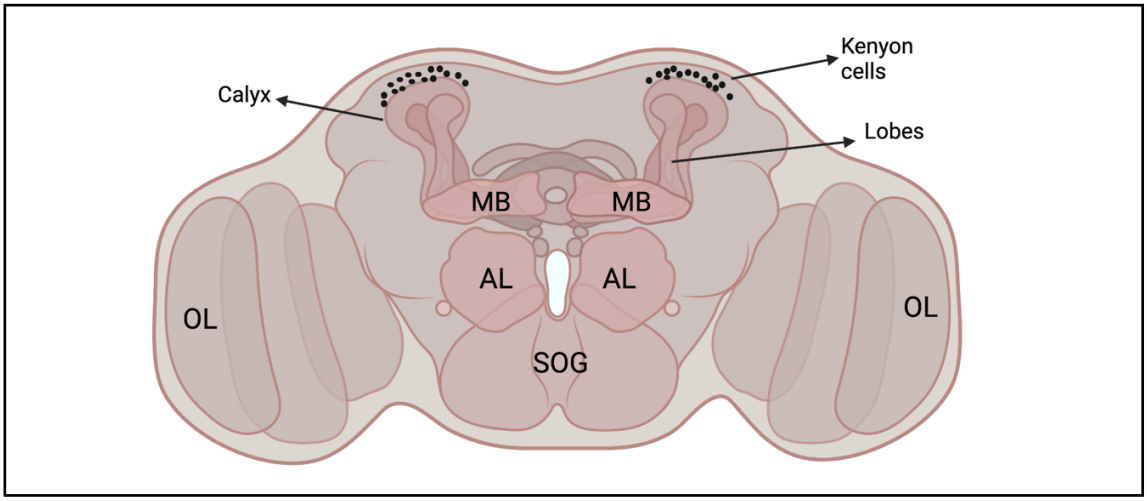


Figure 4.2 Simplified structure of the *Drosophila* brain showing location of Kenyon cells, the intrinsic neurons of the mushroom bodies.
The mushroom bodies (MB) consist of clusters of intrinsic neurons called Kenyon cells. Kenyon cells extend their dendrites to form the calyx and their axons project through a tract known as the peduncle, which branch off into different lobes. MB = mushroom body, AL = anterior lobe, SOG = subesophageal ganglion, OL = optic lobe.

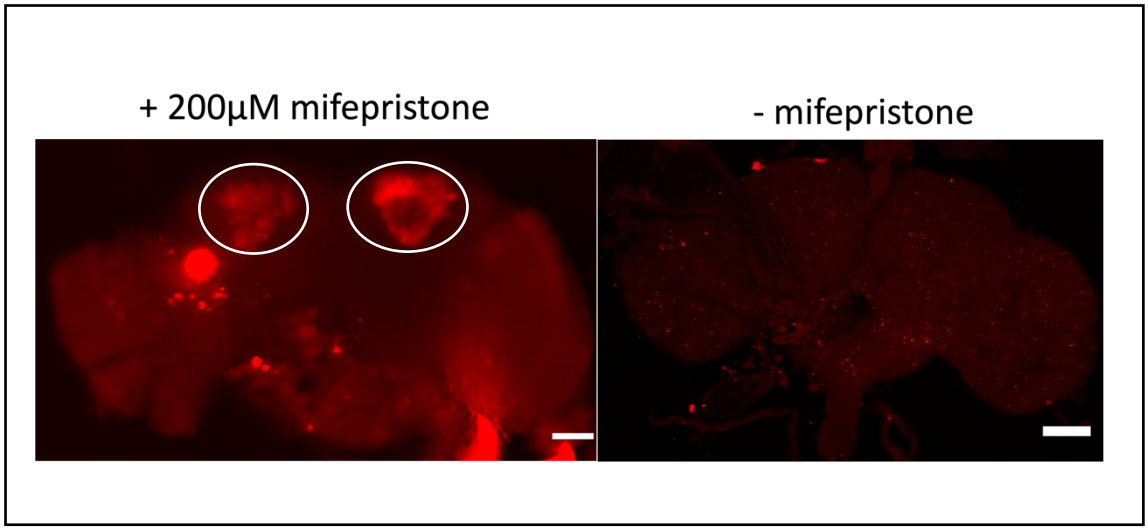


Figure 4.3 The inducible mushroom body driver MB-GeneSwitch driving expression of a UAS-mCherryNLS signal in the Kenyon cells.
A recombinant fly expressing the mifepristone inducible MB-GeneSwitch driver combined with a UAS-mCherryNLS signal was fed on 200 µm of mifepristone (25 °C) for 72 hours to induce expression. MB-GeneSwitch drives expression in the Kenyon cells of the mushroom bodies (white circle). The driver is not leaky, with no expression in the absence of mifepristone. Scale bar = 50 µm

The recombinant MB-GeneSwitch, UAS-mCherryNLS fly was crossed to Aβ, Aβ + UAS-p35, UAS-p35 and w^{1118} for the control, and the female progeny were aged at 25 °C on 200 μm mifepristone food to induce expression (Figure 4.4A). Adult brains were dissected on day 3, day 14 and day 30 of expression and the number of Kenyon cells per mushroom body of each brain hemisphere was counted using the Trackmate plugin in ImageJ (see materials and methods for full details). The average number of Kenyon cells for each genotype on each day was plotted and analysed using linear regression so that the rate in decline of neurons could be quantified (Figure 4.4B). There appeared to be no visual change in the number of Kenyon cells present between day 3 and day 30 in all genotypes and the rate in decline of the Kenyon cells was not significantly different between any of the genotypes (Figure 4.4B and C, not significant). These results suggest that Aβ does not accelerate neuronal loss in the mushroom bodies and that the rescue seen with p35 overexpression is not due to a rescue in cell death, at least in the mushroom bodies.

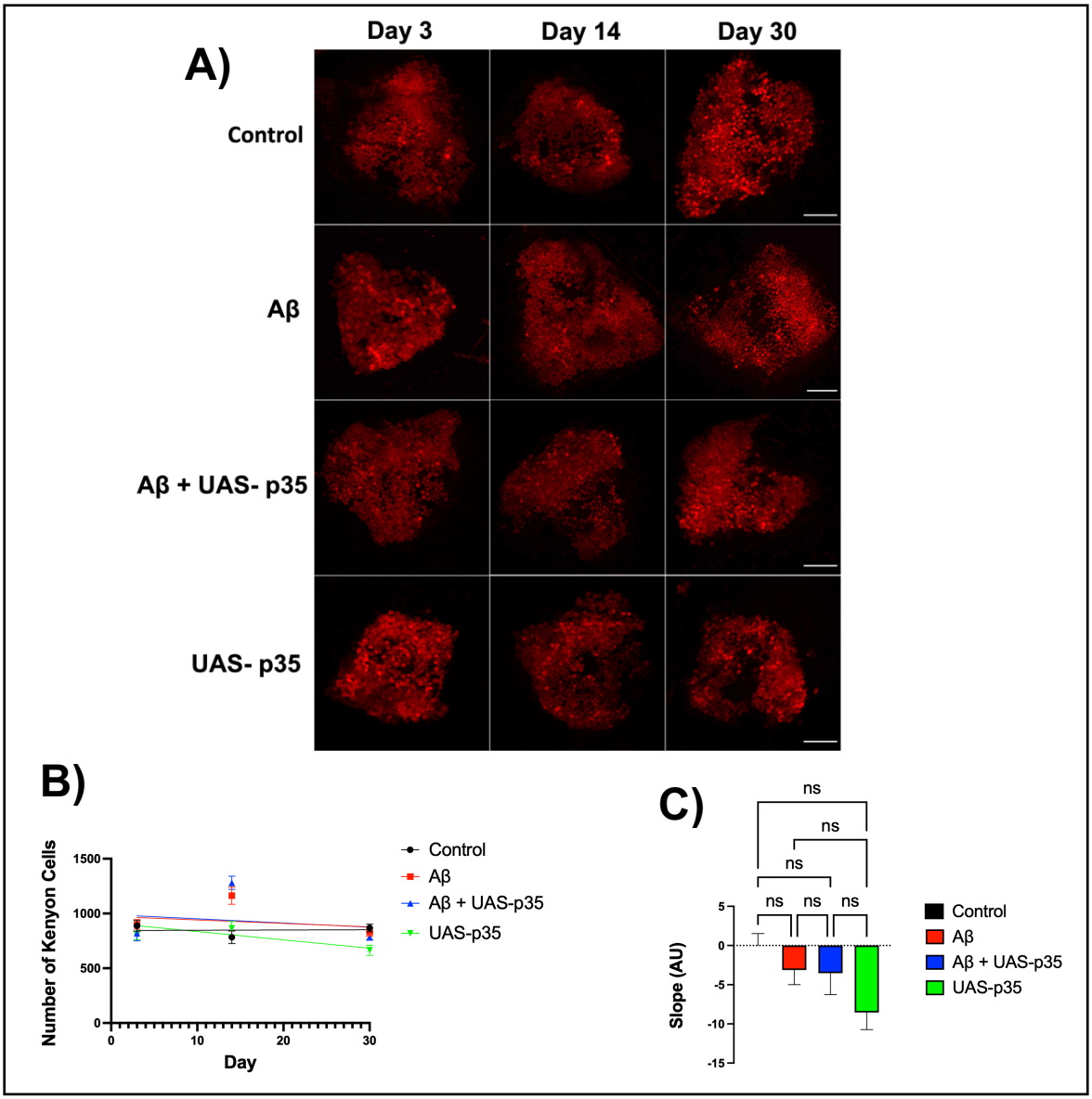


Figure 4.4 Aβ does not induce significant neuronal loss of Kenyon cells at 25 °C. A) Representative immunofluorescence images of the number of Kenyon cells in a mushroom body cluster in one hemisphere of the adult brain. Female recombinant flies expressing the inducible MB-Gene switch driver combined with a UAS-mCherryNLS signal was fed on 200 µm mifepristone food (25 °C) and dissected on days 3,14 and 30. Scale bar = 50 µm. **B) Linear regression lines showing average number of Kenyon cells (and S.E.M) per mushroom body hemisphere.** Average number of Kenyon cells were counted using Trackmate in ImageJ. **Day 3**; control n=9, Aβ n=11, Aβ + UAS-p35 n=9, UAS-p35 n=4. **Day 14**; control n=10, Aβ n=5, Aβ + UAS-p35 n=7, UAS-p35 n=10. **Day 30**; control n=16, Aβ n=10, Aβ + UAS-p35 n=11, UAS-p35 n=13. n = number of mushroom body hemispheres. **C) Bar chart showing the gradients (and S.E.M) of each slope representing the rate in decline of Kenyon cells over time.** There is no significant difference between the rate of decline of Kenyon cells between all genotypes (One-way ANOVA with Tukey's multiple comparisons test).

4.3.3 Investigating apoptosis using the GC3ai reporter

Overexpression of the caspase inhibitor p35 did not correlate with any evidence of rescuing neuronal loss in the mushroom bodies (Figure 4.4C, not significant). As the mushroom bodies is only a small area of the brain I decided to investigate if there was any evidence of apoptosis occurring in the amyloid fly in the whole brain using a different method.

Despite there being no large-scale loss of neurons in the mushroom bodies, it was possible that small numbers of apoptotic events were occurring. Given the failure of the Tuxworth group to detect apoptosis with more traditional reporters (see above), I decided to employ a newer, more sensitive genetically-encoded reporter of effector caspase activity that detect both early and late activity – the GC3ai reporter (Schott *et al.*, 2017). In *Drosophila* the effector caspases Dcp1 and DrICE cleave amino acid sequences 'DEVD' in target proteins. The GC3ai reporter contains a DEVD sequence that needs to be cleaved for the reporter to fluoresce GFP. Therefore, when effector caspase activity occurs the DEVD sequence is cleaved and the GC3ai reporter fluoresces.

First, to confirm that the GFP sensor was working correctly, I expressed the UAS-GC3ai reporter in the eye disc using the strong GMR-Gal4 driver (Figure 4.5). Gal4 at high doses is toxic and induces some apoptosis, as demonstrated previously in the eye disc (Kramer and Staveley, 2003), and which should be detectable using this reporter. Third instar larvae eye discs were dissected and stained for anti-GFP which labels both active and inactive conformations of the GC3ai reporter. The signal from the endogenous fluorescence of folded GFP, which represents the active GC3ai reporter, can be detected using the 488 nm laser without any antibody. Fluorescent endogenous GFP was seen in a small number of cells within the eye disc at a higher rearing temperature (27 °C) but not compared at 18 °C, consistent with apoptosis occurring in the eye disc (Figure 4.5) (Schott *et al.*, 2017). The GMR domain is labelled red by the detection of GC3ai expression with anti-GFP, which recognises

both inactive and active conformations of the sensor. This positive control indicated that the tool is working correctly as reported.

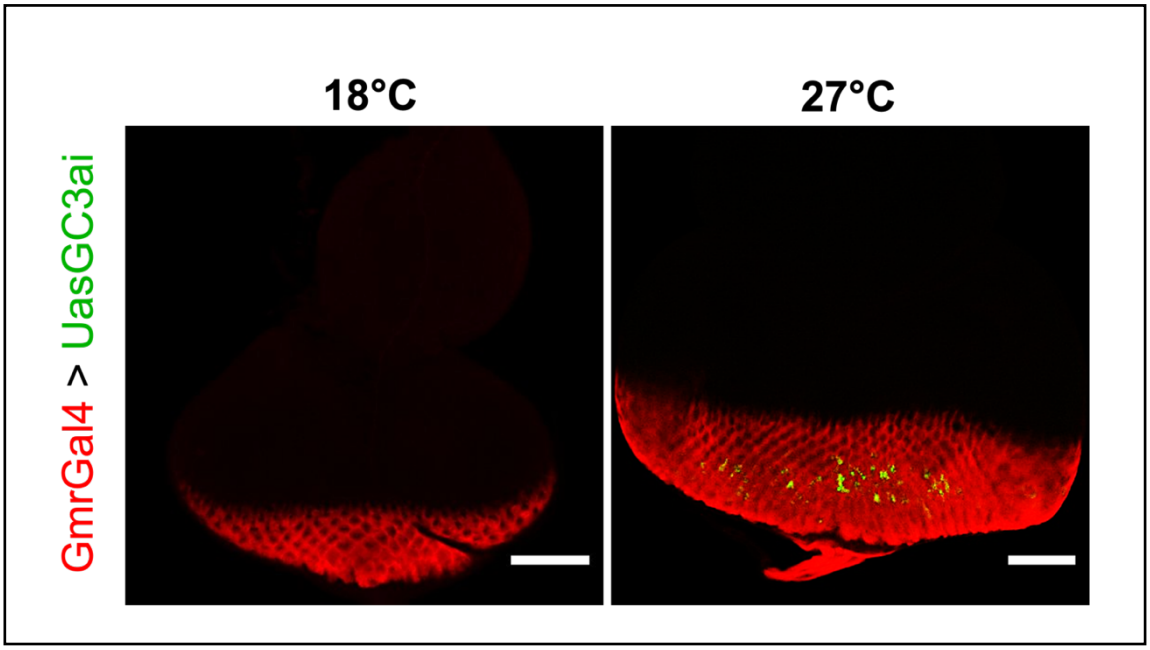


Figure 4.5 The GFP sensor GC3Ai reporting apoptosis induced by Gal4 in the *Drosophila* eye disc.
The GFP sensor GC3ai was expressed in the eye using the GMR-Gal4 driver. Third instar larvae were dissected and stained for anti-GFP (red) which recognises both active and inactive forms of GC3ai. The GMR domain is labelled red by detection of GC3ai expression with anti-GFP, and endogenous GC3ai fluorescence is shown by the green. Apoptosis is induced at higher temperatures (27 °C) compared to no observed apoptosis occurring at 18 °C in the eye disc. Scale bar = 50 μm.

4.3.4 Aβ does not increase apoptosis in the amyloid fly

After confirming the GC3ai reporter was working as reported, I next wanted to use this tool to investigate if there was any evidence of apoptosis occurring in the amyloid fly and if this was dependent on the ATM-Chk2-p53 pathway.

The transgenes and the GC3ai reporter were expressed in adult neurons using the Elav-Gal4 ($Gal80^{ts}$) driver at 27 °C for 14 days (D1- 1 day after eclosion), and adult brains were dissected and stained for anti-GFP (red) (Figure 4.6). As anti-GFP labels both active and inactive conformations of the GC3ai reporter, I expected every neuron in the brain to be GC3ai positive (red), as Elav-Gal4 drives expression in all neurons. However, upon analysis, I observed a stochastic expression of the GC3ai reporter (red staining) (Figure 4.6, yellow arrow). Detection of the endogenous GFP signal (Figure 4.6, blue arrows) revealed visual evidence of apoptosis at day 14 in the control fly (ElavGal4; $Gal80^{ts}$ + UAS-GC3ai). Green autofluorescence was also detected in the fly brain (Figure 4.6, white circle).

The GC3ai reporter was expressed alongside Aβ and aged for 14 days and analysed in the same way. The levels of apoptosis, indicated by the number of GFP dots present in the mid brain were not elevated in the Aβ expressing fly, suggesting that Aβ does not induce apoptosis in this model (Figure 4.7A & B). Knocking down *ATM, Chk2* or *p53* made no difference to the apoptosis observed, suggesting a potential p53 independent cell death mechanism occurring here in the *Drosophila* brain (Figure 4.7A and B, not significant). In conclusion, no evidence of apoptosis can be detected using the GC3ai reporter in the amyloid fly.

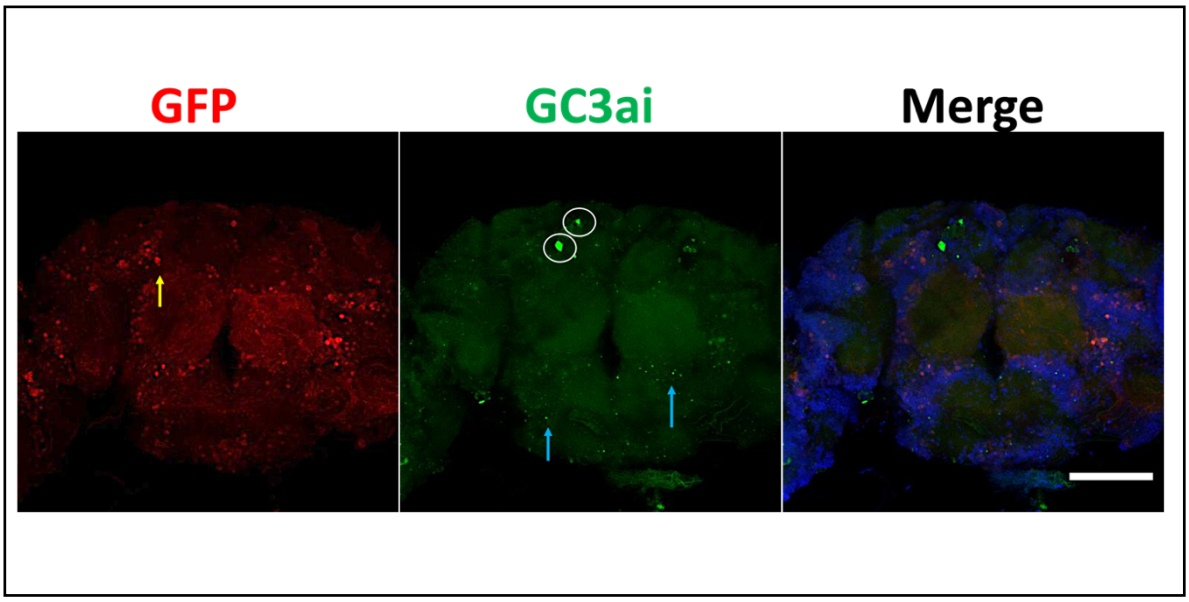
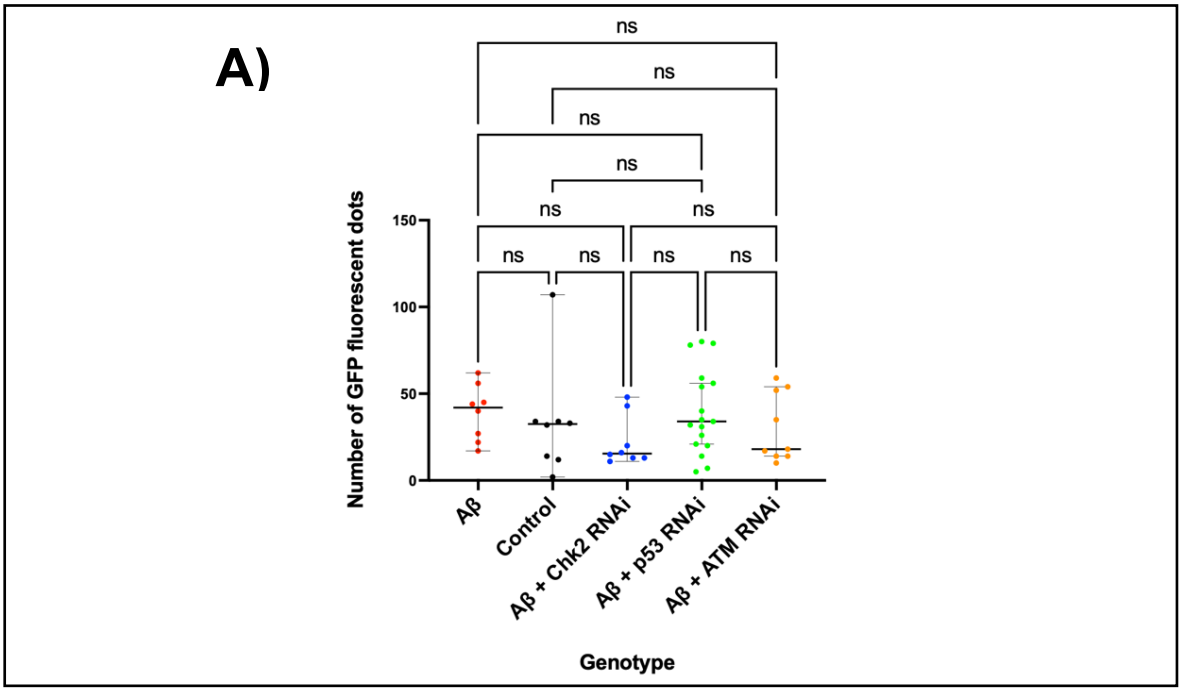


Figure 4.6 Representative images of the GC3ai reporter expressed under the Elav-Gal4 (Gal80^{ts}) driver at 27 °C on day 14. The GFP reporter GC3ai was expressed in adult *Drosophila* neurons using the Elav-Gal4 Gal80^{ts} driver. Adults were aged for 14 days at 27 °C and their brains dissected and stained with anti-GFP (marks active and inactive GC3ai). Red staining represents the Elav domain where the GC3ai reporter is expressed (yellow arrow). Stochastic expression of the GC3ai reporter is observed at 27 °C. Active GC3ai is represented by endogenous GFP fluorescence (blue arrow). Green autofluorescence is observed in the fly brain (white circle). Scale bar = 50 µm.



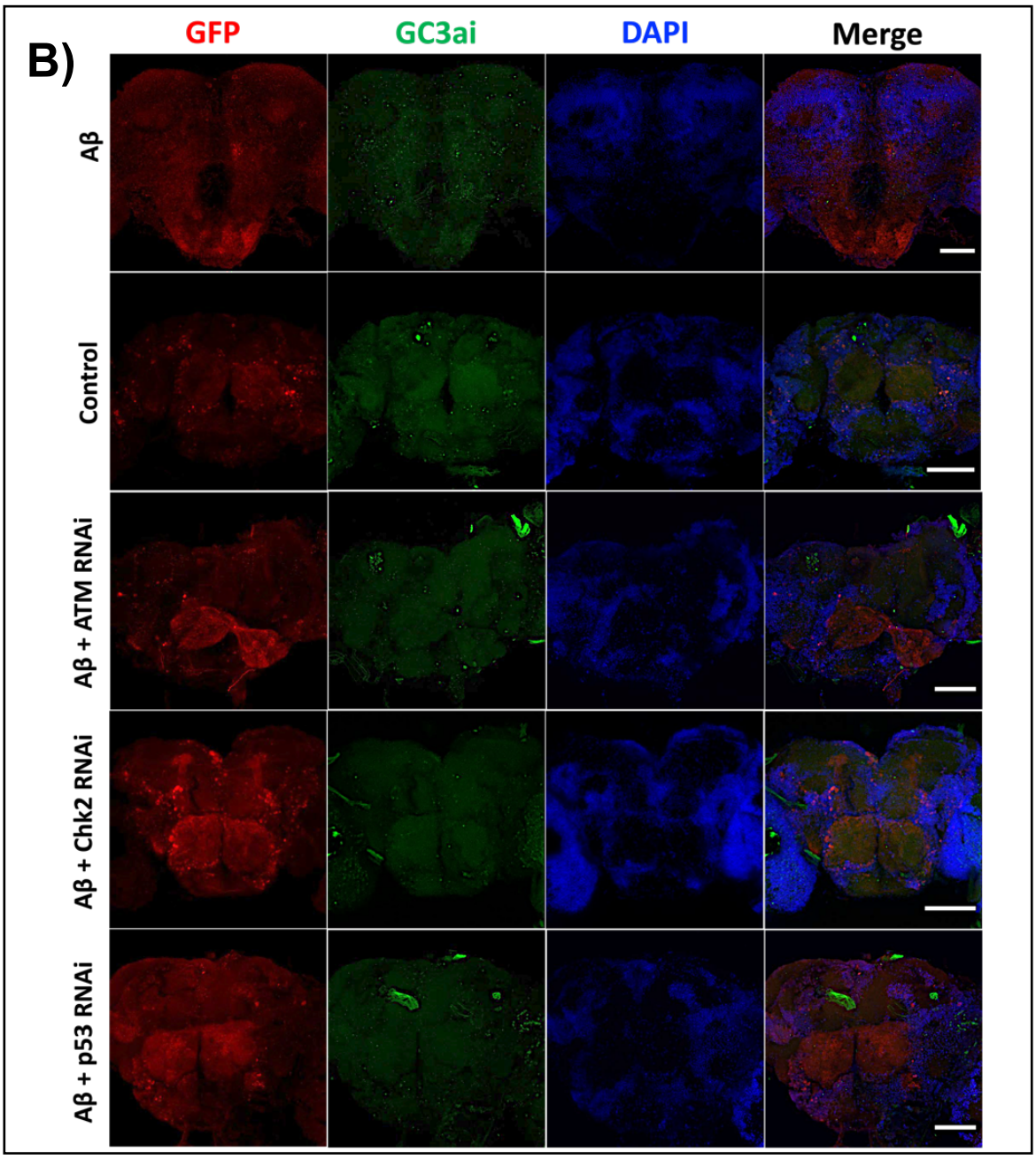


Figure 4.7 Caspase activity is not elevated in Aβ fly using the GC3ai reporter, or dependent on the ATM-Chk2-p53 pathway.
A) Scatter dot plot showing number of GFP fluorescent dots at day 14. Analysis of the number of green, fluorescent dots reveals no significant difference between any of the genotypes at day 14 (Kruskal-Wallis test with Dunn's multiple comparison test, not significant). Black line represents median, error bars represent 95% confidence interval. **B) Immunofluorescence images showing caspase activity, indicated by endogenous GFP fluorescence, is not increased by Aβ**. No obvious increase of GFP signal was observed with Aβ or decreased upon *ATM*, *Chk2* or *p53* knockdown. Scale bar = 50 μm.

4.4 Discussion

4.4.1 Overexpression of the caspase inhibitor p35 is neuroprotective

One of the major mechanisms p53 controls is apoptosis. Apoptosis mediated by p53 is predominantly via upregulation of pro-apoptotic Bcl-2 proteins to activate the intrinsic pathway and upregulation of death receptors to sensitise cells to death ligands, activating the extrinsic pathway (Aubrey *et al.*, 2018). Apoptosis is one of the major mechanisms of cell death discussed in AD, with evidence of apoptotic morphology and an upregulation of pro-apoptotic proteins including Bcl-2 proteins, caspases and death ligands (De La Monte, Sohn and Wands, 1997; MacGibbon *et al.*, 1997; Nagy and Esiri, 1997; Su, Deng and Cotman, 1997; Kitamura *et al.*, 1998; Selznick *et al.*, 1999; Stadelmann *et al.*, 1999; Su *et al.*, 2001, 2003; Zhao *et al.*, 2003).

In *Drosophila* seven members of the caspase family exist – 3 initiator caspases and 4 effector caspases (Kumar and Doumanis, 2000). The main initiator caspase Dronc is homologous to mammalian caspase- 9 and activates the effector caspases, DrICE and Dcp-1, homologous to mammalian caspase 3 (Kornbluth and White, 2005). To investigate if apoptosis was occurring in our amyloid fly model I overexpressed the baculovirus protein p35, which inhibits the activity of the major effector caspases DrICE and Dcp-1 activity but not the main initiator caspase Dronc (Hay, Wolff and Rubin, 1994; Hawkins *et al.*, 2000; Meier *et al.*, 2000; Lannan, Vandergaast and Friesen, 2007).

Overexpressing the caspase inhibitor p35 significantly slowed the decline in motility induced by Aβ, which is seen in our amyloid toxicity model in *Drosophila* (Figure 4.1A and B, ***p=0.008). A general decline in motility is seen in the control fly (ElavGal4;Gal80^{ts} x w^{1118}) due to normal ageing and potential Gal4 toxicity (Kramer and Staveley, 2003), however there was no difference in performance between the

control and those expressing UAS-p35 alone (Figure 4.1A and B, not significant), suggesting inhibiting caspase activity does not have a general protective effect against age related motor decline, and it is specifically neuroprotective to the Aβ induced toxicity.

Our results indicate that inhibiting caspase activity elicits a neuroprotective effect in our amyloid model, in agreement with previous studies using p35 overexpression and caspase inhibitors to rescue Aβ induced neurological phenotypes (Tare *et al.*, 2011; Hong *et al.*, 2012). These studies used the strong GMR-Gal4 driver and the standard Elav-Gal4 driver to overexpress Aβ alongside p35 or using a caspase inhibitor. My results differ here using adult-onset expression in comparison to expression throughout development. Inhibiting caspase activity with p35 overexpression during development in *Drosophila* has been shown to induce abnormal signalling and developmental defects (Pérez-Garijo, Martín and Morata, 2004). I restricted p35 overexpression to the mature adult nervous system to again prevent any confounding phenotypes from inhibiting apoptosis in the developing nervous system

My results here indicate that Aβ mediates neurodegeneration through caspase activity. Inhibiting caspase activity has already proven to be neuroprotective in an AD mouse model (Flores *et al.*, 2018) and other models of neurodegenerative diseases, including Parkinson's disease (Schierle *et al.*, 1999), Huntington's disease (Ona *et al.*, 1999) and ALS (Li *et al.*, 2000). Furthermore, caspase inhibition improves neurological function in acute CNS diseases including ischemia (Friedlander, 2003), suggesting that apoptosis could underlie the toxicity in many neurological diseases.

4.4.2 Aβ does not induce neuronal loss in the mushroom bodies

Although I have shown overexpressing a caspase inhibitor is neuroprotective in our *Drosophila* amyloid toxicity model, I still needed to show apoptosis was elevated in the amyloid expressing fly. Traditional methods of detecting apoptosis in AD models

or post-mortem AD brain material include TUNEL and observation of apoptosis hallmarks (discussed in section 1.6.5). TUNEL detects breaks non-specific to apoptosis and because apoptosis is a quick process, relying on observing apoptosis hallmarks could mean you miss them, resulting in a false result.

As detecting active apoptosis occurring has proven difficult, I tried to simplify detecting apoptosis by investigating if overexpression of p35 correlates with a visual rescue in neuronal loss. To observe visual neuronal loss, I created a recombinant fly expressing the mifepristone inducible MB-GeneSwitch driver combined with a UAS-mCherryNLS, to allow counting of a small subset of neurons- the Kenyon cells in the mushroom bodies (Figure 4.4A). The number of Kenyon cells did not appear to drastically reduce over the time course of the experiment for any of the genotypes (Figure 4.4B), and there was no significant difference between any of the genotypes (Figure 4.4C), indicating Aβ does not accelerate neuronal loss in the mushroom bodies, nor is overexpressing p35 protective in the loss of Kenyon cells. Although our results show that inhibiting caspase activity does not correlate with a protection in Kenyon cell loss in the amyloid toxicity model, it has been shown that an upregulation of caspases does not always result in cell death straight away, with evidence of caspase activation occurring months before neuronal loss in a mouse model of ALS and AD (Irizarry *et al.*, 1997; Pasinelli *et al.*, 2000). This could explain why inhibiting caspase activation did not correlate with a reduction in neuron loss.

Counting the number of Kenyon cells with the ImageJ TrackMate plugin presented some limitations (*full details of counting method in materials and methods*). The threshold for each mushroom body had to be chosen manually since selecting a single threshold for all samples resulted in a massive under and over estimation. The explanation for this could be that a primary antibody was not used against the mCherry signal as it was stable after fixation. This meant upon imaging I noticed a slight degradation of the signal over time. It was not practical to dissect all brains and image them all on the same day, thus some brains would have been more recently mounted compared to older brains, resulting in slight inconsistencies of signal. After 3 days of expression the mean number of Kenyon cells per hemisphere was ~800-

900 in each genotype, as reported by the TrackMate plug in. This was considerably lower than the estimated number of 2000 Kenyon cells per mushroom body hemisphere (Aso *et al.*, 2009). However, the estimated number of 2000 neurons was reported in a study using 25 different mushroom body drivers but it did not include the driver I used here. Hence, it is likely to that MB-GeneSwitch drives expression in only a subset of Kenyon cells.

I failed to detect any significant increase in neuronal loss occurring in the amyloid model, but the mushroom bodies are a small region of the brain and may not be representative of what is occurring on a wider scale throughout the brain. Although extensive neuronal loss is observed in AD post-mortem brains, significant neuron loss in mouse models is not consistently observed (McGowan, Eriksen and Hutton, 2006; Götz, Bodea and Goedert, 2018). In contrast to rodent models, neuronal loss in *Drosophila* can be observed when modelling Aβ toxicity. However, no previous study has tried to individually count number of neurons in the *Drosophila* brain. Previous studies have reported 'neuronal loss' in *Drosophila* by observation of the rough eye phenotype when Aβ is expressed under the control of the GMR-Gal4 driver, an indirect method of observing neuronal loss (Finelli *et al.*, 2004; Greeve *et al.*, 2004; Crowther *et al.*, 2005; Speretta, Thomas R. Jahn, *et al.*, 2012). As explained previously, GMR-Gal4 drives very strongly in several cell types in the developing eye and induces apoptosis in the absence of Aβ (Kramer and Staveley, 2003; Schott *et al.*, 2017) It is not a directly comparable system to adult expression at low levels in the brain, as used here. Neuronal loss has also been quantified by the area of vacuolation, a sign of neurodegeneration in *Drosophila,* but this again was not using adult onset only expression of Aβ (Iijima *et al.*, 2004, 2008; Crowther *et al.*, 2005).

A similar experiment to mine has previously been done where they used tomato expression, driven by the Q system, to mark neurons in the central part of the brain (Niccoli *et al.*, 2016). Simultaneously, they expressed Aβ pan-neuronally in the brain using Elav-GeneSwitch to restrict expression to adult neurons, a similar approach to what I have done here using the MB-GeneSwitch driver. To analyse neuronal loss

the total fluorescence intensity of tomato staining was determined, and they found a significant decrease upon Aβ expression. It must be noted though that they used the more toxic Arctic mutant of Aβ and they expressed Aβ pan-neuronally compared to my experiment where I restricted expression to a small subset of neurons. Upon reflection, using an RFP antibody in my experiment against the mCherry signal could have prevented the signal from degrading and allowed me to quantify the total fluorescence in each genotype.

4.4.3 Aβ does not induce apoptosis in the amyloid fly

A more specific reliable way of detecting apoptosis is looking for active caspase activity. Previous methods in the Tuxworth lab have attempted to use immunohistochemistry to stain for one of the effector caspases in *Drosophila* Dcp1 in the brains of Aβ expressing flies, but this revealed no evidence of apoptosis. TUNEL and acridine orange staining, which are not specific to apoptotic cells but widely used to visualise cell death, were also tried, and revealed no death. It was not clear whether the methods failed to detect apoptosis or instead, that there was little or no apoptosis occurring. I wanted to revisit this since the p35 expression experiment pointed strongly towards apoptosis underlying the decline in motility in the amyloid expressing flies (Figure 4.1). Here, I used a GFP reporter (GC3ai) which detects the activity of effector caspases Dcp1 and DrICE (Schott *et al.*, 2017). The GC3ai reporter has a higher sensitivity than anti-Dcp1 in fixed tissues and can also be used for *in vivo* imaging of real time apoptosis in the fly (Schott *et al.*, 2017). The GC3ai reporter also excludes the potential issue of antibody problems as it relies on the endogenous GFP fluorescence without the need for an antibody penetrating deep into the brain.

I was not able to detect evidence of elevated caspase activity in the brains of the Aβ expressing flies (Figure 4.7A and B). Evidence for some apoptosis occurring (endogenous GFP) was found in the control flies, potentially due to normal

accumulation of DNA damage during aging, but this was not enhanced by Aβ expression (Figure 4.7A and B). I did uncover a few issues with the GC3ai method. Firstly, apoptosis is a fast process with estimation of completion within 2-3 hours (Elmore, 2007). It is unlikely that I would capture this event with a snapshot at day 14 expression over such an extended period. Additionally, the problem of capturing apoptosis is exacerbated by the fact caspase 3 is rapidly degraded, highlighting again the problem of timing the assay to capture the correct data (Tawa *et al.*, 2004). To investigate if the GC3ai method was functional and detectable I could have used overexpression of the Hid protein, an activator of apoptosis in *Drosophila,* as a positive control (Goyal *et al.*, 2000).

Other problems experienced using the GC3ai reporter were uncovered in the analysis of the data. The GC3ai toolkit was originally reported to be used in the eye disc using the driver GMR-Gal4, and I was able to repeat that original report here as a positive control (Figure 4.5) (Schott *et al.*, 2017). In the original paper caspase activity was quantified by normalising the overall signal for endogenous GFP fluorescence to the volume of the GMR domain (red staining) for each eye disc. As I used a different driver here (ElavGal4-Gal80^{ts}) I could not analyse our results in the same way. Surprisingly, the Elav-Gal4 (Gal80^{ts}) driver resulted in stochastic expression of the GC3ai reporter, evidenced by the fact that not every neuron in the brain was marked with red fluorescence (Figure 4.6). It is reported that Gal80^{ts} is maximally repressed at 29 °C (McGuire *et al.*, 2003) and most uses of Gal80^{ts} do use that temperature or higher to induce expression. However, as explained previously, I chose to age the flies at 27 °C to balance Aβ toxicity with minimising the toxic effects of Gal4 on neurons. My staining for the GC3ai reporter indicates is that that the Gal4 system is likely to be only partially induced at 27 °C. To compound the issue of stochastic expression of the reporter, endogenous autofluorescence occurs in the brain meaning that the green channel could not be segmented easily. This resulted in me relying on an alternative method to analyse the endogenous GFP by counting the number of GFP ‘dots’ in the mid-brain using manual thresholding and cell counter in ImageJ.

Increased caspase activity is an indication of an initiation of apoptosis. Despite the fact I showed that inhibiting caspase activity in the amyloid model was neuroprotective, I failed to show any evidence of increased caspase activity and thus no evidence of elevated apoptosis in the amyloid fly. Our findings disagree with the observation that Aβ can induce apoptosis, as seen *in vitro* (Harada and Sugimoto, 1999; Marín *et al.*, 2000; Awasthi, Matsunaga and Yamada, 2005; Takada *et al.*, 2020). However, addition of Aβ oligomers *in vitro* at high concentrations does not necessarily reflect what is occurring *in vivo*, particularly if expression in our system is low-level and stochastic. Previous findings of increased caspase activity in *Drosophila* models of amyloid toxicity is limited, but Dcp1 and active caspase-3 have been identified in the eye and brain, respectively, using different forms of Aβ than used here in my experiment (Tsuda *et al.*, 2017; Wu *et al.*, 2017).

4.4.4 Does Aβ induces apoptosis via a p53 independent mechanism?

Even though caspase activity was not increased in the amyloid-expressing fly, it was not decreased when *ATM, Chk2* or *p53* expression was knocked down (Figure 4.7A and B). One possibility is that cell death could be occurring in a p53-independent manner in the amyloid fly. It has previously been shown that apoptosis in *Drosophila* can occur in a caspase dependent but Chk2 and p53 independent manner, which is blocked by p35 expression, suggesting p53 independent cell death still requires caspase activity (Wichmann, Jaklevic and Su, 2006; McNamee and Brodsky, 2009).

4.4.5 Possible non-apoptotic role for caspases

As I have shown no evidence of an increase in apoptosis occurring in the amyloid-expressing flies, I must consider other possibilities of how caspase activity could induce neurotoxicity. Here, I propose the involvement of caspase activity promoting Tau pathology, which could be blocked by p35 expression. Caspase activation is found in tangle bearing neurons in AD (Rohn, Head, Su, *et al.*, 2001; Su *et al.*, 2001; Gastard, Troncoso and Koliatsos, 2003) and can cleave Tau in particular at the

Aspartate residue 421 (Canu *et al.*, 1998; Gamblin *et al.*, 2003; Rissman *et al.*, 2004). There is evidence to suggest caspase activation is involved in the early stages of Tau pathology and cleaved Tau by caspases aggregates quicker than full length tau and accelerates formation of NFTs (Gamblin *et al.*, 2003; Rissman *et al.*, 2004). Additionally active caspase 3 colocalises with cleaved Tau in pre-tangle bearing neurons but not mature tangle bearing neurons (Rissman *et al.*, 2004). It could be possible that neurotoxicity via p53 signalling in the amyloid fly is due to caspase activation through increased transcription of pro-apoptotic proteins of the Bcl-2 family, leading to Tau cleavage and Tau pathology. This could be investigated using an antibody that recognises Tau truncated at Asp421, to see if there is an decrease upon p35 expression (Canu *et al.*, 1998; Fasulo *et al.*, 2000; Gamblin *et al.*, 2003; Rissman *et al.*, 2004). Other alternative roles for caspases are discussed in final discussion 7.0.

4.5 Conclusions

My results here show that inhibiting caspase activity via p35 is neuroprotective in our amyloid *Drosophila* model. However, no increase in apoptosis in the amyloid expressing fly could be detected, suggesting that the neuroprotective effect seen with caspase inhibition is not due to a reduction in apoptosis. It is possible that caspase activity could be eliciting neurotoxicity via a different non cell death mechanism.

5.0 Downstream targets of p53 – cell cycle re-entry

5.1 Introduction

Alongside apoptosis being investigated as a potential downstream mechanism of p53 mediated neurotoxicity, abnormal cell cycle re-entry was investigated as an alternative pathway.

Neurons are traditionally viewed as post-mitotic, terminally differentiated cells. However, neurons abnormally re-entering the cell cycle from G_0 is observed in various chronic neurodegenerative diseases including Parkinson's disease, ALS and AD, and after acute CNS injuries such as ischemia, SCI and TBI (Love, 2003; Velardo *et al.*, 2004; Di Giovanni *et al.*, 2005; Wang *et al.*, 2009). A plethora of evidence exists showing ectopic cell cycle activation in neurons can induce cell death (Al-Ubaidi *et al.*, 1992; Feddersen *et al.*, 1992; Wang *et al.*, 2009). As previously discussed, targeting the ATM-Chk2-p53 pathway is neuroprotective in a *Drosophila* amyloid toxicity model (Taylor and Tuxworth, 2019; Taylor *et al.*, 2022). In response to DNA damage p53 is activated by both ATM and Chk2 to induce cell cycle arrest to allow DNA repair (Feroz and Sheikh, 2020). DNA damage, which accumulates in neurodegeneration, is a stimulus for neuronal cell cycle re-entry and this is dependent on ATM activity (Kruman *et al.*, 2004; Fielder, Von Zglinicki and Jurk, 2017). Potentially in pathology, p53 signalling is perturbed to induce cell cycle re-entry and trigger cell death, rather than induce cell cycle arrest to protect the genome. Taken together, these observations suggest that the neuroprotective effect of targeting the ATM-Chk2-p53 pathway in the *Drosophila* amyloid toxicity model, might be mediated by reducing abnormal cell cycle re-entry in the CNS. Therefore, I wanted to investigate if cell cycle re-entry was occurring in the amyloid toxicity model to determine whether cell cycle re-entry and therefore could be contributing to the loss of neural function.

5.2 FLY-FUCCI reporter system

FLY-FUCCI (fluorescent ubiquitination-based cell cycle indicator) is a tool that allows the distinguishment between the cell cycle phases G1, S and G2 (Zielke *et al.*, 2014) (Figure 5.1). In *Drosophila*, the FLY-FUCCI system relies on fluorochrome-tagged degrons from E2F1 and Cyclin B proteins which are degraded by the ubiquitin ligase complexes $CRL4^{Cdt2}$ and APC/C, respectively, at different points in the cell cycle. APC/C and $CRL4^{Cdt2}$ have differential activity throughout the cell cycle, allowing for degradation of Cyclin B and E2F1 at different points. The transcription factor E2F1 accumulates in G1 to prepare the cell for S phase transition and then is degraded during S phase by $CRL4^{Cdt2}$ (Shibutani, Swanhart and Duronio, 2007; Shibutani *et al.*, 2008) . Cyclin B promotes the transition from G2 to mitosis and is degraded by the APC/C complex in mitosis (King *et al.*, 1995). The FLY-FUCCI system labels Cyclin B with RFP and E2F1 with GFP. In G_0/G1 CyclinB-RFP is degraded by the APC/C complex, labelling the cell green. As cells progress to S phase, GFP-E2F1 is degraded by the S phase ubiquitin ligase $CRL4^{Cdt2}$, labelling cells red, due to the presence of CyclinB-RFP. After cells enter G2 and progress to early mitosis, both GFP-E2F1 and CyclinB-RFP are present, labelling the cell yellow. APC activity starts to rise mid-late mitosis, degrading RFP and labelling the cell green.

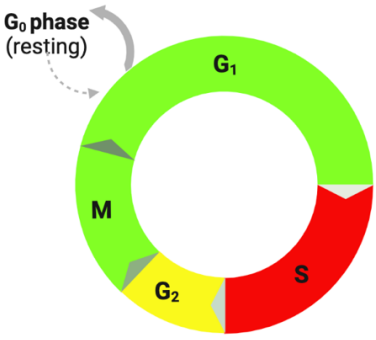


Figure 5.1. FLY-FUCCI reporter allows different phases of the cell cycle to be labelled.
FLY-FUCCI is a system that allows cells in different phases of the cell cycle to be labelled. Cells in G_0/G1 and late mitosis are labelled green due to high activity of the APC/C ubiquitin ligase complex degrading CyclinB-RFP. Progression into S phase labels cells red due to high activity of $CRL4^{Cdt2}$ degrading GFP-E2F1. As cells enter G2 and early mitosis, both GFP-E2F1 and CycB-RFP are present, labelling the cell yellow.

5.3 Results

5.3.1 FLY-FUCCI labels cells in G1, S and G2

The FLY-FUCCI reporter was used to investigate if there was any evidence of aberrant cell cycle progression in *Drosophila* neurons expressing Aβ 1-42. To confirm the FLY-FUCCI reporter was correctly working, the ubiquitous driver Actin-Gal4 was used to drive the FLY-FUCCI reporter throughout development at 27 °C. Third instar larvae were dissected, and their wing discs were stained for GFP and RFP (Figure 5.2). The wing disc was chosen for a positive control to observe cycling cells due to their high cell turnover and proliferation that occurs during development (Neto-Silva, Wells and Johnston, 2009). As expected, cells in all phases of the cell cycle were observed with green cells in G_0/G1 or late mitosis (Figure 5.2, red arrow), red cells in S phase (Figure 5.2, green arrow), and yellow labelling those in G2/early mitosis (Figure 5.2, yellow arrow).

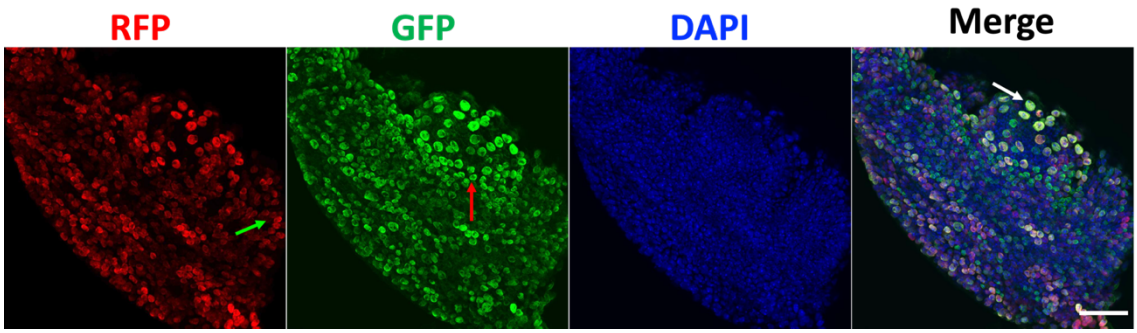


Figure 5.2 FLY-FUCCI reporter labelling cycling cells in a *Drosophila* larval wing disc.
A) Immunofluorescence image of a *Drosophila* larval wing disc expressing the FLY-FUCCI reporter showing cycling cells in different phases of the cell cycle. FLY-FUCCI reporter was expressed ubiquitously using the actin-Gal4 driver as a positive control. Third instar larvae were dissected, and their wing discs stained for RFP (red), GFP (green) and DAPI (Green). Cells in G_0/G1 or late mitosis are labelled green (red arrow), S phase are labelled red (green arrow) and G2 are labelled yellow (yellow arrow in merge channel). Scale bar = 20 μm.

5.3.2 No cell cycle re-entry is observed in the amyloid fly

After confirming the FLY-FUCCI reporter was working as reported (Zielke *et al.*, 2014), I next wanted to investigate if there was any evidence of cell cycle events occurring in the amyloid-expressing flies. The mushroom body driver Ok107-Gal4 was chosen to drive expression of FLY-FUCCI +/- Aβ 1-42. Ok107-Gal4 drives expression strongly in the Kenyon cells, the intrinsic neurons that make up the mushroom body structures (Aso *et al.*, 2009). This driver was chosen for several reasons: I wanted to drive expression in a smaller subset of neurons in a defined lobular area of the brain which can be identified easily and imaged identically. Additionally, the mushroom bodies are involved in memory and learning, making them a relevant area to neurodegenerative diseases (Modi, Shuai and Turner, 2020). Ok107 is also a strong driver which ensured that if effects on cell cycle were present, I would maximise my chance of detecting them.

Flies were aged for 14 days at 27 °C (D1-1 day after eclosion) and adult brains were dissected and stained for GFP and RFP. Day 14 was chosen for dissection as this is the time when the steep decline in motility occurs in our amyloid *Drosophila* model using previous tracking data (Figure 3.3A). At day 14, all Kenyon cells expressing the FLY-FUCCI reporter, with and without Aβ 1-42 expression, were labelled green which indicates the cells are either in G_0, G1 or late mitosis (Figure 5.3A). Although FLY-FUCCI cannot distinguish between these phases, it is likely that the GFP positive neurons are in G_0, as there is no other evidence that cell cycle progression is occurring in this experiment (no yellow or red staining). As there was no evidence of cell cycle progression events at 14 days of expression, I decided to age the flies for 28 days to investigate if longer transgene expression and exposure to Aβ would induce abnormal cell cycle events (Figure 5.3B). At day 28 all Kenyon cells were still labelled green with no evidence of cell cycle progression, as seen before at day 14 (Figure 5.3B). Although there was no evidence that abnormal cell cycle re-entry had occurred in the amyloid fly, there appeared to be a consistent visual reduction in the volume of the mushroom body region labelled by GFP and DAPI in flies expressing FLY-FUCCI + Aβ 1-42 compared to the FLY-FUCCI control alone (Figure 5.3A and

B). This could indicate some neuronal cell loss is occurring. Although no nuclear RFP staining was present in both genotypes, there was some non-specific staining seen in the red channel (Figure 5.3A, red arrow). To confirm this was non-specific staining due to the RFP antibody, as previously done before*, Drosophila* brains expressing the FLY-FUCCI reporter were dissected at day 14 and stained with the red fluorescent secondary antibody but with no anti- RFP. This removed all background red staining, suggesting the primary RFP antibody is binding non-specifically to produce the background signal (images not included).

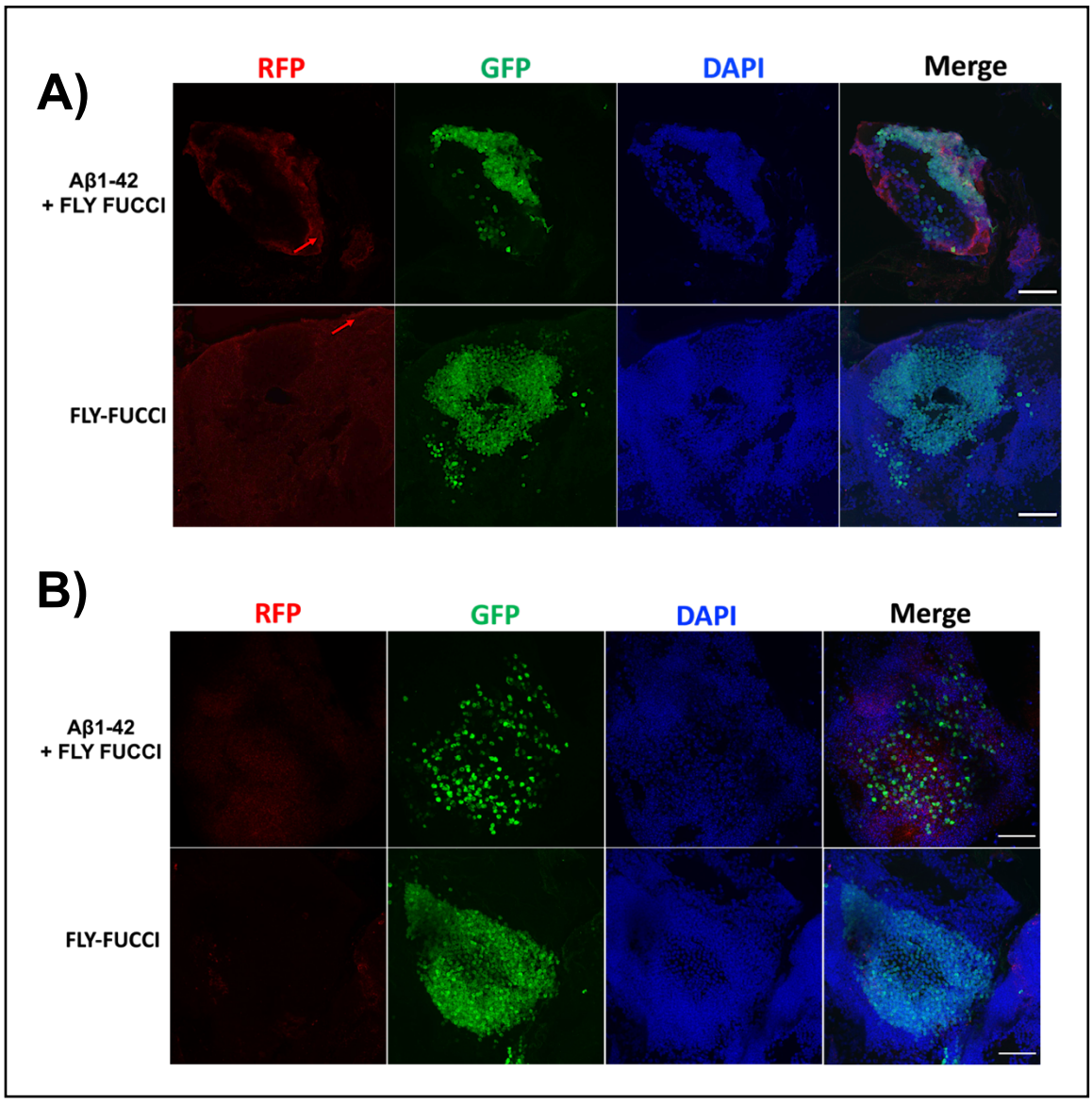


Figure 5.3. No evidence for cell cycle re-entry is observed in the *Drosophila* amyloid toxicity model using FLY-FUCCI.
A) Representative immunofluorescence image of adult *Drosophila* brains expressing the FLY-FUCCI reporter with and without 14 days of Aβ1-42 expression. FLY-FUCCI reporter was expressed in the mushroom bodies using the strong Ok107-Gal4 driver. Flies were aged for 14 days and stained for RFP (red), GFP (green) and DAPI (Green). Kenyon cells expressing the FLY-FUCCI reporter remained green, indicative of G_0, G1 or late mitosis. No evidence of any S or G2 phase cells were observed. Background red staining was observed due to non-specific binding of the primary RFP antibody (red arrow). **B) Representative immunofluorescence image of adult *Drosophila* brains expressing FLY-FUCCI reporter with and without 28 days of Aβ1-42 expression.** FLY-FUCCI reporter was expressed in the mushroom bodies using the strong Ok107-Gal4 driver. Flies were aged for 28 days and stained for RFP (red), GFP (green) and DAPI (Green). Kenyon cells expressing the FLY-FUCCI reporter remained green, indicative of G_0, G1 or late mitosis. All scale bars = 20 μm.

5.4 Discussion

5.4.1 No evidence of abnormal cell cycle re-entry in the amyloid fly

Abnormal cell cycle re-entry is seen in neurological diseases including chronic neurodegenerative diseases such as Parkinson's disease, ALS and AD but also after acute insults, such as SCI and TBI (Wang *et al.*, 2009). Evidence of G1, S, G2 and mitotic markers have been reported in post-mortem AD brains, however there is no evidence of a completed mitosis (Lee *et al.*, 2009; Wang *et al.*, 2009). Ectopic neuronal cell cycle re-entry induces cell death, thus it has been hypothesised that pathological neurons re-enter the cell cycle, do not complete it, and then die (Fielder, Von Zglinicki and Jurk, 2017). DNA damage is a stimulus for neuronal cell cycle re-entry, a mechanism dependent on ATM activity (Kruman *et al.*, 2004). p53 is downstream of ATM in the DDR and under normal conditions p53 controls cell cycle arrest events in response to DNA damage. However, in AD it is reported that p53 is conformationally changed and dysfunctional and consequently impaired signalling occurs (Uberti *et al.*, 2006; Lanni *et al.*, 2008; Abate *et al.*, 2020). In pathology could induction of the DDR, and consequently activation of the ATM-Chk2 pathway, lead to activation of dysfunctional p53 and abnormal cell cycle re-entry events to induce death? I therefore wanted to investigate if cell cycle re-entry was occurring in the amyloid fly to investigate if this could be contributing to the toxic effect ATM-Chk2-p53 activation has on neuronal survival and function.

Previous attempts in the Tuxworth lab to investigate cell cycle re-entry in our amyloid model have involved staining for bromodeoxyuridine (BrdU), 5'Ethynyl-2'-deoxyuridine (EdU) and proliferating cell nuclear antigen (PCNA) using immunohistochemistry. All these methods have shown no evidence of abnormal cell cycle re-entry events. Here I used the FLY-FUCCI reporter to attempt to identify any cell cycle events occurring in the amyloid fly. One potential benefit of using the FLY-FUCCI system vs. antibody staining for cell cycle-specific proteins is that it allows for

clear distinguishing of G1, S and G2. The cell cycle is highly dynamic and is controlled by Cyclin/Cdk activity. At different points in the cell cycle, specific cyclins and Cdks are temporally expressed to control different phases. However, overlap of Cyclin/Cdk expression does occur as the cell cycle phases are transitioning. Additionally, FLY-FUCCI can be used during live imaging (Zielke *et al.*, 2014).

My data here suggest no evidence of any abnormal cell cycle events occurs in the amyloid-expressing fly, shown by an absence of any red or yellow staining (Figure 5.3A and B). Although the larval wing disc was used as the positive control, it may have been useful to use an Actin-Gal4 driver to express the FLY-FUCCI line ubiquitously as an additional positive control. This would have allowed us to observe the FLY-FUCCI reporter working in adult *Drosophila* brains in cycling glial cells. It would have been easy to discriminate the cycling glia from neurons as glia have smaller nuclei.

Although cell cycle re-entry has been observed in AD mouse models (Yang *et al.*, 2006) there have been no reports from *Drosophila* models of amyloid toxicity. One report using expression of Tau in the *Drosophila* brain did report cell cycle re-entry (Khurana *et al.*, 2006), but previous attempts by our group to repeat these findings failed when Tau expression was restricted to adult neurons. Previous work has shown that Aβ itself can act as a mitogen and induce neuronal cell cycle re-entry *in vitro* (Copani *et al.*, 1999; Giovanni *et al.*, 1999; Wu *et al.*, 2000; Varvel *et al.*, 2008). My results here do not agree with these findings that Aβ can induce cell cycle re-entry. However, previous studies have only shown this to be true with *in vitro* cell culture, using high concentrations of amyloid beta. Additionally, different forms and fragments of Aβ have been used between my experiment and others. Here, I use the Aβ12L version in which tandem dimers of Aβ1-42 are connected by a flexible linker sequence in between (Speretta, Thomas R. Jahn, *et al.*, 2012). Previous studies have used Aβ 1-42 oligomers and the protein fragment Aβ 25-35, making direct comparison difficult (Copani *et al.*, 1999; Giovanni *et al.*, 1999; Wu *et al.*, 2000; Varvel *et al.*, 2008). One other potential difference is that expression of Aβ in my system is restricted to the mature adult brain to avoid confounding effects on immature neurons

Here my data reveals green labelled Kenyon cells in flies expressing FLY-FUCCI +/- Aβ1-42 at both day 14 and day 28, indicating neurons are either in G_0, G1 or late mitosis (Figure 5.3A and B). The disadvantage of FLY-FUCCI is that it does not distinguish between these 3 phases, so it is possible that some neurons were in G1 when the brains were fixed but no neurons had proceeded far enough in G1 towards G1/S to be labelled red. This could be confirmed or rejected by staining for anti-cyclin D1 antibodies, a protein which initiates G1/S progression.

An alternative explanation for my results is that I may have missed cell cycle re-entry events labelling neurons red or yellow and cell death of the neuron had already occurred. I observed a consistent reduction in GFP-positive Kenyon cells in the mushroom body areas of flies expressing FLY-FUCCI with Aβ (Figure 5.3A and B), indicating some neuronal loss may have occurred as seen in other *Drosophila* amyloid toxicity models (Finelli *et al.*, 2004; Greeve *et al.*, 2004; Iijima *et al.*, 2004, 2008; Crowther *et al.*, 2005; Speretta, Thomas R. Jahn, *et al.*, 2012). If this was true, then the loss of green positive Kenyon cells would have coincided with a loss of DAPI also. This was difficult to observe as DAPI stains all neurons and glia in the brain, making it hard to distinguish the boundaries of the mushroom body area only. To confirm, I could have stained for the mushroom body axonal marker, Fas II. This would have allowed us to see if any reduction in green was also matched by a reduction in Fas II positive cells, indicating a loss of mushroom body neurons. However, given I saw no signs of cell cycle re-entry, this was not considered a valuable experiment, given limited time. Additionally, simultaneous experiments were on going to count neuronal loss in the mushroom bodies (see section 4.3.2).

5.5 Conclusions

The aim of this experiment was to investigate if cell cycle re-entry was occurring in the amyloid toxicity model in *Drosophila*, to explore whether it could contribute to the deleterious effect seen in the amyloid model. A combination of different methods has now been attempted previously in the Tuxworth lab using BrdU, EdU and PCNA staining and now here using the FLY-FUCCI system. No evidence of abnormal cell

cycle re-entry has been observed in the Drosophila amyloid model and therefore it is unlikely to be a factor in the neuroprotective effect seen with inhibiting *ATM, Chk2* or *p53*.

6.0 Alternative targets of Chk2 – Tau phosphorylation

6.1 Introduction

Alongside the experiments investigating the potential mechanisms downstream of p53, we simultaneously attempted to investigate Tau phosphorylation as another potential downstream target of Chk2.

Aberrant Tau phosphorylation is found in many neurodegenerative diseases collectively known as the tauopathies (Arendt, Stieler and Holzer, 2016). In AD hyperphosphorylated Tau makes up one of the pathological hallmarks - extracellular neurofibrillary tangles, which are suggested to contribute to the neurodegeneration (Kosik, Joachim and Selkoe, 1986; Bancher *et al.*, 1989). The number of NFTs formed of hyperphosphorylated Tau correlates strongly with the cognitive decline and neuronal loss seen in AD, compared to Aβ plaque deposition (Bondareff *et al.*, 1989; Arriagada *et al.*, 1992; Gomez-isla *et al.*, 1997; Giannacopoulos, 2003). Additionally, mutations in the Tau gene can induce neurodegeneration in the absence of Aβ plaques (Lee and Leugers, 2013). An abundance of *in vitro* evidence exists indicating that Aβ induced neurotoxicity is dependent on the presence of Tau, as discussed in detail in the introduction (see section 1.3.4) (Rapoport *et al.*, 2002; Ittner *et al.*, 2010; Vossel *et al.*, 2010; Jin *et al.*, 2011; Shipton *et al.*, 2011; Seward *et al.*, 2013; Zempel *et al.*, 2013). Additionally, reduction in Tau prevents Aβ induced learning and memory defects *in vivo* (Rapoport *et al.*, 2002; Roberson *et al.*, 2007; Leroy *et al.*, 2012). The above evidence suggests Tau is a crucial mediator of neurotoxicity.

To date, 57 phosphorylation sites have been identified on Tau extracted from AD brain material *post mortem*, which can be phosphorylated by many different kinases (Hanger, Anderton and Noble, 2009; Wesseling *et al.*, 2020). Hyperphosphorylation of Tau reduces its microtubule binding ability and some of these sites are particularly important for controlling this binding of Tau to microtubules, including Ser262 and

Ser356, Ser396,Thr231 and Ser235 (Biernat *et al.*, 1993; Bramblett *et al.*, 1993; Drewes *et al.*, 1995; Sengupta *et al.*, 1998; Buée *et al.*, 2000). Of these, phosphorylation at Ser262 (pSer262) is described as one of the predominant phosphorylation sites which determines microtubule binding of Tau (Biernat *et al.*, 1993; Scott *et al.*, 1993; Sengupta *et al.*, 1998). Many Tau kinases have been identified to be able to phosphorylate at this site, including GSK3β, CK1, PKA and the DNA damage kinase Chk2 (Hanger, Anderton and Noble, 2009; Mendoza *et al.*, 2013). Additionally, pSer262 is essential for Tau toxicity and Aβ-induced neurodegeneration (Nishimura, Yang and Lu, 2004; Iijima-Ando *et al.*, 2010; Iijima, Gatt and Iijima-Ando, 2010). Furthermore, overexpression of Chk2 enhances Tau induced neurodegeneration in *Drosophila,* which is dependent on pSer262 (Iijima-Ando *et al.*, 2010).

Chk2 phosphorylates 7 sites on Tau *in vitro* (Ser214, Ser262, Ser289, Thr403, Ser409, Ser433, Ser435) which are found to be phosphorylated in AD. Therefore, Tau phosphorylation could be another potential mechanism in which Chk2 activation mediates neurotoxicity in our amyloid toxicity model. I wanted to investigate whether activation of the DDR and subsequent activation of the ATM-Chk2 pathway could lead to toxic Tau phosphorylation events. Due to all of the above evidence in the literature I decided to focus on pSer262 (Mendoza *et al.*, 2013). This would allow us to determine whether Tau phosphorylation at Ser262 could be contributing to the deleterious effect ATM-Chk2 activation has on neural survival and function (Taylor *et al.*, 2022).

6.2 Results

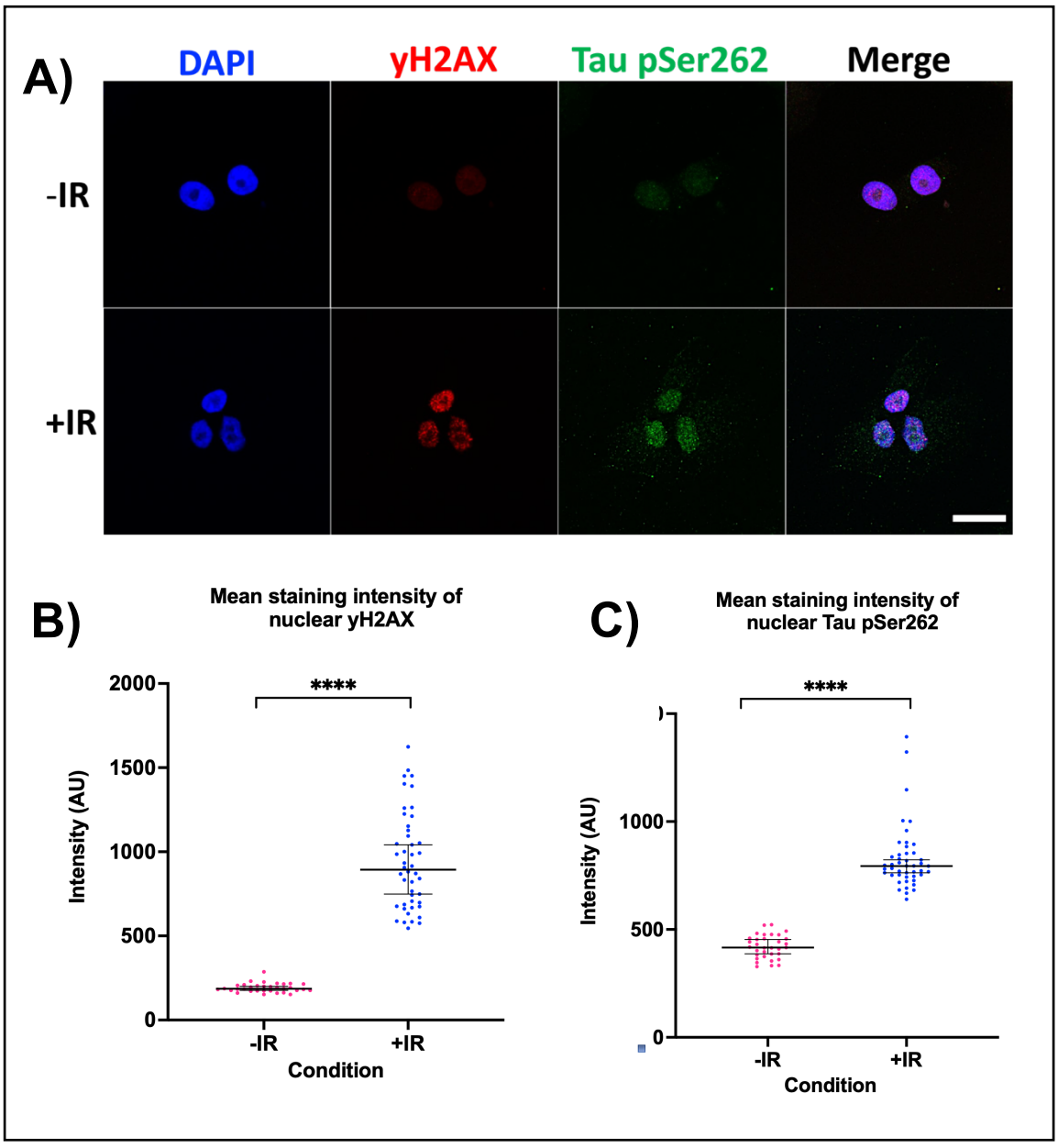
6.2.1 Tau phosphorylation at Serine 262 in response to DNA damage

To activate the DDR, SK-N-AS human neuroblastomas cells were exposed to 8 Gy X-irradiation (IR). Due to Tau being predominantly expressed in neurons, SK-N-AS cells were chosen as this is a human cell line capable of being differentiated into neurons *in vitro*. IR induces DSBs through direct damage to the sugar backbone but also indirectly through the generation of free radicals via hydrolysis of water molecules near to the DNA (Cannan and Pederson, 2017). ROS induces DNA damage through oxidation of bases or causing SSBs. DSBs can arise from this if SSBs are found close together, or during repair of the oxidised base via BER (Cannan and Pederson, 2017).

Irradiated cells were fixed after 1 h of recovery at 37 °C to allow the response to the DSBs to be mounted, then stained with anti-γH2AX, which detects phosphorylated histone H2A at Serine 139. This phosphorylation event is mediated by ATM in response to DSBs at thousands of histones surrounding the break, and hence is a commonly used marker of detecting DSBs (Kinner *et al.*, 2008). As expected, large numbers of characteristic γH2AX-positive foci were detected, which resulted in a significant increase in γH2AX staining intensity after IR compared to non-irradiated cells (Figure 6.1A & B, ****$p<0.0001$). In contrast, non-irradiated cells showed no distinct γH2AX foci (Figure 6.1A).

The irradiated cells were also stained with a phospho-specific antibody to Tau pSer262. The mean fluorescence staining intensity of nuclear Tau pSer262 for each cell was quantified using ImageJ (*see methods for full details*). This revealed a clear increase in Tau pSer262 levels after IR compared to -IR (Figure 6.1A and C ****$p<0.0001$). There was a clear, strong positive correlation between the intensity of nuclear Tau pSer262 staining and γH2AX staining (Figure 6.1D, $r^2 = 0.5313$, ****$p<0.0001$). Unexpectedly, pSer262 Tau was almost exclusively nuclear in

localisation. In contrast, using an antibody that recognises Tau independent of phosphorylation state (total Tau), revealed that total Tau was distributed throughout the cell, including the cytoplasm and nucleus (Figure 6.2A). The intensity of total Tau staining in the nucleus did not change after IR (Figure 6.2B, p=0.1965, not significant). This suggests that the increase in nuclear Tau pSer262 after irradiation is likely a result of phosphorylation of Tau already present in the nucleus, and not Tau translocating into the nucleus.



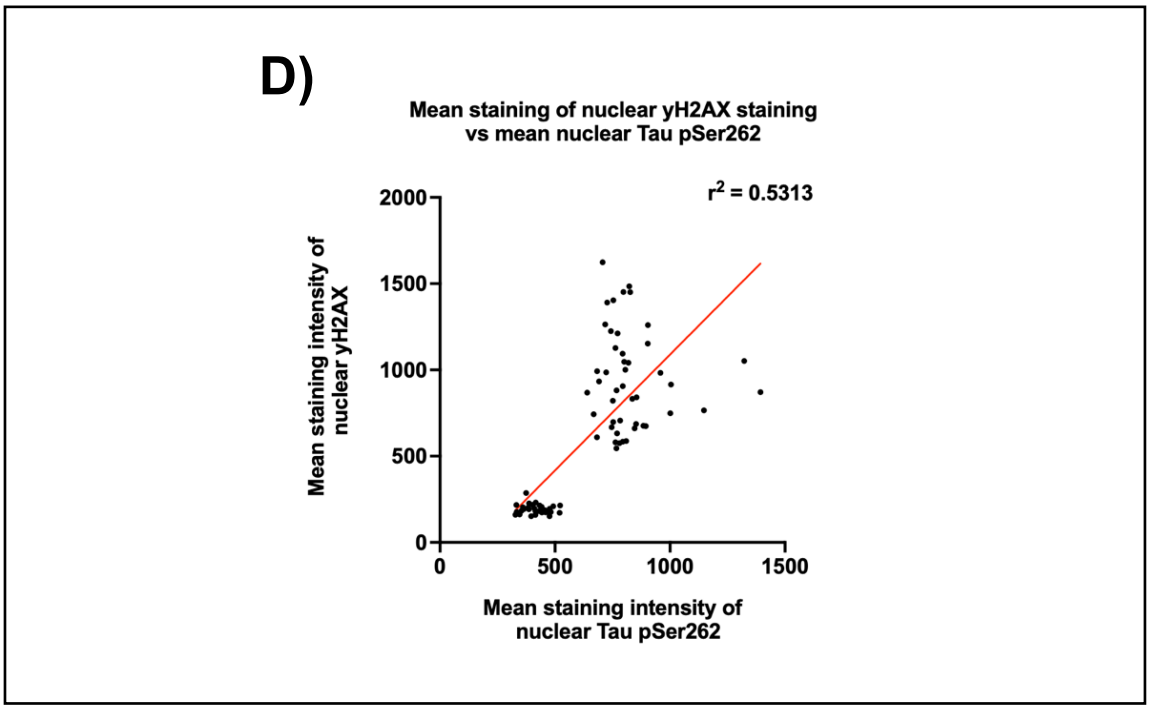


Figure 6.1 Irradiation of cells causes nuclear Tau phosphorylation at Ser262 A) Immunofluorescence images of cells after IR reveals phosphorylation of nuclear Tau at Ser262. 8 Gy of IR were applied to neuroblastomas. After 1 h cells were stained with DAPI (blue), γH2AX (red) and Tau pSer262 (green). Staining intensity of Tau pSer262 increases after IR and is predominantly nuclear. Scale bar = 20 μm **B) Scatter dot plot showing staining intensity of γH2AX +/- IR.** γH2AX staining intensity increases after IR (n=46) compared to -IR (****p<0.0001, n=33). Statistical analysis to compare Tau pSer262 staining was calculated using a Mann-Whitney test. Black line represents median, error bars represent 95% confidence interval. **C**) **Scatter dot plot showing staining intensity of nuclear Tau pSer262 +/- IR.** Analysis of nuclear Tau pSer262 staining intensity of the same cells reveals an increase after IR compared to -IR (****p<0.0001). Statistical analysis to compare Tau pSer262 and γH2AX staining was calculated using a Mann-Whitney test. Black line represents median, error bars represent 95% confidence interval. **D) Correlation graph showing relationship between nuclear staining of Tau pSer262 and H2AX phosphorylation.** A strong positive correlation exists between nuclear Tau pSer262 staining and H2AX phosphorylation induced by IR (r^2 = 0.5313, ****p<0.0001, n=79). Correlation significance was determined using a Pearson's correlation.

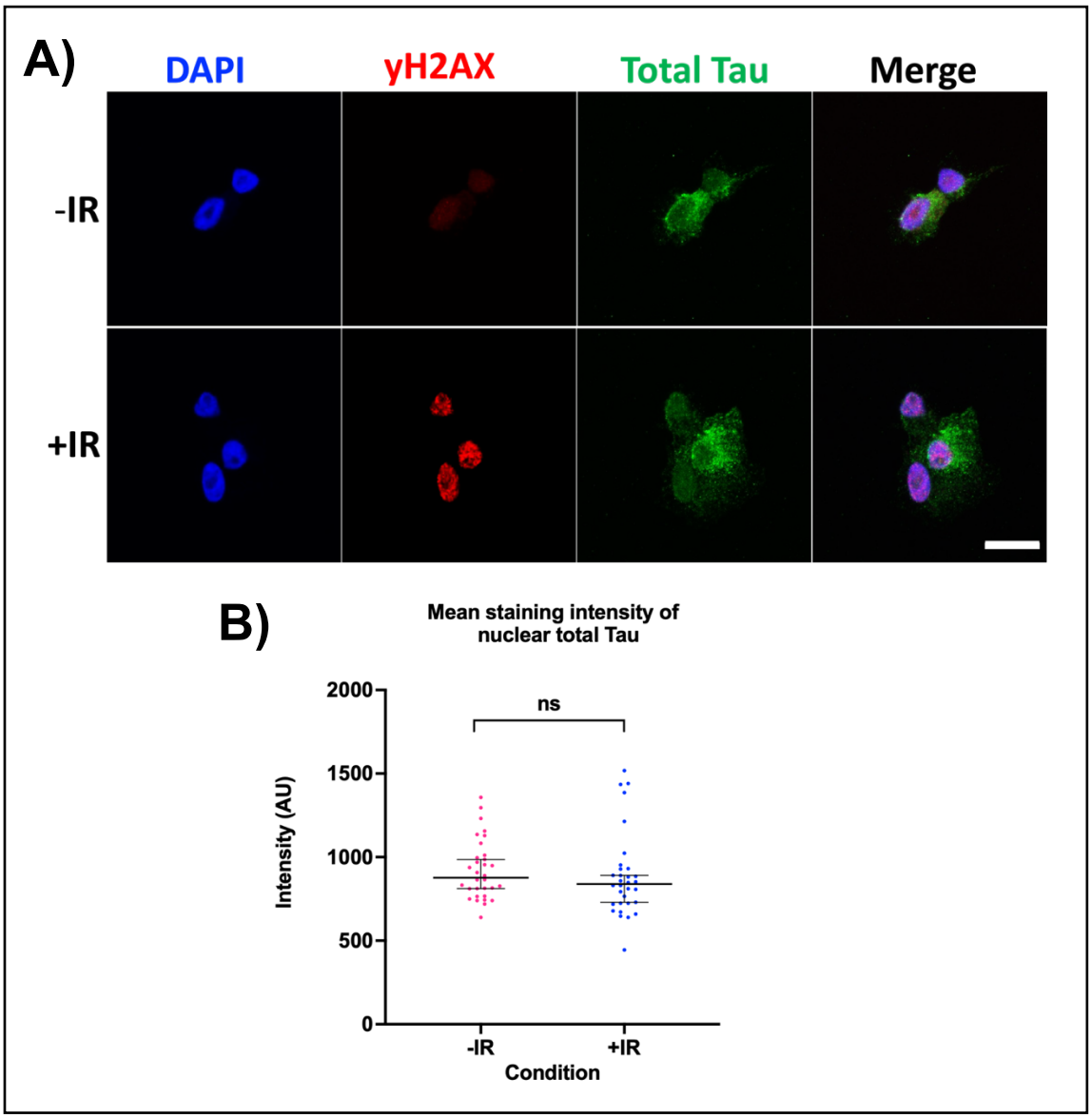
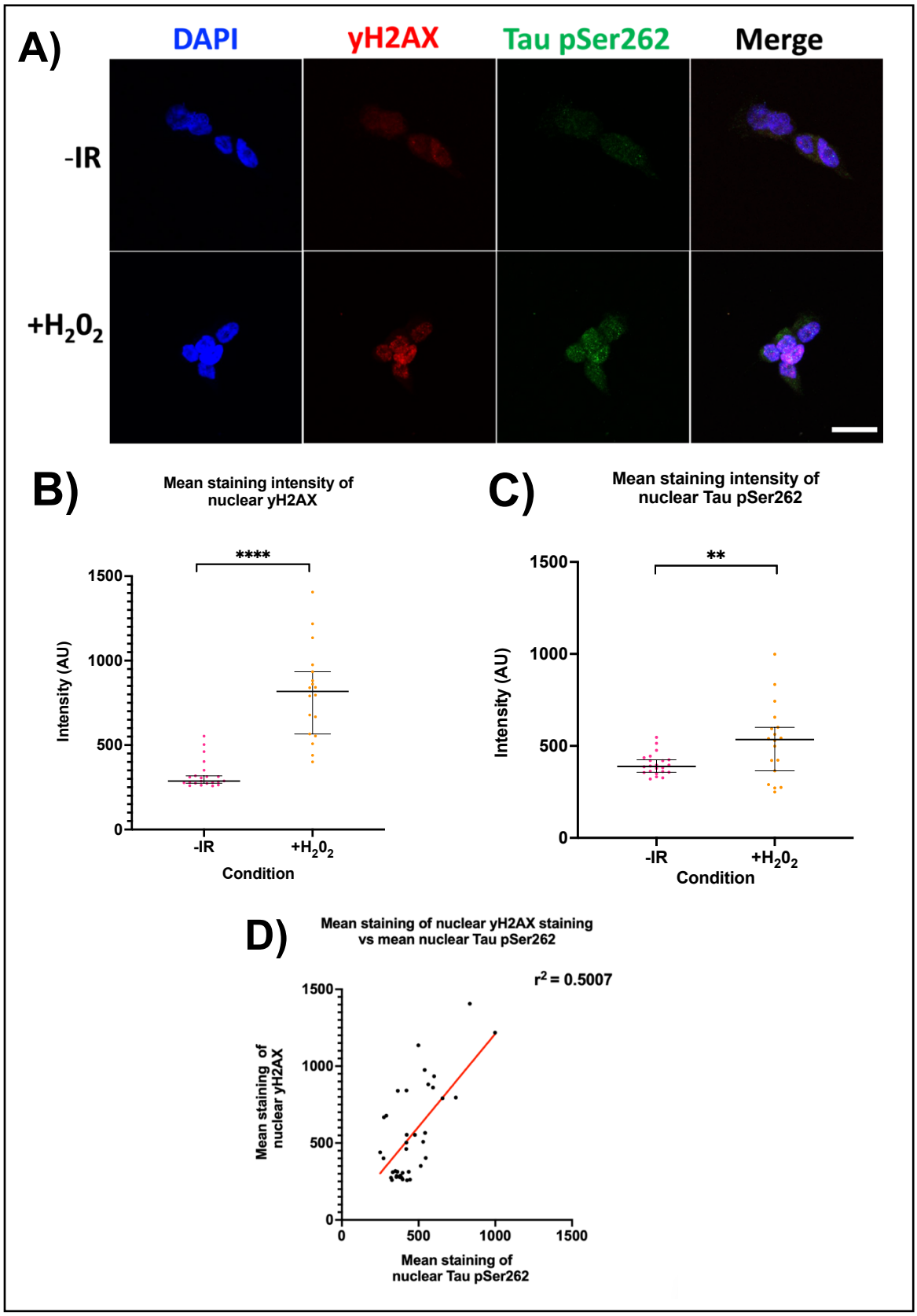


Figure 6.2. Irradiation of cells does not change the cellular distribution of total Tau.
A) Immunofluorescence images of cells after IR reveals no change to distribution of total Tau. 8 Gy of IR were applied to neuroblastomas. After 1 h cells were stained with DAPI (blue), γH2AX (red) and total Tau (green). Total Tau is localised throughout the cell which does not change after IR. Scale bar = 20 μm. **B) Scatter dot plot showing staining intensity of total Tau +/- IR.** There was no statistically significant change between the staining of nuclear total Tau +/-IR (n=32, not significant, Mann-Whitney test). Black line represents median, error bars represent 95% confidence interval.

After observing an increase in nuclear phosphorylation of Tau at Ser262 after activation of the DDR *via* IR, I next decided to activate the DDR pathway *via* an alternative stimulus: oxidative stress. Oxidative stress is elevated in AD and is another potential cause of the DNA damage that accumulates in AD (Zhao and Zhao, 2013). Accumulating Aβ deposits are a known source of ROS, potentially due to their interactions with metal ions. The interaction of Aβ with metal ions allows electron transfer to occur to the metal ions, producing free radicals such as ROS in the reaction (Cheignon *et al.*, 2018). ROS can induce DNA lesions such as SSBs or oxidise DNA bases which are repaired via BER. BER involves SSB intermediates, which if found clustered together could convert to DSBs (Srinivas *et al.*, 2019). Moreover, ATM is directly activated by raised levels of ROS (Xie *et al.*, 2021).

To induce oxidative stress, neuroblastomas were exposed to 200 μM of H_20_2 for 30 min, to generate ROS. Cells were then fixed and stained with anti-γH2AX and anti-Tau pSer262. Incubation of cells with 200 μM of H_20_2 was sufficient to induce DNA damage, seen by a visible increase in γH2AX positive foci and staining intensity compared to the control group (-IR) (Figure 6.3A and B, ****p<0.0001). Staining for anti-Tau pSer262 revealed an increase in nuclear Tau pSer262 after induction of oxidative stress, and again, this was a predominantly nuclear event (Figure 6.3A & C, **p=0.0100). As seen with IR, there was a strong positive correlation between staining intensity of nuclear Tau pSer262 and γH2AX foci (Figure 6.3D, r^2 = 0.5007, ****p<0.0001). It is plausible that chronic activation of the DDR via elevated levels of ROS leads to this nuclear phosphorylation event on Tau at Ser262, affecting Tau dynamics and microtubule binding and, ultimately, long-term consequences for neuronal function.


A)
DAPI
yH2AX
Tau pSer262
Merge
-IR
+H202
B)
Mean staining intensity of nuclear yH2AX
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Intensity (AU)
1500
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500
0
-IR
+H202
Condition
C)
Mean staining intensity of nuclear Tau pSer262
**
Intensity (AU)
1500
1000
500
0
-IR
+H202
Condition
D)
Mean staining of nuclear yH2AX staining vs mean nuclear Tau pSer262
r2 = 0.5007
Mean staining of nuclear yH2AX
1500
1000
500
0
0
500
1000
1500
Mean staining of nuclear Tau pSer262

Figure 6.3. Oxidative stress causes nuclear Tau phosphorylation at Ser262. **A) Immunofluorescence images of cells after inducing oxidative stress reveals phosphorylation of nuclear Tau at Ser262**. 200 μM of H_20_2 was added to neuroblastomas for 30 min. Cells were then stained with DAPI (blue), γH2AX (red) and Tau pSer262 (green). Staining intensity of Tau pSer262 increases with H_20_2 compared to without (-IR group) and is predominantly nuclear. Scale bar = 20 μm. **B) Scatter dot plot showing staining intensity of γH2AX +/- H_20_2**. There is an increase in γH2AX staining intensity after inducing oxidative stress with H_20_2 (n=18) compared to -IR (****p=<0.0001, n=22). Statistical analysis to compare γH2AX staining was calculated using a Mann-Whitney test. Black line represents median, error bars represent 95% confidence interval. **C) Scatter dot plot showing staining intensity of nuclear Tau pSer262 +/- H_20_2.** Analysis of nuclear Tau pSer262 staining intensity of the same cells reveals an increase after inducing oxidative stress with H_20_2 compared to -IR (**p=0.0100). Statistical analysis to compare pSer262 staining was calculated using an unpaired T-test. Black line represents median, error bars represent 95% confidence interval. **D) Correlation graph showing relationship between nuclear staining of Tau pSer262 and H2AX phosphorylation induced by applying H_20_2**. A strong positive correlation exists between nuclear Tau pSer262 staining and H2AX phosphorylation induced by H_20_2 (r^2 = 0.5007, ****p<0.0001, n=40). Correlation significance was determined using a Pearson's correlation.

6.2.2 Nuclear Tau Ser262 phosphorylation depends on ATM

As I have confirmed that inducing DNA damage increases phosphorylation of nuclear Tau at Ser262, I wanted to investigate if this phosphorylation event was dependent on activation of ATM to see if this phosphorylation event was specific to activation of the DDR and the ATM pathway.

In order to investigate if the increase in nuclear Tau pSer262 after IR was sensitive to ATM activity, 10 μM of the ATM inhibitor (ATMi) KU-60019 (IC_{50}= 6.3nM) was applied 1 h prior to IR (Golding *et al.*, 2009). KU-60019 is a very potent and highly specific inhibitor of ATM that works by acting as a competitive ATP inhibitor, preventing the S1981 autophosphorylation event, that is required for activation (Figure 6.5A) (Shiloh and Ziv, 2013). As before, after IR the cells were left 1 h after IR and then fixed and stained with anti-γH2AX. As previously, γH2AX foci was induced after irradiation (Figure 6.5B and C, ****p<0.0001). Inhibiting ATM prior to irradiation resulted in a significant decrease in γH2AX foci staining compared to irradiated cells alone (Figure 6.5B and C, ****p<0.0001). This was expected since H2AX is directly phosphorylated on Ser139 by ATM in response to DSB detection (Kinner *et al.*, 2008).

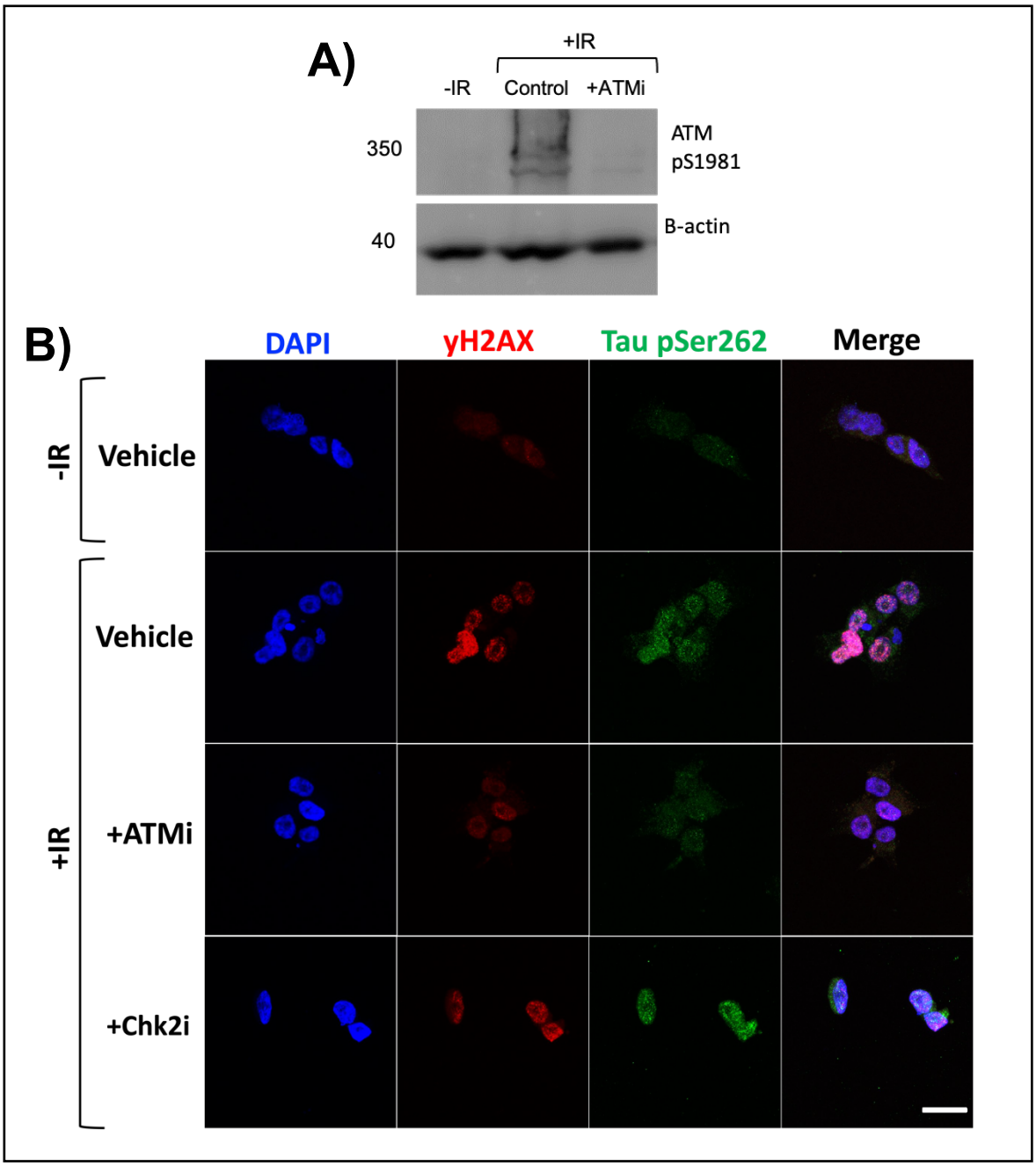
As before, the irradiated cells were then stained with anti-Tau pSer262, and the mean nuclear fluorescence quantified. Like previously, IR led to an increase in the nuclear Tau pSer262 levels compared to -IR (Figure 6.5B and D, ****p<0.0001) but ATM inhibition significantly reduced this phosphorylation event (Figure 6.5B and D, ****p<0.0001). My findings indicate that the nuclear phosphorylation of Tau Ser262 after IR is dependent on ATM activation and suggested the neuroprotective effect seen when targeting the ATM-Chk2 pathway could be due to a decrease in nuclear Tau Ser262 phosphorylation. However, it is unlikely that ATM directly phosphorylates this site on Tau. ATM phosphorylates serine or threonine residues followed by a glutamate (SQ or TQ motifs) (Matsuoka *et al.*, 2007; Pines *et al.*, 2011). The amino acid sequence surrounding Tau Ser262 does not resemble the consensus sequence used by ATM (Pines *et al.*, 2011). Instead, a kinase activated downstream of ATM is

likely to be responsible for this nuclear phosphorylation event on Tau in response to DNA damage. One likely candidate is Chk2, which is phosphorylated and activated by ATM in the DDR and has been shown to be capable of phosphorylating Tau on Ser262 *in vitro* (Iijima-Ando *et al.*, 2010; Mendoza *et al.*, 2013). Moreover, reducing *Chk2* expression genetically via RNAi is neuroprotective in an amyloid toxicity Drosophila model (Taylor *et al.*, 2022).

To test that the nuclear Tau phosphorylation event I see in irradiated cells was indeed Chk2 dependent, I used the same molecular inhibitor approach as for ATM – 1 h prior to irradiation, 10 μM of the Chk2 inhibitor (Chk2i) CCT241533 (IC_{50} = 3nM) was applied to neuroblastoma cells. CCT241533 is a selective and potent ATP inhibitor of Chk2, therefore inhibiting Chk2 autophosphorylation events which are needed for full Chk2 activation (Anderson *et al.*, 2011). A confirmation of Chk2 inhibition following Chk2i treatment of cells was attempted but multiple commercial anti-pChk2 antibodies failed to work as advertised and did not recognise pChk2 in western blots of irradiated cells (the positive controls).

After IR, neuroblastoma cells were left 1 h and then fixed and stained with anti-γH2AX as before. There was a significant increase in staining intensity of γH2AX foci upon irradiation compared to non-irradiated cells (Figure 6.5B and C, ****p<0.0001). Chk2 inhibition did not significantly affect γH2AX foci staining when compared to irradiated cells alone (Figure 6.5C, p=0.1311, not significant). This was expected since Chk2 is downstream of ATM in the DDR and therefore inhibiting Chk2 should not affect H2AX phosphorylation. As before, the irradiated cells were then stained with anti-Tau pSer262, and the mean nuclear fluorescence quantified. As seen before, IR led to an increase in nuclear Tau pSer262 levels compared to -IR (Figure 6.5B and D, ****p<0.0001), but unexpectedly Chk2 inhibition did not reduce the Tau pSer262 staining (Figure 6.5B). There was no statistically significant difference in nuclear Tau pSer262 staining between the irradiated cells and those with Chk2 inhibited prior to irradiation (Figure 6.5D, p>0.9999, not significant). One explanation could be that the inhibitor may have failed to inhibit Chk2 activity using these doses and method of application, despite colleagues using the same inhibitors successfully

in other systems at these doses (see section 6.3.2 for discussion on this). A second Chk2 inhibitor was used (BML-227), which is an ATP competitive inhibitor of Chk2, but this resulted in rapid blebbing of the neuroblastoma cells (results not shown). My findings indicate that the increase in phosphorylation of nuclear Tau on pSer262 after IR is not dependent on Chk2 activity. However, my results cannot be considered conclusive since the failure of the anti-pChk2 antibodies to detect their target means I cannot definitively confirm the CCT241533 inhibitor worked in these cells.



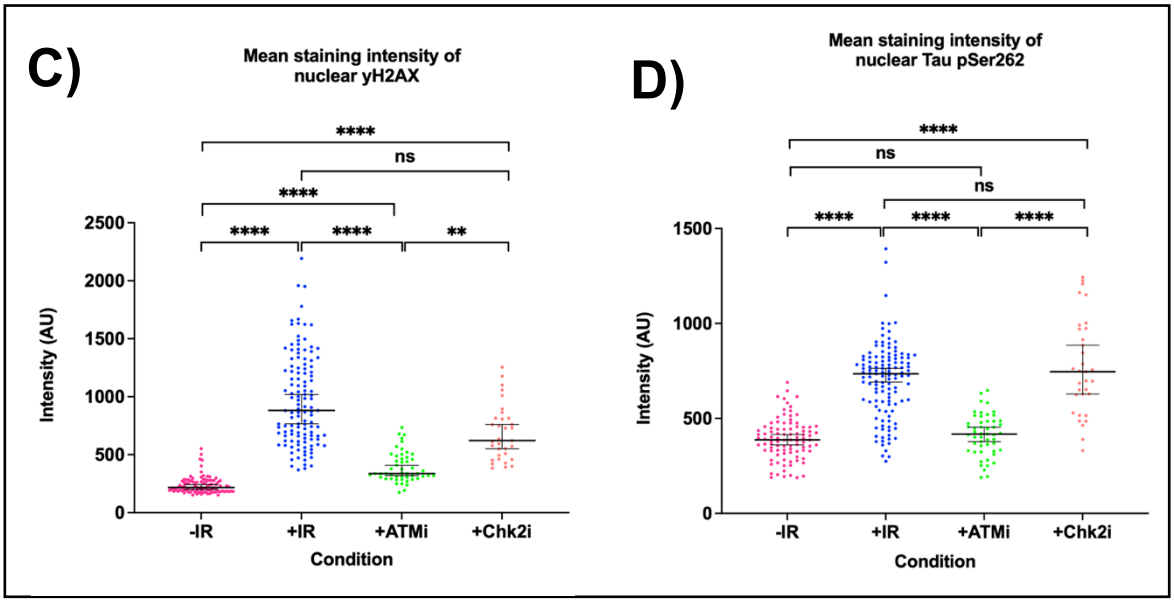


Figure 6.5 Nuclear Tau phosphorylation at Ser262 after IR is dependent on ATM activation but not Chk2 activation.

A) Western blot confirming the ATM inhibitor blocks ATM activation. The ATM inhibitor blocks the autophosphorylation event on ATM at Ser1981, required for activity. **B) Immunofluorescence images of cells reveals nuclear Tau pSer262 increases after IR but decreases with ATMi.** 1 h prior to 8 Gy of IR, 10 μM of the ATMi or Chk2i were added to cells. Vehicle control is DMSO alone. 1 h after IR, neuroblastomas were stained with DAPI (blue), γH2AX (red) and Tau pSer262 (green). The increase in nuclear Tau pSer262 staining intensity seen after application of irradiation (+IR) reduces with ATMi prior to irradiation (+ATMi). Scale bar = 20 μm. **C) Scatter dot plot showing staining intensity of γH2AX +/-IR with +/- ATMi or Chk2i.** Staining intensity of γH2AX increases after +IR (****p<0.0001, n=125) compared to -IR (n=95) which is decreased when ATM is inhibited before IR (****p<0.0001, n= 51). Inhibiting Chk2 before IR had no effect on γH2AX phosphorylation compared to +IR (n= 33, not significant). Black line represents median, error bars represent 95% confidence interval. **D) Scatter dot plot showing staining intensity of nuclear Tau pSer262 +/-IR with +/-ATMi/Chk2i.** Analysis of nuclear Tau pSer262 staining intensity of the same cells reveals an increase after IR (****p<0.0001) compared to -IR. Nuclear Tau pSer262 induced by IR decreases with ATMi (****p<0.0001) but not Chk2i (not significant). All statistical analysis to compare Tau pSer262 staining and γH2AX staining was calculated using a Kruskal-Wallis test with Dunn's multiple comparisons test. Black line represents median, error bars represent 95% confidence interval. Groups +/- IR presents data from 3 independent experiments. Groups +ATMi / +Chk2i presents data from 2 independent experiments.

6.2.3 Nuclear Tau pSer262 is not dependent on AMPK

Knowing that nuclear Tau Ser262 phosphorylation was sensitive to ATM, I decided to look at another downstream kinase of ATM known to be able to phosphorylate Tau at Ser262: AMPK (Thornton *et al.*, 2011). AMPK controls metabolic pathways in the cell in response to low ATP levels. AMPK is activated by increased AMP levels (low ATP) in response to stimuli such as hypoxia or oxidative stress (Kim *et al.*, 2016). Binding of AMP changes the structure of AMPK allowing full access of upstream kinases to phosphorylate Thr172 in the kinase loop, which is needed for complete activation. AMPK can be activated by ATM in response to DSBs and oxidative stress, both of which are elevated in AD (Fu *et al.*, 2008; Alexander *et al.*, 2010). AMPK is also activated by exposure to amyloid beta peptides (Thornton *et al.*, 2011).

I have also shown that both IR and oxidative stress can increase nuclear Tau pSer262, making AMPK a candidate. In order to investigate if nuclear Tau pSer262 was sensitive to inhibitors of AMPK, 1 h prior to irradiation 10μM of the AMPK inhibitor (AMPKi) SBI-0206965 (IC_{50} = 170 nM) was applied to the neuroblastomas (Ahwazi *et al.*, 2021). AMPK activity is triggered by binding of ATP which induces phosphorylation of threonine 172 in the kinase activation loop via upstream kinases (Dite *et al.*, 2018). SBI-0206965 inhibits AMPK activation and signalling by overlapping with the ATP binding site (Dite *et al.*, 2018). As previously, cells were left 1 h after IR and then fixed and stained with anti-γH2AX and anti-Tau pSer262. As seen before, there was a significant increase in the staining intensity of γH2AX foci after irradiation compared to non-irradiated cells (Figure 6.6A and B, ****p<0.0001). As expected, AMPK inhibition did not significantly affect γH2AX foci staining when compared to irradiated cells alone (Figure 6.6A and B, p=0.2610, not significant).

Similarly, to Chk2i, the intensity of nuclear Tau pSer262 did not significantly change when AMPK was inhibited prior to irradiation compared to +IR alone (Figure 6.6A and C, p>0.9999, not significant). My results suggest that the increase in nuclear Tau pSer262 after IR is not dependent on AMPK. However, my results cannot be considered conclusive as I did not definitively show that the AMPKi worked

successfully. On reflection, to confirm if the AMPKi had worked successfully I would have carried out a western blot using an antibody against phosphorylated acetyl-CoA carboxylase (ACC1), a downstream target of AMPK, which would have decreased upon AMPKi inhibition, as previously seen (Dite *et al.*, 2018). An antibody against pThr-172 could not be used as even upon inhibition with SBI-0206965, an increase in pThr-172 was still observed which was suggested to be because the application of SBI-0206965 did not suppress upstream kinase activity (Dite *et al.*, 2018).

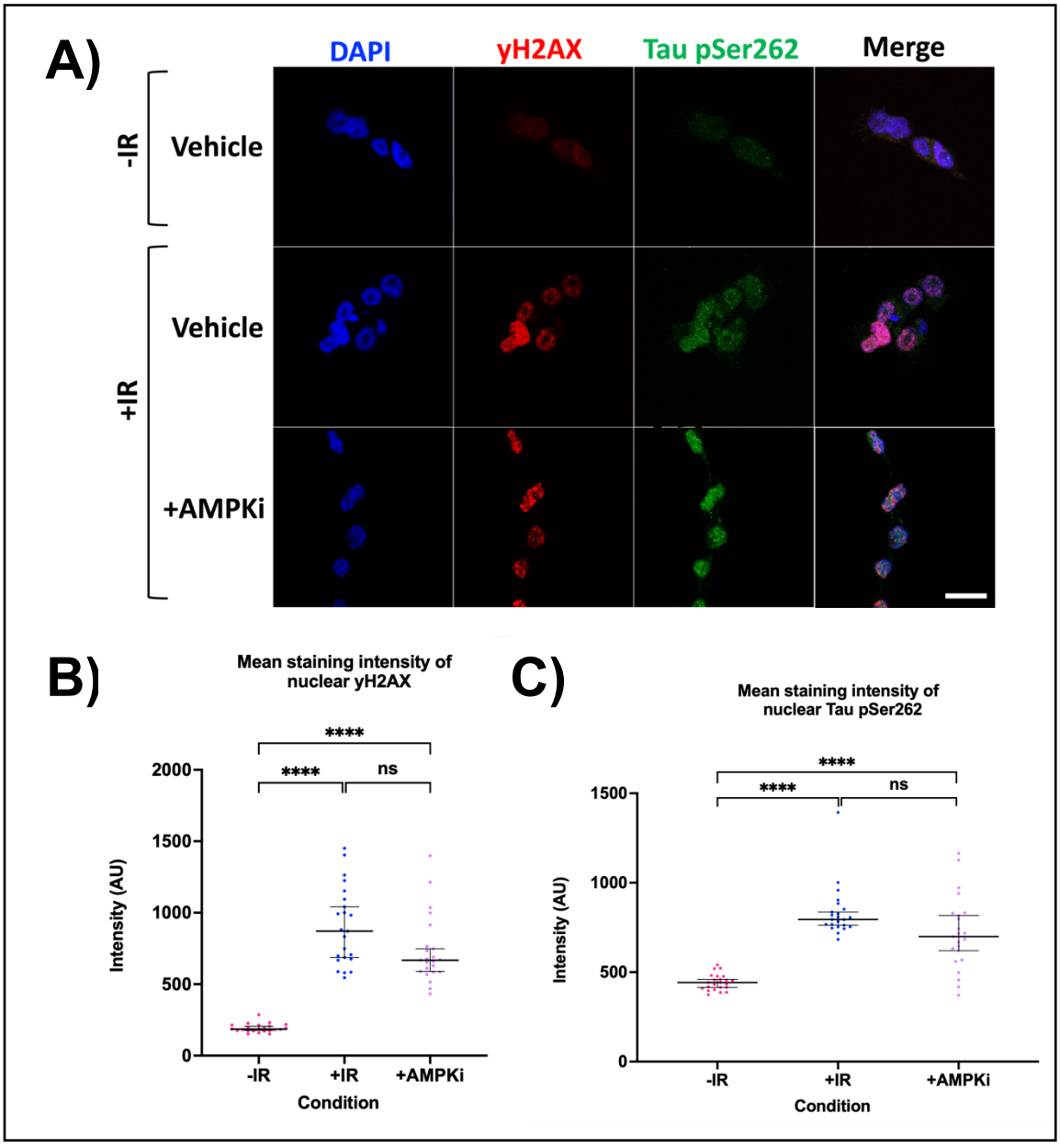


Figure 6.6 Nuclear Tau pSer262 after IR is not dependent on AMPK activation. A) Immunofluorescence images of cells reveal nuclear Tau pSer262 after IR is not AMPK dependent. 1 h prior to 8 Gy of IR, 10 μM of AMPKi was added to cells. Vehicle control is DMSO alone. 1 h after IR cells were stained with DAPI (blue), γH2AX (red) and Tau pSer262 (green). The increase in staining intensity of nuclear Tau pSer262 seen after application of irradiation (+IR) does not reduce with AMPKi prior to irradiation (+AMPKi). Scale bar = 20 μm. **B) Scatter dot plot showing staining intensity of γH2AX +/- IR.** Staining intensity of γH2AX increases after IR (****p<0.0001, n=23) compared to -IR (n=23) but inhibiting AMPK made no significant change to γH2AX phosphorylation when inhibited prior to IR (n=23, not significant). Black line represents median, error bars represent 95% confidence interval. **C) Scatter dot plot showing staining intensity of nuclear Tau pSer262 +/- IR with AMPKi.** Analysis of nuclear Tau pSer262 staining intensity of the same cells reveals an increase after IR (****p<0.0001) compared to –IR**.** Nuclear Tau pSer262 induced by IR does not change with AMPKi inhibited prior to irradiation (not significant). Black line represents median, error bars represent 95% confidence interval. All statistical analysis to compare Tau pSer262 staining and γH2AX staining was calculated using a Kruskal-Wallis test with Dunn's multiple comparison test.

6.2.4 Other Tau phosphorylation events are not induced by irradiation

Cellular stress in the form of DNA damage has previously been shown to be a stimulus for the accumulation of phosphorylated Tau. Formaldehyde exposure induces nuclear Tau phosphorylation at Thr181 and Ser396 (J. Lu *et al.*, 2013), and exposure to Aβ oligomers induces nuclear Tau phosphorylation at Tyr216 (Noel, Barrier and Ingrand, 2016). Formaldehyde induces DNA damage by causing DNA-protein crosslinks and Aβ peptides induce DSBs *in vitro* and *in vivo* and also are a source of ROS (Suberbielle *et al.*, 2013; Cheignon *et al.*, 2018; Tuxworth *et al.*, 2019). Other stimuli such as oxidative stress and heat shock also cause translocation of dephosphorylated Tau into the nucleus, indicating Tau could be involved in a nuclear function in response to environment stress (Sultan *et al.*, 2011). DNA

damage can be considered a form of cellular stress. Therefore, I wanted to investigate if DDR activation induces general widespread nuclear Tau phosphorylation or whether it was specific to Ser262.

I chose to investigate two Tau sites known to be abnormally phosphorylated in AD: Ser 396 and Ser 404, which are recognised by the well characterised PHF-1 monoclonal antibody (Greenberg *et al.*, 1992). Moreover, Ser396/Ser404 phosphorylation has been shown to be induced by osmotic stress (Stoothoff and Johnson, 2001). To investigate if nuclear Tau phosphorylation at Ser396/404 could be induced by DDR activation, cells were irradiated, fixed, and stained as previously. Cells were stained with anti-γH2AX (green) and anti-pSer396/404 (PHF-1; red). As seen previously, irradiation induced foci to form and an increase in staining intensity with anti-γH2AX was seen 60 min after IR compared to -IR (Figure 6.7A and B, ****$p<0.0001$). A weak signal was detected in the nucleus using anti-pSer396/404, which did not significantly change after irradiation (Figure 6.7A and C, $p=0.0619$, not significant). My results indicate that the previously identified site Ser396/404 which could be phosphorylated in response to osmotic stress, is not induced in response to other cellular stress such as DNA damage here. My results so far suggest Tau phosphorylation in response to DNA damage activation does not result in general widespread phosphorylation and could indicate pSer262 plays a particular role in response to DNA damage.

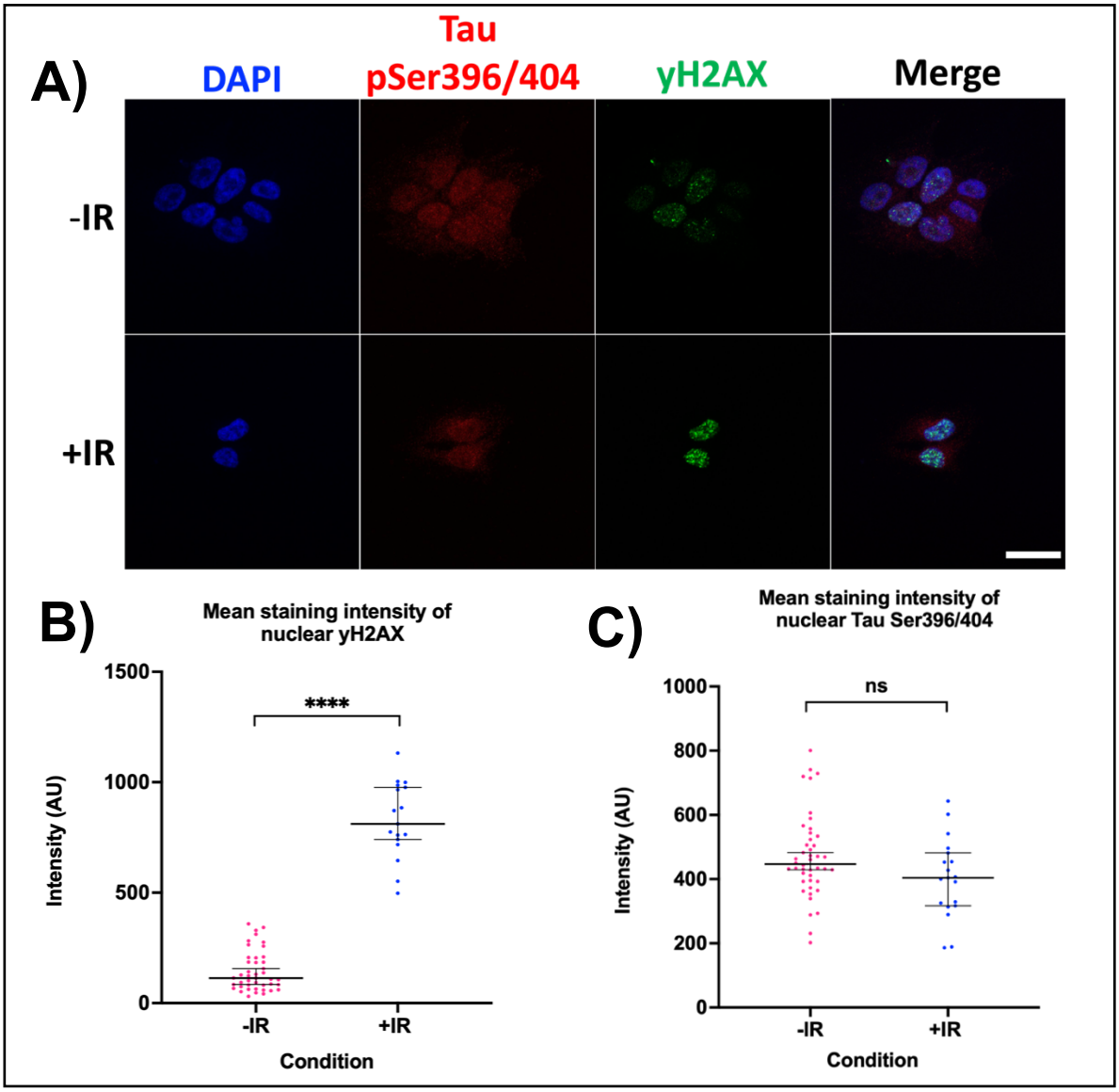


Figure 6.7 Tau pSer396/404 is not induced by irradiation.
A) Immunofluorescence images of cells reveal no change in pSer396/404 after irradiation. 8 Gy of IR were applied to neuroblastomas. 1 h after IR cells were stained with DAPI (blue), γH2AX (green) and Tau pSer396/404 (red). A weak signal of pSer396/404 is detected in the nucleus which doesn't change after IR**.** Scale bar = 20 μm. **B**) **Scatter dot plot showing staining intensity of γH2AX +/-IR.** Staining intensity of γH2AX increases after +IR (n=44) compared to -IR (****p<0.0001, n=19). Black line represents median, error bars represent 95% confidence interval**. C) Scatter dot plot showing staining intensity of nuclear Tau pSer396/404 +/- IR.** Analysis of nuclear Tau pSer396/404 staining intensity of the same cells reveal no significant change after IR compared to -IR (not significant). Black line represents median, error bars represent 95% confidence interval. Statistical analysis to compare Tau pSer262 staining and γH2AX staining was calculated using a Mann-Whitney test.

6.2.5 Antibody batch failure

Post- the COVID 19 shutdown, a new batch of Tau pSer262 antibody was ordered from Invitrogen and used to restart experiments. The experiments were repeated identically to previously, but with the new batch of antibody the initial increase in phosphorylation of nuclear Tau Ser262 after IR could not be detected (Figure 6.8A and C, p=0.4601, not significant). The staining intensity of nuclear Tau pSer262 before and after IR did not significantly change (Figure 6.8A and C), and in marked contrast to the original experiments. However, as previously seen before, there still was a significant reduction in nuclear pSer262 staining when ATM was inhibited prior to irradiation, compared to irradiated cells alone (Figure 6.8C, ****p<0.0001), but the magnitude of the decrease in staining intensity was much smaller than seen previously, when staining almost completely disappeared (compare figure 6.8A with 6.5B). Chk2 inhibition also significantly decreased the pSer262 staining which was not seen before (Figure 6.8A and C, ****p<0.0001). Again, the magnitude of the decrease was small. It must be noted that the n numbers for the Chk2 group in the previous experiment (n=33) was a lot smaller compared to this experiment (n=80) which could affect significance.

Due to the contradictory immunofluorescence results after changing antibody batch, I wanted to test the specificity of the new batch. Site directed mutagenesis was used to mutate the Ser262 residue in a GFP-tagged 2N4R Tau cDNA cloned into a Lentiviral vector (pLenti-DsRed_IRES_MAPT:EGFP) (Rousseaux *et al.*, 2016). The serine 262 (codon TCC) was mutated to an alanine (codon GCC) to generate a non-phosphorylatable site. Successful sequence alteration was confirmed by Sanger sequencing. The wildtype (S262) and the mutated plasmids (S262A) were then transfected into A549 lung carcinoma epithelial cells. After a survey of commonly used cell lines, A549 cells were chosen due to their low levels of endogenous Tau. Cells were transfected 1 day prior to fixation and stained with anti-total Tau (which detects Tau independent of its phosphorylation state) and anti-GFP. Cells that had successfully taken up the plasmid were identified using the expression of the GFP (Figure 6.9A). As expected, total Tau levels were higher in the transfected cells when

compared to non-transfected A549 cell, where a weak signal was detected, reflecting the low levels of endogenous Tau (Figure 6.9A). Two antibodies were tested for their specificity for Tau pSer262: the Invitrogen antibody used for the initial experiments and a second from Signalway. Unexpectedly, both antibodies produced a strong signal against the cells expressing wildtype Tau or the mutated Tau S262A (Figure 6.9B & C). The mutagenised vector was Sanger sequenced for a second time to confirm the presence of the S262A mutation, which proved to be correct. My conclusion was that neither the new Invitrogen anti-pSer262 batch nor an alternative antibody sourced from the Signalway antibody was specific to Tau pSer262.

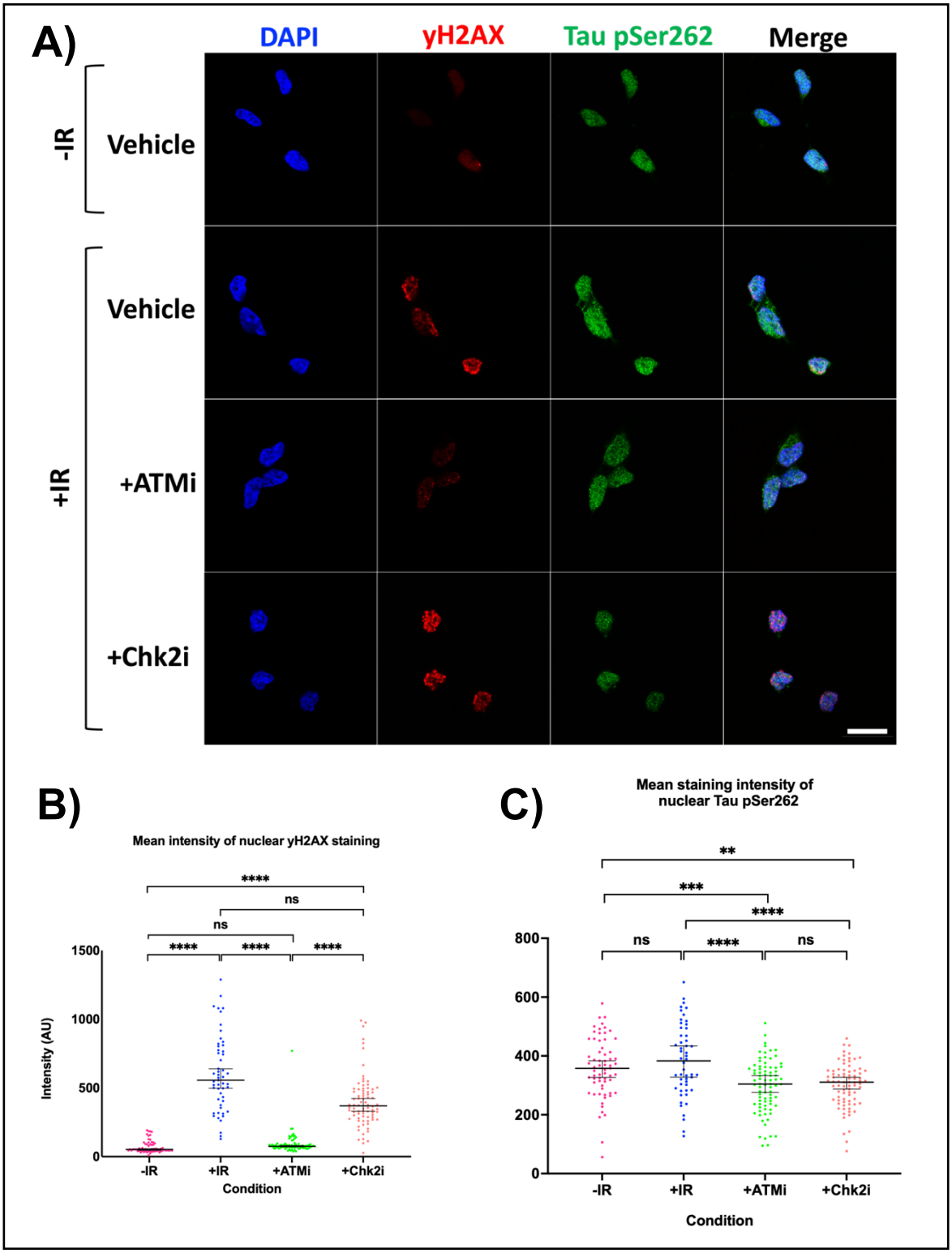

A)
DAPI
yH2AX
Tau pSer262
Merge
-IR
Vehicle
+IR
Vehicle
+ATMi
+Chk2i
B)
Mean intensity of nuclear yH2AX staining
Intensity (AU)
1500
1000
500
0
****
ns
ns
****
****
****
-IR
+IR
+ATMi
+Chk2i
Condition
C)
Mean staining intensity of nuclear Tau pSer262
800
600
400
200
0
**
***
****
ns
****
ns
-IR
+IR
+ATMi
+Chk2i
Condition

Figure 6.8 New antibody batch of Invitrogen Tau pSer262 did not detect the increase in nuclear Tau pSer262 after IR.
A) Immunofluorescence images of cells reveal no change in nuclear Tau phosphorylation at Ser262 after IR with a new Invitrogen antibody batch. 1 h prior to 8-Gys IR, 10 of the ATMi or Chk2i were added to cells. Vehicle control is DMSO alone. 1 h after IR, cells were stained with DAPI (blue), γH2AX (red) and Tau pSer262 (green) from the new batch. Staining intensity of nuclear Tau pSer262 did not change with +IR or decrease upon ATMi. Scale bar = 20 μm. **B**) **Scatter dot plot showing staining intensity of γH2AX +/-IR.** Staining intensity of γH2AX increases after IR (****p<0.0001, n=52) compared to -IR (n=65) and decreases with ATMi (****p<0.0001, n=81) but not Chk2i (p=0.1079, not significant, n=80). Statistical analysis to compare γH2AX staining was calculated using a Kruskal-Wallis test with Dunn's multiple comparison test. Black line represents median, error bars represent 95% confidence interval. **C**) **Scatter dot plot showing staining intensity of nuclear Tau pSer262 +/- IR and +/- ATMi/Chk2i.** Analysis of nuclear Tau pSer262 staining intensity of the same cells reveal no significant change after IR compared to -IR (not significant), however the staining intensity of pSer262 does significantly decrease compared to +IR alone when either ATM (****p<0.0001) or Chk2 are inhibited prior to irradiation (****p<0.0001). Statistical analysis to compare Tau pSer262 staining was calculated using a one-way ANOVA with Tukey's multiple comparison test. Graphs here includes data from 2 independent experiments collated. Black line represents median, error bars represent 95% confidence interval.

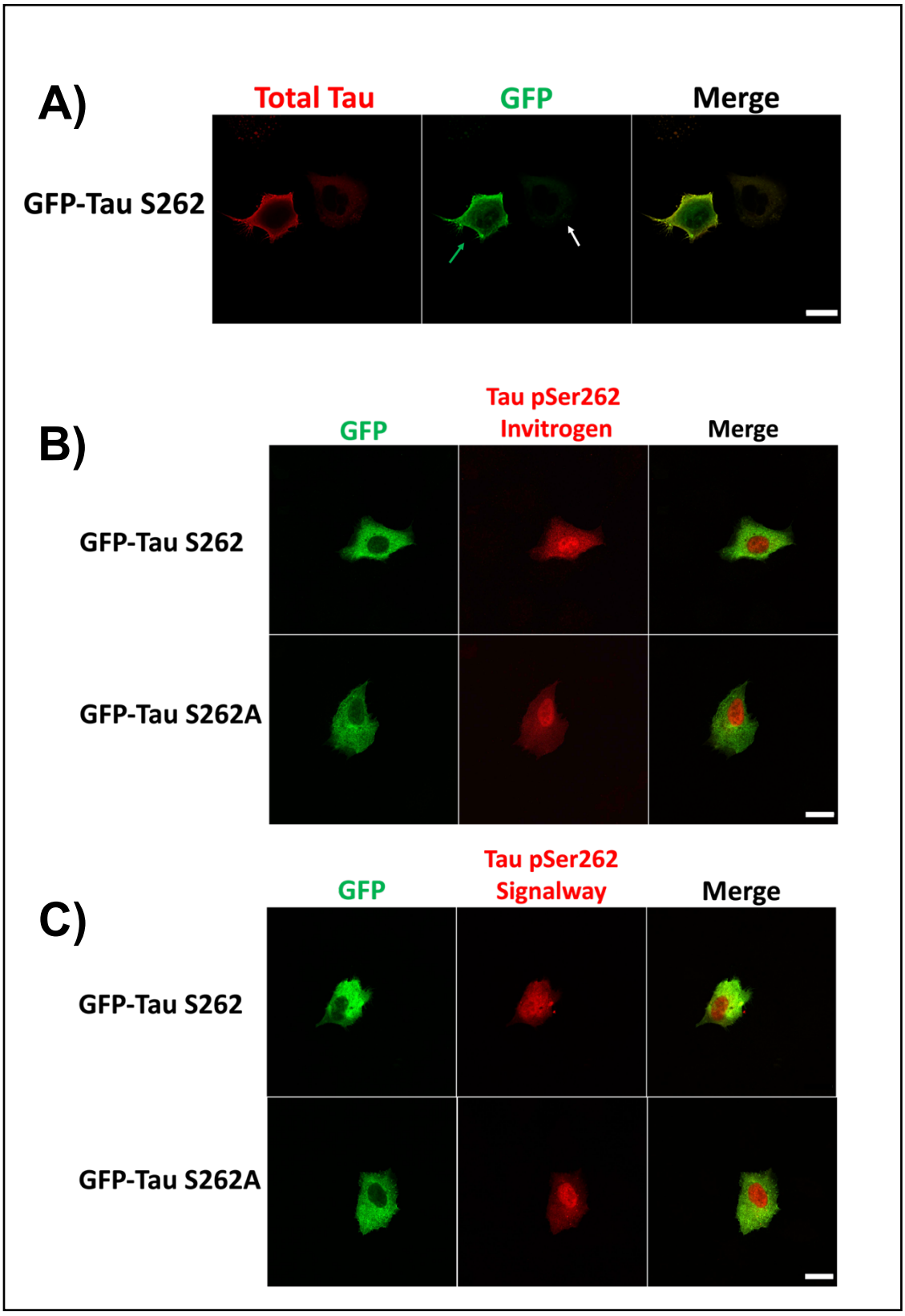

A)
Total Tau
GFP
Merge
GFP-Tau S262
B)
Tau pSer262
Invitrogen
GFP
Merge
GFP-Tau S262
GFP-Tau S262A
C)
Tau pSer262
Signalway
GFP
Merge
GFP-Tau S262
GFP-Tau S262A

Figure 6.9 Invitrogen and Signalway antibodies to Tau pSer262 are not specific. A) Immunofluorescence images showing an example of successful transformation of A549 cells with wildtype GFP-tagged Tau. 1 day prior to staining, A549 cells were transfected with a wildtype GFP-tagged Tau. Cells were then stained for total Tau (red) and GFP (green). Green arrow points to a transfected cell, white arrow points to an untransfected cell. **B) The new Invitrogen batch of anti- Tau pSer262 is not specific to pSer262.** Site directed mutagenesis was used to mutate the codon for serine 262 to an alanine on Tau, to generate a non phosphorylatable site (GFP-TauS262A). Cells were then transfected with the mutated or wildtype GFP-tagged Tau plasmid and stained for anti-GFP (green) and anti-Tau pSer262 (red) from Invitrogen. A strong signal was detected in the S262A cells in both the cytosol and the nucleus, indicating Invitrogen pSer262 antibody is not specific to this site. **C) Signalway anti-Tau pSer262 is not specific to pSer262.** Cells were transfected with the wildtype plasmid containing GFP-Tau S262 or the mutant plasmid containing GFP-Tau S262A. Cells were then stained with anti-GFP (green) and anti-Tau pSer262 (red) from Signalway. A signal was detected in the S262A cell in both the cytosol and the nucleus using the Signalway pSer262 antibody, indicating this antibody was also not specific. All scale bars = 20 μm.

6.3 Discussion

6.3.1 Phosphorylation of Tau Ser262

Phosphorylation of Tau on Ser262 was originally discovered to be an essential site in controlling the ability of Tau to bind to microtubules (Biernat *et al.*, 1993; Sengupta *et al.*, 1998). Over the years, phosphorylation of Tau on Ser262 has received much attention as one of the of the earliest Tau phosphorylation events to occur in AD pre-tangles, suggesting it is important in early-stage disease (Augustinack *et al.*, 2002; Lauckner, Frey and Geula, 2003). Moreover, phosphorylation at Tau Ser262 is involved in initiating phosphorylation at other AD related sites and is needed for Tau induced neurodegeneration and Aβ induced Tau toxicity (Nishimura, Yang and Lu, 2004; Iijima-Ando *et al.*, 2010; Iijima, Gatt and Iijima-Ando, 2010). I chose to investigate the phosphorylation site Ser262 because of these reasons and because overexpression of Chk2 in *Drosophila* expressing human Tau enhanced neurodegeneration and phosphorylation at this site was critical for this toxicity (Iijima-Ando *et al.*, 2010). Furthermore, knockdown of *Chk2* was protective against the toxicity of amyloid beta in *Drosophila* (Taylor *et al.*, 2022).

My initial findings here show that activation of the DDR and consequently activation of the ATM/Chk2 pathway leads to a nuclear Tau phosphorylation at Ser262 in human neuroblastoma cells (Figure 6.1 and 6.3). This phosphorylation event was dependent on ATM activity but was not affected by treating cells with the Chk2 inhibitor (Figure 6.5). As nuclear Tau phosphorylation was not dependent on Chk2 activation, my data suggests it is unlikely that this phosphorylation event is a major contributing factor to the neurotoxic effect of expressing Aβ in our *Drosophila* amyloid toxicity model. However, the loss of antibody specificity of the key reagent means these findings cannot be considered conclusive.

6.3.2 Nuclear Tau pSer262 in response DNA damage is ATM dependent

In response to DDR activation via irradiation and induction of oxidative stress, phosphorylation of nuclear Tau at Ser262 increased in neuroblastoma cells (Figure 6.1 and 6.3). Tau phosphorylation at Ser262 in response to irradiation was dependent on ATM activity (Figure 6.5). This suggests that activation of the DDR could be the initial starting point leading to the abnormal phosphorylation of Tau at Ser262 in AD. As previously stated, it is unlikely ATM directly phosphorylates Tau Ser262 as the consensus sequence of ATM is not found surrounding Serine 262, which requires a serine or threonine residues followed by a glutamate (SQ or TQ) (Matsuoka *et al.*, 2007). Instead, a kinase activated downstream of ATM is likely to be responsible for this nuclear phosphorylation event on Tau.

Chk2 lies downstream of the ATM kinase in the DNA damage response pathway, and can indeed phosphorylate Tau at Ser262 *in vitro* (Mendoza *et al.*, 2013), therefore it was hypothesised that Chk2 could be responsible for Tau Ser262 phosphorylation. However, application of the Chk2i before activation of the DDR did not change the phosphorylation of Tau seen at Serine 262 in response to irradiation (Figure 6.5). My results indicate that Chk2 does not phosphorylate Serine 262 which is different to previous studies that have used an *in vitro* phosphorylation system (Mendoza *et al.*, 2013). My results may differ to previous studies that have used an *in vitro* system as this involves adding together large quantities of kinase and purified Tau and this does not necessarily reflect what may happen in a cell (Mendoza *et al.*, 2013). Additionally, I cannot confirm the Chk2 inhibitor was definitively working in this system, as many phospho-specific Chk2 antibodies failed in western blots. However, the method of application and dose used (10 μm) has routinely been used by my colleague, Prof. Zubair Ahmed on both retinal ganglion cells and dorsal root ganglion cell cultures *in vitro* which he has proven successfully inhibits Chk2 activity using phospho-specific Chk2 antibodies in western blots.

Other Tau sites found abnormally phosphorylated in AD (Ser396/404) that can be induced in response to other stress stimuli such as osmotic stress, did not increase

after activation of the DDR (Figure 6.7) (Stoothoff & Johnson, 2001). Although after activation of the DDR Ser396/Ser404 staining did not change, I cannot be confident that the antibody was working as I did not perform a positive control due to the COVID-19 lockdown resulting in time constraints and redirecting priorities to finish my main experiments. If I had more time, I would have induced cellular hyperosmotic stress using sorbitol to observe an increase in phosphorylation at Ser396/404, as seen before using a western blot (Stoothoff and Johnson, 2001). Despite missing this positive control, the PHF-1 antibody is an extremely well-characterised monoclonal antibody which has been previously been validated using site directed mutagenesis (Otvos *et al.*, 1994).

6.3.3 Role of nuclear Tau phosphorylation

My initial findings here revealed an increase in phosphorylation at Tau Ser262 in response to DNA damage but, very unexpectedly the phosphorylated Tau was almost exclusively nuclear. Most publications on Tau discuss the function of Tau in the context of microtubule cell biology. However Tau is indeed found in nuclei of both neuronal and non-neuronal cells, suggesting additional roles (Maina, Al-Hilaly and Serpell, 2016). There have been many suggested roles for nuclear Tau. Tau can bind non-specifically to the A/T rich minor groove of DNA, using both the proline rich domain and the microtubule binding domain, and protects it from heat shock induced damage, insult from free radicals and thermal denaturation (Hua and He, 2003; Wei *et al.*, 2008; Sultan *et al.*, 2011). Nuclear Tau has been suggested to play a role in nucleolar organisation and silencing of some genes via heterochromatinisation (Sjöberg *et al.*, 2006). Indeed, one suggested mechanism for Tau-induced neurodegeneration is *via* loss of heterochromatin and consequently increased transcription of normally silenced genes (Frost *et al.*, 2014; Frost, Bardai and Feany, 2016). Tau is also involved in transcriptional activation of transposable elements, possibly also as a result of unwinding of heterochromatin (Sun *et al.*, 2018). In AD and multiple neurodegenerative diseases, activation and dysregulation of transposable elements are seen and these events are associated with neuronal

death and abnormal cell cycle alterations (Sun *et al.*, 2018; Saleh, Macia and Muotri, 2019).

One suggested mechanism in which Tau promotes neurodegeneration is via heterochromatin relaxation (Frost *et al.*, 2014). As the DDR is active in multiple neurodegenerative diseases, including many tauopathy models (Khurana *et al.*, 2012; Colnaghi *et al.*, 2020) it would have been interesting to investigate the hypothesis - is phosphorylation at Ser262 in response to DNA damage is responsible for Tau mediated heterochromatin relaxation and ultimately neurodegeneration? The effect of Tau Ser262 phosphorylation on its binding to DNA could have been investigated using an electrophoretic mobility assay. Phosphorylated Tau has reduced DNA binding (Y. Lu *et al.*, 2013; Camero *et al.*, 2014) but it is not clear if phosphorylation at Ser262 affects DNA binding. My GFP-Tau S262 and S262A constructs would be useful tools for these future experiments, potentially using GFP-trap beads to purify the Tau from cells to measure DNA binding by an electrophoretic mobility shift assay**.** It is possible that the mutant Ser262A Tau would remain bound to DNA after irradiation of cells or exposure to amyloid beta oligomers, with likely transcriptional changes that could be identified by RNA seq. Several genes are upregulated with Tau transgenic expression, and are associated with heterochromatin loss and Tau induced neurodegeneration including *Ago3* and *CG15661* which have homologous genes in humans (Frost *et al.*, 2014). If these genes were upregulated in the same manner after irradiation, but not in the Tau Ser262A-expressing cells, this would suggest this phosphorylation event in response to DNA damage is important for regulating the chromatin relaxation and aberrant gene transcription that underpin Tau induced neurotoxicity.

6.3.4 Antibody batch failure

My initial findings that nuclear Tau phosphorylation at Ser262 is induced in response to DDR could not be repeated with a new antibody batch of anti-pSer262 (compare figure 6.1 with 6.8). The specificity of the new batch of Invitrogen antibody was tested and it was found not to be specific to Tau phosphorylated at Ser262 (Figure 6.9B). A second brand of anti-pSer262 was also non-specific from Signalway (Figure 6.9C).

The Invitrogen anti-Tau pSer262 has been used by various other groups over several years for immunohistochemistry, immunocytochemistry, and western blot. It was extremely disappointing that a different batch from the same company tested non-specific to Tau pSer262 (Figure 6.9B). Invitrogen supply antibody testing data for its use in immunohistochemistry and western blot only, but there is no data available to prove its antigen specificity using site directed mutagenesis, as I have done here. Previous studies using this Invitrogen antibody in immunocytochemistry reveal Tau pSer262 located in the cell body as well as dendrites of primary mouse cortical neurons, however no nuclei co-stain was used and only one representative image is shown (Tseng *et al.*, 2017; Borin *et al.*, 2018). In primary rat hippocampal neurons stained with Invitrogen anti-Tau pSer262 does in fact, appear to colocalise with DAPI staining (Park *et al.*, 2012).

The only way to move my experiments forward would have been to make my own phospho-specific antibody to pSer262. Given that making phospho-specific antibodies can be difficult (and sometimes impossible) and due to COVID-19 lockdown significantly disrupting my lab time I decided to bring this chapter to an end and redirect priorities. Given that I only had two commercially available antibodies to Tau pSer262 (Invitrogen and Signalway) and neither proved to be specific, the only way to move my experiments forward and be confident in my staining would have been to make my own phospho-specific antibody to Tau pSer262. Given that making phospho-specific antibodies can be difficult (and sometimes impossible) and due to COVID-19 lockdown significantly disrupting my lab time I decided to bring this chapter to an end and redirect priorities.

6.4 Conclusions

The first aim of my project was to ask whether activation of the ATM-Chk2 pathway in the DDR results in phosphorylation of Tau at Ser262 and if so, whether this event could contribute to neurotoxicity induced by amyloid beta in *Drosophila*. As nuclear Tau phosphorylation was not sensitive to the Chk2i, at least in neuroblastoma cells, my data indicates that inhibiting this phosphorylation event is unlikely to be a factor in the neuroprotective effect seen with inhibiting Chk2 in this model (Taylor *et al.*, 2022).

7.0 Discussion

The aim of this research was to investigate the potential cellular events that mediate neuroprotection in our *Drosophila* model of chronic amyloid toxicity when the ATM-Chk2 signalling pathway is inhibited. The importance of the DNA damage response (DDR) in the nervous system during pathology may appear controversial. There are several inherited human diseases caused by defects in DNA repair proteins. These include ataxia-telangiectasia (A-T) caused by mutations in *ATM*, and ataxia-telangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome (NBS) caused by mutations in *Mre11* and *Nbs1*, respectively, which are both components of the DSB-sensing MRN complex (Kulkarni and Wilson, 2008). All present with severe, early-onset neurological abnormalities; A-T and ATLD presenting with neurodegeneration and NBS with microcephaly (Kulkarni and Wilson, 2008). In contrast, when DDR genes such as *ATM, Mre11 and Nbs1* are knocked down in our *Drosophila* model of chronic neurodegeneration they elicit neuroprotection, which is the complete opposite to the predicted outcome. One explanation for this is that these DDR inherited diseases are autosomal recessive disorders where there is either a complete loss of protein function or a massive reduction in protein levels of activity in homozygous patients. In our system we use RNAi lines to knock down protein levels, which is more reminiscent of the heterozygous carriers for DDR disorders who display no neurological symptoms.

The neurological abnormalities that manifest in these DDR diseases suggest that the immature nervous system might be more vulnerable to DNA damage. There are several potential explanations for their high reliance on the DDR. In early development neurogenesis relies on high rates of neural stem cell proliferation, which is associated with replication associated DNA damage (O'Driscoll and Jeggo, 2008; Mckinnon, 2013; O'Driscoll, 2017). This means neural progenitors are likely to be highly reliant on repair systems which, if defective, would lead to accumulation of DNA damage into the adult nervous system, and potentially result in neurodegeneration (Rolig and McKinnon, 2000). It is also suggested neural progenitors have a lower threshold for apoptosis (Lee *et al.*, 2012), so if repair

proteins are defective or have reduced activity and DNA damage accumulates, this may exacerbate neural cell death and result in microcephaly. Finally, DSBs have a crucial role in mediating learning and memory by inducing the expression of early genes (Suberbielle *et al.*, 2013; Madabhushi *et al.*, 2015). Unrepaired or mis-repaired breaks could lead to transcriptional mutations during development which could induce cellular abnormalities, presenting in neurological defects.

7.1 Potential non-apoptotic roles of caspases in neurodegeneration

The main findings of my research are that *p53* knockdown elicits the same neuroprotective effect seen when *ATM* or *Chk2* is knocked down in our amyloid toxicity model in *Drosophila*. As p53 is downstream of both ATM and Chk2 it is likely that at least part of the mechanism of neuroprotection when I target the Chk2 pathway is via p53 signalling. Inhibiting caspase activity by expressing the baculovirus inhibitor, p35, also elicited a neuroprotective effect, suggesting a potential role of caspases in neurodegeneration. Despite this, I could not detect any evidence of elevated apoptosis occurring. Is it possible that activation of p53 by ATM and Chk2 mediates neurotoxicity through a caspase-dependent but non-apoptotic function? This could be investigated further by looking at the interaction of caspase with Tau (*as previously discussed in section 4.4.5*).

Alternative substrates of caspases includes APP. Cleavage of APP by caspases releases a highly toxic C terminal fragment which contributes to Aβ induced cell death (Gervais *et al.*, 1999; Lu *et al.*, 2000, 2003). More recently it was found that this C terminus fragment induces synaptic injury (Park *et al.*, 2020). Synaptic loss is observed in AD and has the strongest correlation with the cognitive decline (Terry *et al.*, 1991; DeKosky, Scheff and Styren, 1996; De Wilde *et al.*, 2016). Could it be possible that inhibiting caspases with p35 protects against the processing of endogenous *Drosophila* APP, and thus protects against synapse loss and neurodegeneration? The larval neuromuscular junction is routinely used to study the

synaptic integrity and physiology in *Drosophila* (Collins and DiAntonio, 2007). However, I use drivers that restrict expression to adults only, meaning studying the larval NMJ may not be particularly useful to study adult-onset neurodegeneration. Recently methods to prevent the larva from pupating have been developed (Perry *et al.*, 2020). Here the larval motor neurons continue to develop for 2-3 weeks. It is plausible this system could be adapted as an amyloid toxicity model to provide an easily accessed and imaged synapse integrity model. One alternative to the larval neuromuscular junction is to dissect the dorsal longitudinal muscles to examine the synaptic structures in adult *Drosophila* (Sidisky and Babcock, 2021). Synaptic integrity could be assessed with immunohistochemistry staining of presynaptic markers Synapsin or Bruchpilot to mark the active zones which could be counted (López-Arias *et al.*, 2017). It would be interesting to investigate if overexpression of p35 alongside Aβ has any benefit to synaptic integrity.

7.2 The potential effect of p53 induced synaptic changes in neurodegeneration

It is also possible that the neuroprotective effect seen when knocking down *p53* in our amyloid model could also be explained by not only by inhibiting caspase activity, but also from regulating changes at the synapse. Out of all the pathological hallmarks in AD, synaptic loss has the strongest correlation with the cognitive decline (Davies *et al.*, 1987; Terry *et al.*, 1991; DeKosky, Scheff and Styren, 1996; De Wilde *et al.*, 2016). It has already been shown that *p53* knockdown enhances neurodegeneration in a *Drosophila* tauopathy model, via alterations in synaptic gene transcription such as those encoding synaptic vesicle exocytosis, pre-synaptic and post-synaptic proteins, which could explain the decline in motor activity observed here in our amyloid *Drosophila* model (Figure 3.1 and Figure 4.1)(Merlo *et al.*, 2014). To investigate if similar synaptic gene changes are occurring in our amyloid model with *p53* knockdown, whole-genome ChIP-ChIP analysis could be used in the same way used by Merlo *et al* to identify if similar gene changes occur.

7.3 Transposable element mobilisation in neurodegeneration

Future work could also focus on alternative downstream mechanisms of p53 than caspase activity and apoptosis that might mediate neurotoxicity. In recent years, transposable elements (TE's) have emerged as potential mediators of neurodegeneration (Ravel-Godreuil *et al.*, 2021). Transposable elements are highly repetitive sequences that can generate new copies of themselves and insert elsewhere into the genome, inducing mutations and genome instability. Transposable elements are normally silenced in humans by epigenetic mechanisms, preventing their expression, but in multiple neurodegenerative diseases activation of TEs has been reported, including in ALS, Parkinson's disease, multiple sclerosis and in various tauopathies, including AD and progressive supranuclear palsy (Ahmadi *et al.*, 2020). It has been shown recently shown that transposable element mobilisation is an essential mediator of Tau induced neurodegeneration in *Drosophila*, and is facilitated by unwinding of heterochromatin (Frost *et al.*, 2014; Guo *et al.*, 2018; Sun *et al.*, 2018). As the DDR is active in many tauopathy models (Khurana *et al.*, 2012; Colnaghi *et al.*, 2020), this heterochromatin unwinding could potentially be mediated by combined phosphorylation of KAP-1 at different residues by ATM and Chk2 (Hu *et al.*, 2012). Additionally, upon DNA damage, p53 mediates transcription which requires epigenetic changes to enable the transcription machinery to access the DNA (Allison and Milner, 2004). Could p53 mediate widespread heterochromatin loss in the amyloid expressing fly, and promote TE activation and neurodegeneration? I began to use a reporter in *Drosophila* named cellular labelling of endogenous retrovirus replication (CLEVR), which detects replication of the transposable element gypsy (gypsy-CLEVR) (Chang *et al.*, 2019). Replication of the endogenous gypsy element remobilises the fluorescent reporter Watermelon to combine with the upstream activating sequence (UAS) and thus driving expression of the watermelon signal after TE replication. I wanted to investigate if transposable element movement was occurring in the amyloid expressing fly and if this is abolished upon p53, ATM or Chk2 knockdown (Chang *et al.*, 2019). I built lines to look at this but after many control experiments and discussions with the developing lab, it became clear that the reporter had become corrupted, so this question remains open.

7.4 Targeting the double strand break repair pathway in therapy

The involvement of p53 in neurodegenerative diseases raises the question of how p53 inhibitors might be used in therapy. Synthetic inhibitors of p53 have successfully been used to protect hippocampal neurons *in vitro* against death induced by DNA damaging agents, by glutamate and by Aβ (Culmsee *et al.*, 2001). p53 inhibitors also have neuroprotective effects in rodent models of Parkinson's disease, stroke and cerebral ischemia (Culmsee *et al.*, 2001; Duan *et al.*, 2002; Leker *et al.*, 2004). It must be noted that the inhibitors were used at low concentrations and with short administration times, with higher concentrations being toxic to neurons (Culmsee *et al.*, 2001; Leker *et al.*, 2004). The long-term use of p53 inhibitors raises the likely possibility of unwanted side effects, such as tumorigenesis, so application of p53 inhibitors is not likely to be approved for anything other than short-term treatments. This effectively rules them out as therapies for slowly progressing diseases like Alzheimer's disease. However, they may have some use for more rapidly progressing neurodegenerative diseases, such as ALS, or for inherited conditions such as neuronal ceroid lipofuscinosis which present in childhood and cause catastrophic early dementia.

If p53 is unlikely to be a suitable target for long-term disease therapy, potentially moving back up the pathway may be more practical. Targeting ATM could prove more problematic as it has more than 700 substrates and regulates many downstream processes, including autophagy and stress signalling. In contrast Chk2 has fewer downstream targets (~25), meaning inhibiting Chk2 may be more useful in therapy. Inhibiting Chk2 using clinically relevant inhibitors has already shown to be effective models of neurotrauma following spinal cord and optic crush injury (Taylor *et al.*, 2022) and gene therapy approaches could be used to try to target Chk2 knockdown specifically to neurons, for instance by combining the neuron-biased AAV9 with a neural specific promoters to express siRNA (Ojala, Amara and Schaffer, 2015; Naso *et al.*, 2017). Again, this approach would be more likely to be used for faster progressing neurodegenerative conditions such as ALS. Whether targeting the

ATM-Chk2-p53 pathway would be neuroprotective in *Drosophila* models of ALS is being investigated currently by the group.

8.0 COVID impact statement

My PhD has been affected by the COVID-19 pandemic in many ways. Due to the lockdown, I was working from home from March 2020 until the start of July 2020. Upon return to the lab, there was still may restrictions up until 2021, meaning lab time and space was limited, resulting in shift patterns and restricted lab access. Social distancing, alongside the working from home guidance that followed the lockdown also meant supervision in the lab was limited and experiments were therefore completed slower than planned. Due to large numbers of flies needed for my experiments, I was also delayed with restarting experiments as I needed to re-expand all my fly lines upon return. Finally, priorities had to be redirected to ensure I had sufficient data for my PhD, and the time was used most effectively. This resulted in some experiments being missed out, and one chapter (the Tau phosphorylation chapter) coming to an earlier than anticipated. The mental impact of COVID during my PhD has also been extremely stressful with the limited time available. I have tried my absolute best to ensure I have produced high quality data with the circumstances presented.

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