The role of the cytoskeleton in Alzheimer’s disease: a Drosophila perspective

MPhil Thesis

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24-09-2015
Abstract

Alzheimer’s disease (AD) pathogenesis is likely to be caused by dysfunction of two neuronal proteins, amyloid-beta (Aβ) and tau. Whilst excellent in vivo assays have been performed, and animal models which develop pathology which resembles AD have been generated, the cellular events that lead to neurodegeneration remain poorly understood, in particular those which involve the cytoskeleton. Microtubules (MTs) are vital for many axonal functions, including growth and transport. MTs have been implicated in neurodegeneration; 44% of cytoskeletal genes have OMIM links to human disorders, and over half of those disorders result in neuronal dysfunction. Aβ and tau are well understood biochemically, but the functional links between these proteins, and how they cause neurodegeneration, remain poorly understood. In the context of AD, Aβ and tau are known to have numerous toxic effects which could have extensive influence on the function of the cytoskeleton; a system which is essential in neurons. Here I utilise Drosophila primary neuron culture in order to determine the subcellular phenotypes associated with the application of Aβ via different genetic and artificial means, in concert with human tau (hTau), in a comparative analysis. I have demonstrated that fly neuron culture is suited in this capacity, and have demonstrated that different methods of Aβ and hTau application to neurons elicit different phenotypes, in particular regarding the timing and extent of MT disorganisation, and suggest that there may be qualitative reasons for the different phenotypes between the approaches taken.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
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<tr>
<td>ADF</td>
<td>Actin depolymerisation factor</td>
</tr>
<tr>
<td>AICD</td>
<td>Amyloid intracellular domain</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
</tr>
<tr>
<td>BPSD</td>
<td>Behavioural and psychological symptoms of dementia</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<tr>
<td>DS</td>
<td>Down's syndrome</td>
</tr>
<tr>
<td>dTau</td>
<td>Drosophila tau</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GDP</td>
<td>Gross domestic product</td>
</tr>
<tr>
<td>HFIP</td>
<td>Hexafluoro-2-isopropanol</td>
</tr>
<tr>
<td>hTau</td>
<td>Human tau</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney Epithelial cells</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
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<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate-buffered saline with 0.3% Triton X-100</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filaments</td>
</tr>
<tr>
<td>p-tau</td>
<td>Hyperphosphorylated tau</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activation sequence</td>
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1. Introduction

1.1 Alzheimer’s is a ubiquitous and fatal neurodegenerative disease

Alzheimer’s disease (AD) is a ubiquitous neurodegenerative disease and the most common cause of dementia, affecting 7% of people ages 65 and over. The risk of developing AD doubles every five years after age 65. Often not considered a lethal disease itself, AD could be considered the fourth biggest killer in Western populations, as a diagnosis of Alzheimer’s disease at age 65 or earlier is associated with a 67% decrease in median remaining lifespan. In the USA, of the top ten causes of death, AD is the only disease which cannot be prevented or cured.

There are an estimated 44 million people worldwide who are currently living with dementia, with most dementias caused by AD. The number of people with AD is expected to double by 2030, and triple by 2050. The ballooning AD population will have a large impact on its economic burden. Presently, AD costs the global economy US $604 billion, which constitutes 1% of global GDP. This cost is expected to increase to US $1 trillion by 2030, due to rapidly expanding ageing populations in newly industrialised countries. Since the publication of the World Alzheimer Report 2010, the largest economic study completed on AD to date, a Chinese study determined the number of AD patients in China to be 9.2 million, which eclipses the estimate of 5.4 million Chinese AD patients in the 2010 study. The global GDP will be ever more severely impacted by rising AD prevalence; the need to understand and treat AD has never been greater.

The grand challenge in AD research is to develop a therapy which can directly halt disease progression, or even reverse it. This aim would be greatly facilitated if a better understanding of the subcellular and molecular mechanisms which lead to pathogenesis could be gained.

In this introduction, I will outline the pathological features of AD, the hypothesised subcellular and molecular events which are thought to cause the disease, hypotheses to be investigated, and the overarching aims for the project.

1.2 Pathological features of AD

1.2.1 Cognitive and behavioural symptoms

The progression of AD typically begins with mild cognitive impairment (MCI) which progresses onto dementia and eventually death. As a neurodegenerative disease, AD causes dementia and short-term memory loss, most frequently in individuals aged 65 and over; age is the main risk factor. Symptoms worsen progressively until the death of the patient. An increase in symptom severity is often indicative of more severe cognitive decline and decrease survival. Symptoms can be placed into two main categories: cognitive symptoms, which concern basic cognitive function,
comprehension and memory, and behavioural symptoms, which include agitation, aggression and altered sleep.\textsuperscript{11} Cognitive and behavioural symptoms can be placed into four clusters (Table 1).

Table 1: Clusters of BPSD symptoms

<table>
<thead>
<tr>
<th>Cluster 1: Depression</th>
<th>Cluster 2: Apathy</th>
<th>Cluster 3: Delusions</th>
<th>Cluster 4: Disinhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anxiety</td>
<td>Pacing, fidgeting, repetitive behaviour</td>
<td>Hallucinations</td>
<td>Elation</td>
</tr>
<tr>
<td>Irritability</td>
<td>Sleep disturbance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agitation / aggression</td>
<td>Appetite disturbance</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Passmore, 2007\textsuperscript{11}

The humanistic burden of AD extends beyond the patient themselves; there is considerable burden placed on caregivers due to the demanding nature of caring for an AD patient. Symptom clusters 1 and 2 caused the most distress to carers, in particular aggression, sleep disturbance, and depression.

1.2.2 Physiological symptoms

The gross anatomical changes to the brain are predominantly ventricle enlargement and a substantial loss of brain mass (up to 30%), as a result of neuronal death.\textsuperscript{12} At the microscopic level, two major lesions are present: extracellular senile plaques, and intracellular neurofibrillary tangles (NFTs). In advanced cases of AD, senile plaques are present throughout the entire brain, from the basal ganglia to the cortex, localised in blood vessels, and throughout the brain parenchyma.\textsuperscript{13} The distribution of senile plaques does not follow a well-defined pattern over the course of the disease; all brain regions are affected.

In contrast, the spread of NFTs throughout the brain follows a well-defined course (Table 2). Layer pre-α of the hippocampus is involved initially, followed by increasing involvement of the limbic system as the disease progresses. In late-stage AD the cortex is involved, wherein executive functions are severely deteriorated. The main components of senile plaques and NFTs are amyloid-beta (A\textsubscript{β}) and tau, respectively.

Table 2: Overview of Braak staging (adapted from Braak and Braak, 1991)
### Amyloid stage

<table>
<thead>
<tr>
<th>Amyloid stage</th>
<th>Tau stage</th>
<th>Regions of NFT presence and severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. LIGHT AMYLOID BURDEN</td>
<td>I</td>
<td>Layer Pre-α of the Transentorhinal region - low number of NFTs</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Pre-α - higher number of NFTs. Few isolated NFTs may be present in the isocortex.</td>
</tr>
<tr>
<td>B. MODERATE AMYLOID BURDEN</td>
<td>III</td>
<td>Pre-α and entorhinal region, subiculum, hippocampus. ‘Ghost tangles’ (extracellular NFTs left behind from the death of the host cell) are present.</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>As above, with greater numbers of ghost tangles in layer Pre-α. Low numbers of NFTs are present in the isocortex.</td>
</tr>
<tr>
<td>C. HEAVY AMYLOID BURDEN</td>
<td>V</td>
<td>Very large numbers of ghost tangles in layer Pre-α. The entire hippocampus is affected, and for the first time NFTs are abundant in the isocortex.</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>Very large numbers of ghost tangles in layer Pre-α and throughout the hippocampus - many of the ghost tangles have been degraded and replaced by glial cells. The isocortex is heavily burdened with NFTs.</td>
</tr>
</tbody>
</table>

1.3 Amyloid precursor protein (APP) and amyloid-beta

The primary component of senile plaques is Aβ, a 4.5 kDa, toxic, highly hydrophobic, aggregation-prone peptide produced by cleavage of amyloid precursor protein (APP). 24 APP is membrane-bound protein found in neurons, and has structural similarities with Notch receptors. 25 It is hypothesises to have roles in cell motility and adhesion- overexpression of APP in MDCK cells caused accelerated healing in a wounding assay via interactions with integrins and FE65. 26

There are two major pathways of APP cleavage: non-amyloidogenic cleavage, and amyloidogenic cleavage. Non-amyloidogenic cleavage is performed by various ADAM metalloproteases, previously categorised as α-secretase. Amyloidogenic cleavage is performed firstly by BACE1 β-secretase, followed by cleavage by an enzyme complex of PEN1, PEN2, nicastrin, and AP-1, also referred to as γ-secretase. The Aβ peptide produced by γ-secretase cleavage of C99 can be 40 or 42 amino acids in length. A and β-secretase-mediated cleavage can produce an additional peptide 3 amino acids in length, called P3 (not shown). 27
There are two main isoforms of Aβ, which are either 40 or 42 amino acids in length. β_{40} and Aβ_{42} are present in the CSF and blood at a 10:1 ratio. Aβ_{42} is the more toxic isoform and has a greater propensity for aggregation than Aβ_{40}, due to the addition of two extra hydrophobic residues at its C-terminus. Cleavage of APP, and the subsequent release of Aβ is thought to be critical for the disease process; Aβ is not toxic if it remains cognate with the rest of APP. At the simplest level, pathogenesis is thought to be caused by a shift in the ratio of Aβ_{40} and Aβ_{42} production and a decrease in the rate of Aβ peptide clearance.

The toxic effects of Aβ are dependent upon its aggregation state. After cleavage from APP, Aβ is initially monomeric. Over a short period of time, oligomers and protofibrils form as the Aβ monomers aggregate. Eventually, these larger Aβ aggregates assemble into large fibrils of indeterminate length via hydrophobic interactions which are possible due to the β-pleated sheets formed in Aβ aggregation. Aggregation is dependent upon amino acids 16 to 22 of Aβ (Aβ_{16-22}). The amino acids in this sequence, KLVFF, are highly hydrophobic and imbue high propensity for aggregation on the rest of the peptide. Disruption or replacement of the KLVFF sequence, through insertions or deletions, prevents peptide aggregation. In addition, blocking of the KLVFF sequence...
prevents aggregation between Aβ monomers. This is the mechanism of action of n-methylated peptides (meptides), potential drug candidates for AD.\textsuperscript{23}

1.4 Aβ in Alzheimer’s Disease

1.4.1 Toxic species of Aβ

The toxicity of Aβ appears to be linked to its aggregation state. The volume of senile plaques and NFTs is not indicative of disease severity, making it unlikely that large aggregates of Aβ or tau are the causative agent of the disease. A greater burden of Aβ or tau does not correlate with greater cognitive decline; senile plaques are often found in the brains of healthy people without any AD symptoms. However, cognitive decline correlates with increased soluble Aβ oligomer concentrations in the brain.\textsuperscript{24} The greater the severity of cognitive decline, the higher the concentration of Aβ oligomers in the post-mortem brain. These findings gave rise to the idea that oligomeric Aβ is responsible for the bulk of Aβ-mediated neuronal damage.

1.4.2 Toxic functions of Aβ

Aβ oligomers produce reactive oxygen species (ROS) \textit{in vitro}.\textsuperscript{21} ROS inflict nonspecific damage to macromolecules. When ROS-inflicted damage outpaces than the cell’s capacity to repair it, cell death results. The production of ROS is highly specific to oligomers; in an experiment where monomeric Aβ was added to DMSO, a ‘burst’ of H\textsubscript{2}O\textsubscript{2} production was observed at the point at which oligomers and protofibrils were forming.\textsuperscript{21}

ROS production is one of many mechanisms by which Aβ oligomers are toxic. Artificial peptides rich in β-pleated sheets (similar to Aβ aggregates) were found to sequester normal cellular protein, leading to an effective loss-of-function of the sequestered proteins.\textsuperscript{25} The proteins most sequestered were those involved in chromatin regulation, RNA and protein synthesis, cytoskeletal organisation, and transport. It is likely that all of the aforementioned pathways are heavily disrupted, as between 10\% and 45\% of the total protein amount of the cell (for these proteins) was sequestered within Aβ-like aggregates.\textsuperscript{25}

Oligomeric and protofibrillar Aβ species can cause extensive disruption to lipid membranes.\textsuperscript{26} Compromise of the cell membrane can rapidly lead to cell death through loss of structural integrity.
1.5 Tau

Tau is a MT-associated protein which is present in the human nervous system as six isoforms. These isoforms vary depending on alternative splicing of MAPT transcripts of exons encoding N-terminal membrane interaction domains, and C-terminal MT-binding domains. Tau is evolutionarily conserved with homologues in mice and fruit flies.

The normal function of tau is hypothesised to be cytoskeletal, as it stabilises MTs by preventing depolymerisation and disorganisation, as well as having overlapping functions with known cytoskeletal proteins. Knockout of the Drosophila tau analogue (dTau) causes defects in MT polarity in fly oocytes. There is debate over whether knockout of tau in mice has a phenotype or not; initially no phenotype was reported for the first MAPT null mice, although subsequent observations axons in MAPT null mice had abnormal morphology and a lower density of MTs than controls.
Figure 3: Tau and its role. Tau is necessary for normal levels of MT stabilisation and polymerisation. A: cartoon of an axon showing parallel bundles of discontinuous MTs. B: Electron micrograph of bundled MTs. C: MT with bound tau.

Figure 4: A diagram of full-length (2N4R) hTau showing various kinases and their phosphorylation sites. The number of kinases which are capable of tau phosphorylation reflect the large number of involved pathways. MT-binding repeats are shaded in red.
The *Drosophila* tau homologue, dTau, is present in one isoform. It has five MT-binding domains and lacks the N-terminal membrane interaction domains. There appears to be functional overlap between human tau (hTau) and dTau, as overexpression of hTau in *Drosophila* results in similar neuronal dysfunction phenotypes to overexpression of dTau. hTau has a lower binding affinity for MTs as it is more prone to phosphorylation, a critical step in AD pathogenesis.

Tau is intrinsically disordered, meaning it can assume several stable conformations. Despite the rest of the structure being disordered, the C-terminal MT binding domains in exons 9 to 12 are highly ordered (Figure 4). There is correlation between the number of MT-binding repeats expressed, and the protein’s affinity for MTs. 4R isoforms bind more strongly to MTs than 3R isoforms. Whether tau is associated with MTs or not depends on its phosphorylation state; tau has a greater propensity to bind MTs when hypophosphorylated, whereas hyperphosphorylation leads to a loss-of-function of MT binding and promotes tau aggregation.

In health, the majority of tau is MT-bound, and is mostly localised with dynamic MTs. Tau is hypothesised to mediate MT spacing, and also binds actin (via its proline-rich domain and MT-binding domains) forming cofilin-containing aggregates called Hirano bodies and spectrin filaments.

Tau has functional overlap with other MAPs. In *MAPT* null mice, upregulation of MAP1A was observed which suggests limited functional redundancy between tau and MAP1A, although axonal growth defects were noted. Knockout of MAP1B along with tau leads to early lethality and prevents normal brain development.

### 1.6 Toxic functions of tau

In AD and tauopathies, post-translational modification of tau results in both loss-of-function and toxic gain-of-function. Tau has 35 potential serine/threonine phosphorylation sites (Figure 4) the phosphorylation of which increases the dissociation of tau from MTs as well as increasing the likelihood of self-aggregation. The aggregation of hyperphosphorylated tau (p-tau) resembles that of Aβ; aggregates are rich in β-pleated sheets comprised of repeated hexapeptide motifs in the MT binding domains.

#### 1.6.1 Loss-of-function

The largest tau aggregates, NFTs, are comprised of p-tau in highly ordered structures termed paired helical filaments (PHFs)- parallel fibrils 10nm in diameter, which cross over every 80 nm. If tau is sequestered in large aggregates, it cannot perform its normal function of MT stabilisation, resulting in a loss-of-function. However, the most toxic species of tau may not be the large aggregates, but rather tau oligomers – another similarity to Aβ. Oligomeric tau appears to undergo toxic gain-of-
function; *in vitro* it has been shown to disrupt artificial lipid membranes and increase phospholipid vesicle leakage.38

**1.6.2 Toxic gain-of-function**

P-tau is capable of sequestering MT-bound, hypophosphorylated tau, and changes its conformation to that of p-tau in a phenomenon known as templating.39 The mechanism of templating bears a stark resemblance to how prion proteins spread, converting normally folded proteins into toxic, misfolded forms. In addition, p-tau can be exocytosed from presynaptic sites and endocytosed again at post-synaptic termini, wherein templating may continue and spread through neuronal networks.40 This method of tau spread may explain the well-characterised spread of NFTs from the entorhinal complex to the isocortex as described by Braak.13

P-tau has recently been shown to trigger erroneous cell cycle re-entry in cultured mouse neurons.41 Activation of cell cycle re-entry in this manner results in apoptosis. P-tau is also required for the nucleation of Hirano bodies: actin, ADF, coflin and p-tau-containing inclusions which have been shown to impede transport along MTs in flies.42 In addition, tau can bind to, and stabilize, F-actin via its MT-binding repeat. Hyperstabilization of F-actin has been proven to be required for tau toxicity in both fly and mouse models.42

**1.7 The relationship between Aβ and tau**

Traditionally, drugs have targeted the symptoms of AD rather than attempting to target the underlying cause of the disease. A prime example are acetylcholinesterase (AChE) inhibitors. Levels of choline acetyltransferase (ChAT) are decreased in AD brains, which is due to degeneration of cholinergic neurons in the nucleus basalis of Meynert.43,44 Drugs were therefore developed to specifically inhibit AChE, the enzyme which degrades acetylcholine in the synaptic cleft. Application of these AChE inhibitors (donepezil, rivastigmine, and galantamine) causes a net increase in the amount of acetylcholine, which partially attenuates AD symptoms. However, AChE inhibitors are only mildly effective and do not prevent cognitive decline, with some patients completely unresponsive to treatment.

Aggregation and the production of Aβ peptides are other drug targets that were, and still are, investigated. Unfortunately, no drugs targeting Aβ processing or homeostasis have worked in clinical trials; recent failures include Semagacestat and Avagacestat (γ-secretase inhibitors) and Bapineuzumab (a monoclonal antibody to Aβ).45,46 Even by drug discovery standards, the lack of new therapies is striking. New and more informed strategies should be investigated. I will now describe the most prominent hypothesis which attempts to explain AD pathogenesis, and provide an outline of a model.
1.8 The amyloid cascade hypothesis

The amyloid hypothesis was first put forward in 1991 by John Hardy and Gerald Higgins, and to date is the most widely accepted hypothesis. The basis for the hypothesis lies in both genetic and histological observations which have been made throughout AD research, which has shown that Aβ is the causative agent in AD pathology, with tau placed downstream. As mentioned, Aβ is produced by β-secretase and γ-secretase cleavage of APP, a stepwise process which also produces AICD. Aβ aggregation into toxic oligomers and protofibrils, as well as amyloid signalling, lead to activation of various kinase enzymes which proceed to hyperphosphorylate tau. P-tau then aggregates and becomes toxic itself. All mutations which have been linked to familial AD influence APP processing; these include mutations within the APP gene itself, or mutations within the presenilin proteins involved in the cleavage of APP. Down’s syndrome (DS) patients possess an extra copy of chromosome 21. The APP gene is located on the 21st chromosome – as three copies are present, more APP is produced so there is more substrate available for the secretase enzymes. DS patients have a high risk of developing AD-like symptoms at age 50, lending further support to Aβ being the causative agent of AD. Possession of the apolipoprotein E4 (ApoE4) allele decreases the age of AD pathogenesis by positively modulating amyloidogenic APP processing.

The central dogma to the amyloid cascade hypothesis is that Aβ is the primary causative agent in AD, and begins a neurodegeneration process heavily involving tau. The cascade model is a promising start to future investigations of AD, but the cellular events leading to neuronal death are poorly understood. However, some insight into cellular mechanisms of AD may be gained via investigating the role of the neuronal cytoskeleton in the disease process.

1.9 The neuronal cytoskeleton in neurodegeneration

The cytoskeleton has been previously shown to have a critical role in the pathogenesis of many neurodegenerative diseases; 44% of cytoskeletal genes have links to human disorders, and over half of those disorders result in neural dysfunction. The involvement of tau, a cytoskeleton-associated protein, and of Aβ, which can also directly modulate the cytoskeleton’s function, provide evidence that the cytoskeleton may be a critical factor in pathogenesis. In the last decade, the role of the cytoskeleton in AD has received too little attention, with experimental approaches hindered by the enormous complexity of cytoskeletal machinery.

Tau has a clear relationship with the cytoskeleton; it binds to MTs directly, stabilises them, and regulates their spacing. In addition, tau-induced hyperstabilisation of actin via cross-linking prevents the fission of mitochondria, leading to increased oxidative stress. Aβ can indirectly interact with the cytoskeleton; Aβ has been shown to cause MT destruction by alterations to Ca²⁺.
homeostasis.\textsuperscript{53} It is possible that MTs are depolymerised by A\textsubscript{β} directly, due to the presence of intraneuronal A\textsubscript{β}.\textsuperscript{54}

In my project I will investigate the subcellular mechanisms of A\textsubscript{β} and tau in regards to their effects on the neuronal cytoskeleton, taking advantage of new genetic and experimental approaches that have become available.

1.10 The use of \textit{Drosophila} in AD research

The immense complexity of the cytoskeleton is difficult to tackle experimentally in higher organisms, and genetically highly amenable invertebrate model organisms provide powerful strategies to gain a fundamental understanding that can then be applied in mammalian research. For AD, the nervous system of the fruit fly \textit{Drosophila} provides fantastic opportunities. Thus, the fly nervous system is well investigated at the level of the cytoskeleton, synapses and mitochondrial dynamics, and a number of AD models are readily established.\textsuperscript{31} In addition, tau is intrinsic in MT stability and organisation and actin regulation. Analysis of the growth cone of the developing neuron is a useful system for deciphering the effects of A\textsubscript{β} and tau on the cytoskeleton, and interactions between the two which may lead to any phenotypes. There are a plethora of advantages to using \textit{Drosophila} in this respect, including the low cost of fly work, the rapid pace of work that can be achieved, and the impressive scope for genetic alterations.\textsuperscript{55} Flies have low gene redundancy and have many cellular and molecular mechanisms that are conserved in more complex organisms which makes them particularly well-suited to studying the fundamental mechanisms underlying disease.\textsuperscript{56} Research in the fruit fly has translational importance when studying the mechanism of conserved cellular processes.

1.11 Project aims

The overarching objective of my project was to use Drosophila neurons in order to generate a model of neurodegeneration which can shed light on the role of the cytoskeleton in Alzheimer’s disease.

My first aim was to establish a reliable cellular system which provides robust readouts of A\textsubscript{β} and tau-mediated effects on the neuronal cytoskeleton to be used as reflectors of neuronal health and as direct readouts for cytoskeletal effects. For this purpose, I have adapted the \textit{Drosophila} primary neuron culture system in my first year. I have tested different methods of treating neurons with A\textsubscript{β}, which include the expression of the A\textsubscript{β} peptide, expression of the C99 fragment of hAPP, and direct application of A\textsubscript{β} peptide onto neurons, along with overexpression of human tau. These studies show that neuron culture is suitable for this purpose, and have pinpointed clear differences between distinct application modes of A\textsubscript{β}, which can be used to evaluate the quality of the approaches taken.
My second aim was to use the cell culture system developed in the first aim to develop and supplement a model of neurodegeneration in AD. This is covered in detail in section 5.4.
2. Materials and methods

2.1 Fly stocks

Table 3: Fly stocks

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<thead>
<tr>
<th>GENOTYPE</th>
<th>SOURCE</th>
<th>REFERENCE</th>
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</thead>
<tbody>
<tr>
<td>WT (OREGON R)</td>
<td>Bloomington stock centre</td>
<td>See appendix A</td>
</tr>
<tr>
<td>SCA-GAL4</td>
<td>Marek Mlodzik</td>
<td>See appendix A</td>
</tr>
<tr>
<td>ELAV-GAL4&lt;sup&gt;ES65&lt;/sup&gt;</td>
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<td>UAS-2N4RTAU&lt;sup&gt;hTauIII-68A&lt;/sup&gt;</td>
<td>Guy Tear</td>
<td>See appendix A</td>
</tr>
</tbody>
</table>

For wild-type flies, flies containing the GAL4 insert of interest were crossed to Oregon R flies, to ensure than both control and experimental flies were expressing GAL4, as GAL4 can be slightly toxic in high concentrations. For stage-specific overexpression of insertions, scabrous-GAL4 (ScaGAL4) and elav-GAL4 were used as drivers. The scabrous promoter is active from embryonic stages 9 to 14<sup>57</sup> and the ELAV promoter is active at all stages. The stocks used are described in table 2. For experiments with 6 hour incubation, males carrying the UAS constructs of interest were crossed to virgin females carrying the scabrous-GAL4 insertion. For experiments with longer incubations, males carrying the relevant UAS constructs were crossed to virgin females carrying the ELAV-GAL4 insertion. The UAS-AB stocks (a gift from Damian Crowther) all have P-element mediated insertions at the same genomic locus. There is a 99% probability of the P-element inserting into this site over any other genomic locus, with the implication being that expression levels between embryos will be the same allowing for greater reproducibility.<sup>14</sup> The same system was used in the UAS-hTau fly stocks donated by Guy Tear.

2.2 Primary culture of Drosophila neurons

Neurons were isolated from Drosophila stage 11 embryos. Egg lays were set up overnight at 22°C. Yeast was added to vials to stimulate egg laying. Embryos were dechorionated in 50% NaClO for 90 seconds before being transferred to agar plates for embryo selection. Embryo stage was determined by autofluorescence of the yolk sac using a Leica<sup>®</sup> DM2500 fluorescence microscope. 30 stage 11
embryos were selected per genotype, and added to microfuge tubes containing 10 µL of culture media. Culture media was prepared from Schneider's Drosophila media (Invitrogen) and 20% non-heat-inactivated foetal bovine serum (Biochrom, AG Seromed, Germany). The media was incubated in the dark for 3 days at 4°C prior to addition of 2 µg/mL insulin (Sigma-Aldrich), and if necessary the pH was set to 6.8-6.9 using glacial acetic acid. Embryos were cleaned with 500 µL 80% ethanol solution and washed with 100 µL of culture media. The culture media was then replaced with 100 µL of dispersion media. 101.5 mL of dispersion media was made from 85 mL distilled water, 15 mL HBSS (Gibco), 1.5 mL penicillin-streptomycin solution (Gibco), 5 mg phenyl-thio urea (Sigma-Aldrich), 1 mg dispase, and 25 µg/mL collagenase (Worthington, Cellsystems). Embryos in dispersion media were homogenised using pestles until the media was cloudy and no embryo fragments could be seen. The dispersion media embryo homogenates were incubated for 4 minutes at 37°C. 200 µL of culture media was added to the homogenates to stop dispersion of neurons after the incubation. Homogenates were centrifuged for 4 minutes at 1500 rpm and the supernatant was removed. The homogenates were then resuspended in 90 µL of culture media and 30 µL of the suspension was added to a culture chamber. Three culture chambers were made for each genotype. The culture chambers consist of two microscopy slides, one of which has a circular hole cut through it, glued together with silicone and treated with acetone. Acetone-treated cover slips were placed on top of the chambers with an airtight seal of petroleum jelly around the edge, to prevent contamination or drying. The sealed culture chambers were oriented cover-slip down to allow neurons to adhere to the coverslip. The culture chambers were then incubated at 26°C in the dark for 90 minutes. After 90 minutes the chambers were turned over so the cover slip faced upwards, to prevent non-neuronal cell types from adhering to the coverslip. The culture chambers were then incubated for varying amounts of time depending on the experiment, which was usually 6 hours, 3 days, or 7 days.

2.3 Preparation of beta-amyloid solution
1 mg Aβ42 (R-peptide) was dissolved in 1 mL hexafluoro-2-propanol (Sigma-Aldrich), split evenly amongst 10 vials (0.1 mL per vial) and lyophilised until no fluid remained. Vials were left open-topped overnight in a laminar flow hood to ensure that all HFIP had evaporated and peptide films had formed. Vials were stored at -80°C. Prior to administration to culture, peptide films were resuspended in DMSO.

2.4 Immunocytochemistry
Following incubation, coverslips were removed from the culture chambers and neurons were fixed for 30 minutes with 4% paraformaldehyde solution in 0.05 M phosphate buffer, pH 7.2. Coverslips were washed twice with PBS-T (phosphate-buffered saline with 0.3% Triton X-100) to remove any
residual paraformaldehyde. Between stainings, cover slips were washed twice with PBS-T. Primary antibodies used included mouse anti-α-tubulin (1:1000, Sigma-Aldrich), rat anti tyrosinated-α-tubulin (1:500, Millipore) and mouse anti-SYNORF1 (1:10, DSHB, University of Iowa). Secondary antibodies were Cy3, FITC, or Cy5 conjugated (donkey, Jackson Immunoresearch) and were used at a concentration of 1:100. Filamentous actin was stained with FITC-conjugated phalloidin (Sigma-Aldrich). Vectashield fluorescence medium (Vector labs) was used when preparing coverslips for slides.

2.5 Imaging and statistical analysis
Images were acquired using an Olympus BX50WI microscope with a 100 X objective lens (numerical aperture 1.25) at either 1 X or 1.6 X zoom with Cy3, FITC, and Cy5 filters. Images in different channels were merged using Adobe Photoshop CS 6, and measurements of axons were taken using ImageJ. Analysis of different images was double-blind, which was achieved by using an ImageJ plugin (a gift from Yutaka Matsubayashi), which shuffled images into a random order and hid the file names, before re-ordering images and their respective measurements back together to eliminate any bias. Axon lengths were measured using tubulin staining, and were measured from the base of the axon at the soma to the end of the most distal MT from the soma. MT organisation was quantified by determining if any MTs in the neuron were non-coalesced and were not parallel to other MTs, using a Boolean system for both the axon shaft and the growth cone. Neurons with any evidence of MT disorganisation were categorised as ‘disorganised MTs’, which avoids subjectivity as to the degree of disorganisation and was proven to be a sensitive and robust measure of MT disorganisation.

GraphPad Prism software was used to perform statistical analysis on data. For numerical data, non-parametric Mann-Whitney U tests were used as the data distribution could not be assumed to be Gaussian. 1-way ANOVA with a Tukey post-hoc test was used when more than two experimental groups were tested on. χ² tests were used to analyse MT disorganisation data as the data was in discrete categories (organised or disorganised MTs). P values reached by Mann-Whitney U tests are abbreviated as P_MW, and P values reached by χ² tests are abbreviated as P_X2.

3. Results

3.1 Choosing subcellular phenotypes to measure Aβ and tau-mediated effects and validation of the system
There are several well-defined subcellular readouts which can be analysed in developing neurons, including axon length, different forms of MT organisation, growth cone area, filopodia number, and
filopodia length. It has been reported that in mouse neurons, Aβ treatment causes MT disorganisation and an increase in growth cone area (E. Chiregatti, personal communication). MT disorganisation is usually accompanied by axon shortening, as the MTs are not coalesced properly, and lose their ability to polymerise in the same direction, which is required for efficient axon extension (N. Sánchez-Soriano, pers. comm.).

The readouts selected were axon length, MT classification, and growth cone area, due to the link between MT disorganisation and axon shortening, and also because these measurements have previously been made in mice (H. Tsushima, pers. comm. Error! Reference source not found.). These measurements allowed for a comparison of Drosophila neuron culture systems to mouse culture systems, as validation. Initially, these measurements were made after six hours at 26°C, which is standard procedure for MT phenotype analysis.

**Figure 5: morphology of 6 hour culture neurons.** Neurons were stained for actin (magenta) and tubulin (green). (A) Shows bundled MTs. (B) shows splayed MTs indicated by arrowheads. (C) Shows bundled looped MTs, and (D) shows non-coalesced (disorganised) MTs. At 6 hours in vitro (6HIV) all neurons can be sorted into each of these categories. Bundled, splayed, and bundled looped MTs are all classed as being organised. (E) Normalised MT morphology in wild type neurons between experimental repeats. The ratio of organised MT structures to disorganised MT structures is the same between each repeat (P_{s2} = 0.98, 3 d.f.). (F) An example of how growth cone area is measured (yellow dotted line). (G) Example of axon length measurement (red dotted line). (H) Example of filipodia quantification (blue asterisks). N = number of neurons analysed. Scale bar = 5 µm.

Initial experiments proved the robustness and reproducibility of the neuronal culture system, and provided valuable baseline data on axon length, MT classifications, and growth cone area. The ratio of bundled, splayed, bundled loop, and non-coalesced MTs was well preserved (figure 5). In addition,
the use of 0.5% DMSO in culture growth media was found to have no effect on MT morphology when compared to 0.05% DMSO; this was important as DMSO was planned to be used as a solvent for Aβ. The results echoed those found in previous experiments in the lab (A. Prokop, Y. Qu, personal communication) (figure 5).

Whilst initial experiments proved the reproducibility of six-hour cultures, this was likely not a long enough time period to elicit AD phenotypes, as AD is a chronic disease. Longer incubations in the order of several days have successfully been used to study cytoskeletal dynamics. Synapses form after one day in this culture system, and presynaptic sites can be readily visualised by staining for presynaptic proteins such as Synapsin. After one day of culture, the growth cone is no longer present; narrow projections of MTs and actin are present instead. However, regions of the axon can still be easily identified, for example the axon shaft and the axon terminal (figure 6).

![Figure 6](morphology_of_three-day_culture_neurons.png)

Figure 6: morphology of three-day culture neurons. (A) A neuron stained for tubulin and actin showing no MT disorganisation. (B) Neuron showing regions of axonal MT disorganisation indicated by white arrowheads. (C) Neuron showing extensive distal disorganisation of the axon indicated by white arrow. (D) Neuron stained for tubulin, f-actin, and synapsin, a presynaptic marker. The location and abundance of orphan presynaptic sites (herein referred to as synapses) can be quantified. Synapse quantity was not used as a readout in these experiments as staining must be completely uniform in intensity between slides. Scale bar = 5 µm.

Other commonly used readout in vitro and in vivo include measurement of the amount of neuritic branching, and axon length. 62
With these initial experiments, the system had been proven to be reproducible, and to possess the capacity to allow the assessment of various cytoskeleton, and cytoskeleton-related, readouts. I was ready to begin experiments which examined the impact of AD proteins on cytoskeletal phenotypes.

3.2 Extracellular Aβ causes axonal abnormalities which worsen over time

To date, Aβ in Drosophila has mainly been applied through targeted intracellular expression in neurons in vivo, and this mode of application does not necessarily reflect the situation in the AD brain (discussed in section 4.2). The only exception that I was aware of when I was performing the experiments (September 2012 – July 2013) was the application of extracellular Aβ peptide to larval fly neuromuscular junctions (NMJs) and the analysis of its impact on electrophysiological recordings. Presently, (July 2015) no such experiments have been performed in Drosophila neuron culture. In contrast, in mouse and commonly-used human neuroblastoma cell culture models, extracellular application of Aβ is the standard method for Aβ delivery. This approach has become feasible in Drosophila through new trends of using primary neurons for the analysis of subcellular functions, and these neurons are ideally suited for the direct application of substances such as Aβ, through the culture media.

I therefore started with this approach, hoping that it would allow me to establish a set of reference data that can then be compared to parallel data generated through genetic approaches (i.e. the expression of Aβ and Aβ progenitors). Furthermore, if the use of extracellular Aβ worked as an experimental approach, it would be a useful strategy that could easily be combined with genetic manipulations of neurons (i.e. the direct application of Aβ to neurons which overexpressed hTau). This would accelerate experiments, as fewer crosses would be needed to generate the desired genotype of flies, and provide a broader range of possibilities to experimentally address cellular mechanisms downstream of Aβ.

I began my initial experiments with 6-hour incubations of Aβ42, as previous experiments performed in mouse neurons have shown that Aβ can induce cytoskeletal defects within this relatively short time frame (E. Chieregatti, pers. comm.), and since culturing Drosophila primary neurons is standard in our laboratory (see section 3.1), so that any results produced can be compared to a wealth of existing data sets concerning a large selection of cytoskeletal regulators. As the aggregation state of Aβ42 is critical to its toxic function, recombinant Aβ42 was procured from R-Peptide. Artificial peptide synthesis has a higher error rate than recombinant synthesis; in a large molecule such as Aβ42, even a low error rate of 1-2% could result in a change to the secondary structure of the peptide and prevent effective aggregation.

To choose the concentration of Aβ for these experiments, a number of considerations were made. Human CSF concentrations of Aβ42 (free and protein-sequestered) of AD patients have been found to
vary between 0.5 to 0.7 nM, to as high as 52 nM when protein-sequestered Aβ is considered. Past experiments performed on cultured primary mouse neurons have used Aβ concentrations in the order of micromoles to determine the extent of tau-dependent cell cycle re-entry. As AD is a chronic disease, and pathogenesis is hypothesised to take several years with very low levels of Aβ, using concentrations in the micromolar range (100 to 1000 times higher than is found in AD brains) appears prone to producing artefactual results from excessive nonspecific Aβ toxicity. Fortunately, 100 nM Aβ has recently been used with success on mouse neurons and was sufficient to trigger changes in cytoskeletal dynamics and organisation (H. Tsushima, pers. comm.). I therefore decided to use 100 nM as the starting concentration.

To perform this experiment, I added 100 nM Aβ to the culture medium immediately after neurons were harvested from stage 11 wildtype embryos. The media was left on these cultures for 6 hrs, after which time the cells were fixed and stained for F-actin and MTs. Aβ-treated neurons and untreated control neurons were assessed for axon length, growth cone area, and MT morphology (as shown in Figure 2). None of these measurements taken showed any difference between experimental and control conditions, suggesting that Aβ had no obvious effect on these cells (figure 7). A number of reasons could potentially explain why no phenotype was seen:

1. Aβ has no effect on fly neurons
2. The Aβ used was not toxic (e.g. the monomeric Aβ added to the cell culture did not have time to aggregate into more toxic oligomers and protofibrils)
3. The concentration of Aβ was not great enough to elicit a phenotype

Figure 7: administration of 100 nM extracellular Aβ does not have any observable cytoskeletal effects after six hours. (A) There was no difference in axon length between controls and neurons (P = 0.8252). (B) Growth cone area also was unaffected by Aβ treatment (P = 0.2889). (C) There was no difference in the proportions of MT organisation classes in growth cones (P = 0.4874, 3 d.f.). Controls were treated with 0.05% DMSO, the same concentration as was used to deliver Aβ peptide. N = number of neurons analysed. Experiments were performed in triplicate; analyses were performed using the mean values for each individual experiment.
4. The incubation period was not long enough to allow for any cellular effects to become apparent. I deemed the last two reasons for a lack of phenotype to be the most probable, as intracellular Aβ has been shown to elicit phenotypes in vivo, and the Aβ would have had sufficient time to aggregate over the course of the cultures. Following on from this, I repeated the experiment but increased the concentration of Aβ₁₄₂ ten-fold to 1 µM. Again, there was no observable phenotype detected in terms of axon length, MT disorganisation, and growth cone area (figure 8). Since an increased concentration of Aβ₁₄₂ had no impact, I tested a longer incubation period. To this end, I cultured neurons for 3 or 7 days, and incubated them for the entire time period with an Aβ₁₄₂ concentration of 100 nM. I used the readouts described for older culture. At 3 days in vitro, I found that MT disorganisation was increased in the distal region of axons, but there was no difference in axon length in these cultures (figure 9).
A longer incubation of 7 days with 100 nM of Aβ42 caused MT disorganisation in the axon shaft as well as in the distal axon (figure 10).

Experiments were performed in triplicate; analyses were performed using the mean values for each individual experiment.

In conclusion, 100 nM Aβ42 is a sufficient concentration to cause neuronal abnormalities after an incubation of 3 days in flies. The toxicity of Aβ42 appears to increase over time, and the longer the cultures were incubated the greater was the amount of aberrations. This result suggests that Aβ toxicity is influenced by incubation time. This may be due to changes in the neurons which are time-
dependent, or a change in the peptide such as its aggregation state having an adverse effect. This is further discussed in section 4.2.

3.3 Targeted intracellular overexpression of Aβ causes severe axonal abnormalities

Following success at inducing an abnormal phenotype via direct administration of Aβ, I sought to replicate these phenotypes via genetic means. GAL4/UAS-mediated intracellular overexpression of Aβ peptides is the most commonly used method of investigating AD phenotypes in vivo.\textsuperscript{54,63} Mechanistically, expression of the Aβ peptide is simple; it negates any need to simulate amyloidogenic cleavage, which historically has required co-overexpression of BACE1 along with human APP due to the absence of endogenous BACE activity in flies.\textsuperscript{70} I therefore selected Aβ overexpression as a potential genetic means of replicating the phenotypes observed when Aβ was directly applied to neurons.

Following standard experimental procedure, I began my initial experiments with 6-hour incubations. To drive early overexpression of Aβ peptides, the scabrous promoter was used to express GAL4, which is active at high levels throughout the timescale of the experiment.\textsuperscript{57} Neurons were harvested from stage 11 embryos, incubated for 26°C, fixed and then stained for F-actin and MTs. Aβ\textsubscript{42} and Aβ\textsubscript{42}Arctic overexpressing neurons and control neurons (which expressed only the GAL4 under the control of scabrous) were assessed for axon length, growth cone area, and MT morphology as shown in figure 11\textsuperscript{Error! Reference source not found.}. 
Aβ42 and Aβ42Arctic overexpression caused ~25% axon shortening compared to controls (figure 11). There was no change to growth cone area or the ratio of non-coalesced MTs to organised MTs.
It remained to be seen whether a longer incubation, and thus a higher cumulative concentration of Aβ, had further toxic effects. The incubation time was increased to 3 days, and GAL4 was expressed under the control of ELAV, a promoter which is active at all embryonic stages and would have a longer period of expression compared to scabrous.

Intracellular expression of Aβ42 and Aβ42Arctic caused a decrease in axon length and a 3 to 4 fold increase in axon shaft and distal axon MT disorganisation after 3 days (figure 12). Expression of Aβ40 only increased MT disorganisation in the axon shaft, and did not increase MT disorganisation in the axon terminal.

In conclusion, the phenotypes associated with intracellular expression of Aβ differed from extracellular application as axon shortening occurred, which was not detected when Aβ was applied extracellularly. In addition, the MT disorganisation phenotypes were more severe and occurred earlier (see section 4.3 for an in depth discussion).

3.4 Expression of the C99 fragment of human APP caused moderate axonal abnormalities

Due to the varying phenotypes caused by the previous method of Aβ delivery, another approach was considered. The intracellular expression of Aβ peptides may potentially cause
artefacts through activation of nonspecific pathomechanisms such as ER stress, which are not likely
to be triggered in the AD brain. An ideal method of simulating amyloidogenic cleavage of APP would
be the expression of the BACE1 cleaved C-terminal fragment of human APP, C99 (figure 1). Cleavage
of C99 via γ-secretase results in the production of Aβ40 as well as Aβ42 (Figure 1: Overview of
amyloidogenic and non-amyloidogenic cleavage.). Flies do not possess BACE1, however they do
possess γ-secretase which is localised to the plasma membrane. Therefore, Aβ production would
theoretically be limited to cleavage of C99 in the plasma membrane, resulting in extracellular
secretion of Aβ40 and Aβ42, as is hypothesised to occur in AD. I therefore used stocks carrying UAS
constructs of C99 conjugated to either fly or human signal peptides at the C-terminus to aid its
insertion into the plasma membrane, wherein it can undergo γ-secretase mediated cleavage.
Therefore, C99 overexpression may be a more physiologically relevant way of modelling Aβ-
mediated pathology using genetic means than expressing Aβ peptides on their own.

I chose to test the effects of C99 expression after 3 days of incubation, since I reasoned that it is
possible that expression of C99 may result in lower Aβ42 concentrations since γ-secretase cleavage of
C99 produces Aβ40 and Aβ42 in a 10:1 ratio. As no MT disorganisation was detected at 6 hours
with intracellular expression or extracellular application of Aβ, it seemed unlikely that C99
expression would cause MT disorganisation at this timescale. Expression of C99 with a fly signal
peptide, (D.mel C99) and C99 with a human signal peptide (H. sapiens C99) was driven by the
GAL4/UAS system under the control of the ELAV promoter. After three days of incubation, I
observed axon shortening in neurons expressing both types of C99 (figure 13).
In conclusion, *D. mel* C99 expression caused MT disorganisation after 3 days, although not to the same extent as intracellular Aβ expression, and in a manner more reminiscent of the extracellular application of Aβ. However, the axon shortening phenotype is more similar to intracellular expression.

### 3.5 Expression of hTau causes MT disorganisation although application of Aβ had no additive effect

I next wanted to determine the effect of hTau overexpression on the subcellular readouts detailed in figures 3 and 4. The rationale for overexpressing hTau was that it has previously been shown to cause neuronal death, synaptic dysfunction and shortened lifespan in flies in a manner which resembles an AD phenotype [32]. In addition to overexpressing human tau, I chose to apply Aβ directly to the neurons, as any cooperative enhancement of phenotype would provide an insight into interactions between Aβ and tau. A 6-hour long incubation was chosen as I had not performed any experiments with hTau so it was essential to determine if there was an early phenotype associated with hTau overexpression to provide a strong basis for further experimentation. 100 nM Aβ$_{42}$ was chosen as the starting concentration despite previous experiments finding that this concentration had no effect at 6 hours of incubation, as the hypothesis was that Aβ would enhance the toxicity of hTau via stimulation of pathways that result in tau hyperphosphorylation and thus dysfunction.

Neurons overexpressing 2N4R$^{R406W}$ tau (human tau with a FTD mutation) were harvested from stage 11 embryos and incubated for 6 hours in media containing DMSO alone or DMSO with 100 nM Aβ$_{42}$, and were fixed before being stained for F-actin and tubulin.

In these experiments, 2N4R$^{R406W}$ tau expression caused an increase in the proportion of non-coalesced MTs to organised MTs but did not cause axon shortening or a change in growth cone area (data not shown in graph, Fig. 14). Additional treatment of these hTau-expressing neurons with 100 nM Aβ$_{42}$ extracellularly had no effect on any of the readouts measured. In conclusion, tau was responsible for MT disorganisation after 6 hours, but Aβ had no additive effect. Certainly, this result needs further validation, but would in first approximation be consistent with the cascade hypothesis (see section 4.3 of the discussion).
Figure 14: 100 nM Aβ42 had no effect on MT disorganisation, although hTau expression caused MT disorganisation after 6 hours. (A) Overexpression of hTau and treatment with 100 nM Aβ42 caused no change in axon length. (B) Overexpression of hTau and treatment with 100 nM Aβ42 caused no change in growth cone area. (C) 100 nM Aβ42 alone had no effect on MT disorganisation ($P_{\chi^2} = 0.8313$). hTau overexpression caused an increase in MT disorganisation ($P_{\chi^2} = 0.0485$) and hTau overexpression with 100 nM Aβ42 also caused an increase in MT disorganisation ($P_{\chi^2} = 0.0432$). There was no difference between hTau overexpression alone and hTau overexpression with Aβ42 application ($P_{\chi^2} = 0.9652$). $N =$ number of neurons analysed. Experiments were performed in triplicate; analyses were performed using the mean values for each experimental repeat.
4. Discussion

4.1 The fly neuron culture system provides measurable readouts for the study of Aβ and hTau

*Drosophila* has been used in numerous attempts to model specific aspects of AD in order to identify key molecular interactions and mechanisms which contribute to the disease phenotype. Most of these studies were in vivo and have used systemic phenotypes in order to deduce potential mechanisms. Here I have attempted to take these disease models to the next level by studying them in cultured fly neurons. This complements the existing approaches by investigating the subcellular mechanisms to a degree that cannot be attained in vivo. A single systemic phenotype, for example, may have several distinct subcellular mechanisms contributing towards it which cannot normally be dissected using in vivo means; cell culture provides a means of breaking down the mechanistic background underpinning complex, larger-scale phenotypes.

The primary aim for the project was to use *Drosophila* neuronal cell culture to observe and quantify the effects of Aβ and tau on the neuronal cytoskeleton, in order to develop a model of neurodegeneration. This goal was achieved due to the advantages offered by the neuron culture system; high sample numbers can be achieved with ease, which allows for the detection of subtle differences between readouts, which may be missed with smaller sample sizes. Capitalising on this advantage, I established that MT disorganisation is a reproducible and robust phenotype in response to both Aβ and tau exposure. My findings recapitulate observations made in mouse neurons which were exposed to Aβ by extracellular application (E. Chieregati, pers. comm.), which strongly suggests that the mechanisms underpinning neurodegeneration are evolutionarily conserved.

The MT disorganisation phenotype is likely to be linked to axonal pathology in the AD brain. A healthy, organised cytoskeleton is vital to neuronal function; neurons must maintain large cellular appendages, up to a meter long, for approximately 80 years (a human lifespan). If the cytoskeleton is compromised, normal function of the neuron is inhibited. Axons in aged and AD-afflicted brains have exhibited phenotypes which relate to MT disorganisation, such as neuritic ballooning and the formation of diverticulae. These bulges in the axon are formed as a result of MT bundling, looping and non-coalescence, which have the potential to impede transport of kinesin and dynein-bound cargoes. The current dogma is that the change of straight ‘tracks’ of MTs into convoluted, chaotic sprawls prevents the normal transport of cargoes by steric hindrance, which appears to be a critically important process in terms of neuronal survival and function. These previous experiments, combined with my own work, allow me to assemble the beginnings of a model.
Of particular note is how the different systems of Aβ delivery to neurons all resulted in MT disorganisation as a common phenotype. However, there were marked differences in the timing at which these phenotypes became detectable, and reproducibly different effects on other quantifiable readouts of the neurons, with axon length being of particular note. This suggests that the mechanisms triggered by the various delivery methods of Aβ are not identical, and that evaluation of each of the approaches taken is possible in terms of their physiological relevance to the disease conditions. I will now outline the deviations in the phenotypes exhibited by each of the modelling approaches and provide potential mechanistic explanations as to why these phenotypes may have manifested.

4.2 Different modes of Aβ application cause deviating phenotypes

Using the versatility of the fly primary neuron system and its powerful, quantitative subcellular readouts, I have taken a comparative approach which utilised different methods of Aβ application. This allowed me to evaluate the efficacy and physiological faithfulness of each method and to determine which system is the most reliable, accurate, and representative of the disease process. The striking similarity between all methods of Aβ application was that MT disorganisation was a common denominator regardless of the application method. The key differences between the methods related to the extent of MT disorganisation and the time that it took for MT disorganisation to occur. Only the genetic means of Aβ application (Aβ intracellular expression and expression of the C99 fragment of APP) resulted in axon shortening. Axon length did not change in neurons which had Aβ applied to them extracellularly. The existence of qualitatively different phenotypes between the application methods use could either have an underlying qualitative cause, wherein the phenotypes are caused by differing molecular mechanisms, or may simply have a quantitative cause in the form of the amount of Aβ produced or its aggregation state.

4.2.1 Intracellular Aβ causes severe cytoskeletal phenotypes

Intracellular expression of Aβ had a strikingly severe phenotype, including MT disorganisation and axon shortening which manifested as early as at 6 hours of incubation. This could be due to numerous factors, including too high expression levels, continuous production of Aβ eliciting increasing toxicity over time, and its cellular location.

The Aβ peptide is produced in a location distinct from the cytoplasm, which could be in either the ER, lysosomes, TGN, or the plasma membrane. To increase the probability of secretion of the peptide in these systems, as happens in the disease process (figure 1), a secretion signal peptide from the fly spn43Ac gene is conjugated to it. Hypothetically expression of the peptide therefore should simulate extracellular Aβ-mediated toxicity. However, explicitly intracellular localisation of Aβ
may have several effects which may not happen when Aβ is outside of the cell; incubation of mitochondria isolated from rat neurons with Aβ showed that mitochondrial respiration is inhibited. This may result in more severe deleterious effects than those that actually happen in disease. Intracellular Aβ is present in AD brains, but it is possible that intracellular Aβ may cause subcellular phenotypes via nonspecific mechanisms, such as ER stress. The implications of these findings are discussed in sections 4.2 and 4.3.

4.2.2 Expression of C99 causes less severe cytoskeletal phenotypes compared to intracellular Aβ

Expression of C99 resulted in a less severe phenotype when compared to intracellular expression of Aβ, which could be explained by a variety of reasons. Firstly, Aβ is only toxic when it is cleaved from C99, which is dependent upon the activity of γ-secretase. Second, γ-secretase cleavage of C99 produces both Aβ_{40} and Aβ_{42} in a 10:1 ratio, so it can be expected that less of the more toxic Aβ_{42} would be produced. Third, the location of γ-secretase mediated cleavage may also be a factor; γ-secretase is present on the plasma membrane and also in late endocytic compartments intracellularly. Finally, Aβ_{40} has been shown to partially attenuate the toxicity of Aβ, in cultured rat neurons. Therefore, in C99 –expressing AD models, Aβ is only produced once the parent C99 molecule comes into proximity with γ-secretase, and even then the amount of Aβ_{42} is comparatively low, and where the Aβ ends up could be either intra or extracellular, or both. It is possible that the milder phenotype C99 elicits compared to Aβ peptide expression is due to a combination of these factors. In both Aβ and C99 expression systems however, Aβ would be present intracellularly at some stage; this may explain why the phenotypes are similar.

4.2.3 Differences between genetic Aβ application and direct treatment with Aβ

The more severe phenotypes detected using the genetic expression systems may be due to changes in the peptide produced. Production of Aβ intracellularly would result in high local concentrations in the subcellular compartment where it is produced; preformed aggregates have been shown to form in acidic late endosomes and lysosomes prior to secretion. Aggregation can occur at lower starting concentrations in these conditions – aggregation increases as a function of concentration, but the presence of amyloid seeds can nucleate aggregation at much lower concentrations than when exclusively monomeric Aβ is allowed to aggregate in solution. The presence of aggregates, some of which would be highly toxic ROS-producing oligomers, in endocytic compartments, provide another potential mechanism for the deviating phenotypes seen between the Aβ application methods. In contrast, aggregation of extracellular monomeric Aβ applied to mouse neuron culture was slower, with 4-5 days incubation at 37°C required for trimer and tetramer formation (H. Tsushima, pers. comm.)
comm.). In summary, the different methods of Aβ application may have had different aggregation characteristics.

The different methods of applying Aβ may cause MT disorganisation by more than one mechanism; of particular note is that Aβ is not the lone potentially pathogenic product of amyloidogenic cleavage. The C-terminal fragment of C99 released after γ-secretase-mediated cleavage is the amyloid intracellular domain (AICD); AICD is also produced by the non-amyloidogenic, α-secretase-mediated pathway. Nuclear signalling appears to primarily be mediated by amyloidogenic cleavage of APP however; blocking amyloidogenic cleavage has been shown to prevent AICD-mediated nuclear signalling. Mouse neurons which overexpressed AICD developed AD-like pathology, possibly by downstream interactions with tau. Overexpression of Aβ alone would not therefore activate any potential pathogenic AICD-dependent mechanisms.

The fly neuron model I use provides the means to isolate and distinguish between these potential mechanisms mentioned above. For example, I can increase the dosage of extracellular Aβ in 3 or 7 days cultures and see if axon shortening results. I can also increase the Aβ dosage via genetic means by increasing the copy number of UAS constructs, or by changing the temperature at which I incubate the neurons; the GAL4 / UAS system is temperature sensitive, and has higher activity and thus a greater level of UAS-mediated overexpression at higher temperatures. I could increase the incubation temperature of UAS-C99 cultures to 29°C and see if the stronger phenotype resembles intracellular Aβ expression. Conversely, I could decrease the incubation temperature of UAS-Aβ neurons and see if the less severe phenotypes associated with C99 expression can be recapitulated.

The localisation of Aβ in all the systems can be determined by treating the cultures with a monoclonal antibody against Aβ such as 6E10. This would allow for spatial location of Aβ to be considered; the intensity of staining could also be used to provide an estimate of the amount of peptide, in addition to other biochemical techniques such as ELISA. In addition, I can investigate individual mechanisms which may differ between approaches. Induction of ER stress using conventional tools could be used to determine whether phenotypes associated with intracellular expression of Aβ are artefacts of ER dysfunction. In vivo ROS sensors can be used to determine the extent of oxidative stress in the different systems, which is associated with Aβ aggregation and toxicity. In addition, I could co-overexpress AICD with Aβ in order to determine whether phenotypic differences between C99 and Aβ overexpression are influenced by AICD.

4.3 What are the potential mechanisms that cause MT disorganisation?
MT disorganisation was a common effect induced by all methods of Aβ delivery, and was also observed upon targeted expression of hTau in fly neurons. MT disorganisation has also been
observed upon Aβ application to primary mouse neurons (E. Chieregatti, pers. comm.) which suggests potential similarities between fly and mammalian neurons, but most importantly, conservation of the cellular and molecular mechanisms governing Aβ-induced MT disorganisation.

MT disorganisation is a common phenotype observed for a loss of a number of MT regulators in fly axons. This disorganisation manifests upon loss of MT plus-end regulators such as EB1, spectraplakins, CLASP or Short stop (Shot); loss of dynein, or loss of MT stabilisers such as tau and MAP1B 61 (N. Sánchez-Soriano, pers. comm.). When homologues of these proteins are lost, in particular spectraplakins, dynein, tau or CLASP, MT disorganisation also occurs 88-90. When the phosphorylation state of doublecortin is changed MT disorganisation also occurs, showing that post-translational modification of MAPs is sufficient to cause loss-of-function 91. All of these examples are of MT binding proteins; this implies that MAPs could be mediators of Aβ and hTau-induced toxicity through common or distinct pathways.

As previously discussed (section 4.2), the different methods of Aβ application may have activated or deactivated distinct molecular mechanisms to cause the deviating phenotypes, but they may also share commonalities, including the presence of Aβ intracellularly, which could have occurred in all systems. Aβ in the cytoplasm is likely to impact on tau phosphorylation through the activation of signalling pathways including GSK-3β, JNK, and the activation of DNA repair kinases such as checkpoint kinase 2 (Chk2) as well as the calcium second messenger system (see introduction) to name a few 92-95. As mentioned, tau phosphorylation causes its detachment from MTs 96. This corroborates with findings that loss of endogenous Drosophila tau causes MT disorganisation (N. Sánchez-Soriano, pers. comm.). In addition, expression of hTau in Drosophila neurons recruited dTau away from MTs 97. Therefore, tau plays a critical role in the observed MT disorganisation phenotypes, and there are a range of genetic tools which may be used in future research. An example is the use of dTau tagged with GFP to visualise the localisation of dTau each of the AD models, and to determine whether application of Aβ causes tau mislocalisation from MTs.

Overexpression of hTau, caused MT disorganisation (see section 3.5). The reason for this is likely to be loss-of-function of endogenous dTau, which may have been recruited away from MTs by hyperphosphorylated hTau (hTau has been shown to bind dTau in vivo). Loss of dTau results in MTs losing stability, leading to MT disorganisation and ultimately compromise of the entire neuronal cytoskeleton.

This reasoning would also explain why extracellular Aβ application did not enhance MT disorganisation in neurons expressing hTau, as dTau was already detached in these neurons and so Aβ-mediated induction of tau mislocalisation would have no additive effect. However, this
experiments have only been performed with a 6 hour incubation period, and longer incubation periods may elicit stronger phenotypes. Further experimental approaches could also be used to test the above hypothesis by asking whether the phenotype caused by dTau deficiency can no longer be enhanced when applying Aβ or hTau. Even if this principal model turns out to be incorrect, all of the other regulators of MT dynamics are well established in my host laboratory and could be used of to either refine the tau loss-of-function model, or to work out alternative models that can better explain the observed phenotypes.

4.4 Development of a cytoskeletal model of neurodegenerative disease

By combining my findings with past hypotheses and current opinions in AD pathogenesis, I have been able to develop a hypothetical model of neurodegeneration in AD which focuses on the cytoskeleton. This model builds on the logic of the amyloid cascade hypothesis, which proposes a broad variety of downstream effects as a result of Aβ toxicity. I hypothesise that tau is the keystone protein in AD pathogenesis, and that it causes the bulk of the neuronal damage which leads to cell death. I have outlined my rationale below.

4.4.1 Overexpression of hTau in the presence of Aβ had no additive effect

When I overexpressed hTau in the presence of Aβ, there was no exacerbation of the phenotypes that I had seen when I subjected neurons to Aβ alone. A possible reason for this is that loss-of-function, and toxic gain-of-function of endogenous tau is responsible for the majority of cytoskeletal disorganisation which was observed, and that Aβ initialises the processes leading to dTau LOF/GOF. This is supported by the finding that tau knockout mice did not develop AD-like symptoms when exposed to Aβ, suggesting that tau is required for Aβ to cause its toxicity.98

My findings are consistent with the idea that tau is downstream of Aβ in the AD pathway, although Aβ is required initially to start the disease process. The existence of tauopathies, in which mutant or misfolded forms of tau cause neurodegeneration in the absence of Aβ, lend further credibility to this notion.

4.4.2 Aβ is required to provide the conditions for a self-perpetuating tauopathy

Hyperphosphorylated tau is capable of templating, as described in section 1.6.2. As p-tau can spread through neurons and across synapses, it is possible that even if upstream Aβ toxicity is decreased or stopped completely, a self-perpetuating tauopathy may remain. This is the basis for the model I have developed in figure 15, which is an adaptation of the amyloid cascade model.
Fig. 15: the adapted amyloid cascade hypothesis model. In this model, Aβ is responsible for the initial events which lead to pathogenesis. Activation of a wide variety of kinases (detailed in figure 4) causes hyperphosphorylation of tau, resulting in a loss-of-function of its normal MT-stabilising role, and a toxic gain-of-function and runaway templating. In this model, even if Aβ production is stopped, or its toxicity attenuated, a runaway tauopathy will remain. In this model, tau is the optimal target for any drugs or interventions.
References


## Appendix A

### References for fly stocks used

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<td>ELAV-GAL4</td>
<td>Damian Crowther</td>
<td>Berger C., Renner S., Luer K., Technau G.M. The commonly used marker ELAV is transiently expressed in neuroblasts and glial cells in the Drosophila embryonic CNS. Dev Dyn. 2007; 236(12):3562-3568</td>
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<td>UAS-C99.LCP2SP.HA</td>
<td>Vitruvian Biomedical</td>
<td>Bloomington stock no. 33785. Available at: <a href="http://flystocks.bio.indiana.edu/Reports/33785.html">http://flystocks.bio.indiana.edu/Reports/33785.html</a></td>
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