Investigations into the effects of the Alzheimer's risk gene BIN1 on tau subcellular location and propagation

By Sema Balaban

Dr. Elizabeth Glennon

Department of Basic and Clinical Neuroscience
Institute of Psychiatry, Psychology & Neuroscience
King's College London
University of London

Thesis in partial fulfilment for the degree of MSc in Neuroscience September, 2018.

Word Count

Word Counts:	*Title*: *Abstract* (max 350): *Main Body* (not including Title, Abstract, References and Appendices; 7,500-10,000):	Word Count 247 8,322

Personal Statement:

Dr Lizzie Glennon has designed this project. She has dissected brains of rat pups to collect primary neurones, transfected the cells with the virus, performed immunocytochemistry and took microscope images. She has also harvested and ran BCA assays on tau samples 1 and 2.

I have performed BCA analysis on tau 3 samples and ran Western-blot analyses on tau samples 1-4, quantified and analysed the blots, designed settings to analyse and analysed images using Nikon Elements, analysed particles from images on Image J and data on Prism using t-tests.

We have carried out the tau ELISA together with my supervisor, where I was responsible for untreated cells, and she was responsible for treated cells.

Table of Contents

Abstract

Recent GWAS studies have identified BIN1 as one of 8 other genes that are associated to late-onset Alzheimer's disease (Kamboh et al., 2012). As this is a gene that has been identified fairly recently, there are gaps in literature as to the nature of the association of BIN1 to AD. Studies have identified tau related changes when BIN1 levels are altered (Chapuis et al., 2013). Hence, this experiment has set out to further investigate how altered levels of BIN1 may contribute to tau subcellular location and propagation. Cortical neurones were dissected from E18 mice pups. BIN1 shRNA and control shRNA was used as viruses in this knock-out study. To understand the effects of BIN1 knockdown on the level of intracellular tau, PSD-95, synaptophysin, phosphorylated and dephosphorylated tau, Western-blot analysis was conducted, to understand the effects of knockdown on co-localization of tau with PSD-95, MAP2, synaptophysin and active synapses, these cells were stained and imaged by microscopy and analysed using image software. Finally, a western blot was conducted to understand the effects of treatment and extracellular tau release on cells with knockdown, followed by an ELISA, which was normalised against the blots. Some highlights of this research was a reduction in the level of tau when BIN1 was knocked down, BIN1 knockdown did not effect tau phosphorylation and tau was localised more with PSD-95 in the absence of BIN1. Also, the experiments found that there was more extracellular tau when BIN1 was knocked down in the treated condition.

Acknowledgements

It has been a difficult transition from a degree in Psychology to Neuroscience. Lizzie, you have endlessly supported me and I am very grateful that you always were there for me, spending hours explaining unfamiliar concepts. Also, thank you for preparing me for a career in neuroscience, and being an amazing and inspirational role model, for pushing me to aim higher every single time in the road to a PhD.

My love, Batuhan, you have listened to me read everything that I have written over and over again. You have supported me through my struggles and motivated me to work harder. Thank you for being there…

Rebecca, thank you for devoting your time to ensure I understood the Western blotting protocol and for extra supplies of running and transfer buffers when I ran out, and for tirelessly showing me the correct tank to use during transfer and supporting me all the time, even when I asked the silliest of questions.

Finally, thank you to my amazing parents for supporting me all along this journey!

Introduction

1.1 The characterisation of Alzheimer's disease

Alzheimer's disease is a neurodegenerative illness that affects millions of individuals over the world, and is expected to exceed 60 million by 2030 (Korolev, 2014) and to double in amount every 20 years (Reitz et al., 2011). It is named after the renowned physician Alois Alzheimer who presented the case of his patient Auguste D. with a record of psychiatric assessments, during which he identified changes relative to memory, organisation and personality (Dahm, 2006). Importantly, histology of her brain revealed atrophy, depletion of neurons, senile plaques and NFTs. One variation of AD is late-onset (LOAD), also referred to as sporadic and is the most common type, where family related early onset only explains 5% of cases (Barber, 2012). Memory functions worsen in time, beginning with the inability to retrieve information learnt recently (Isik, 2010)

1.2 Pathology

1.2.1 Tau

Located on chromosome 17, tau is of one of a category of proteins known as Microtubule-Associated proteins (MAPs), and has six isoforms and binds by a proline rich domain to SH3 domains of other proteins (Kolarova et al., 2012). MAPT encodes tau, the gene sequence codes for a projection domain (n-terminal), a proline rich domain and the c-terminal, which consists of Microtubule binding repeats (MTRs) and a tail, and is susceptible to a number of post-translational modifications (PTMs) such as acetylation, phosphorylation, ubiquitination and truncation, all of which may play a role in disease (Ittner & Ittner, 2018)

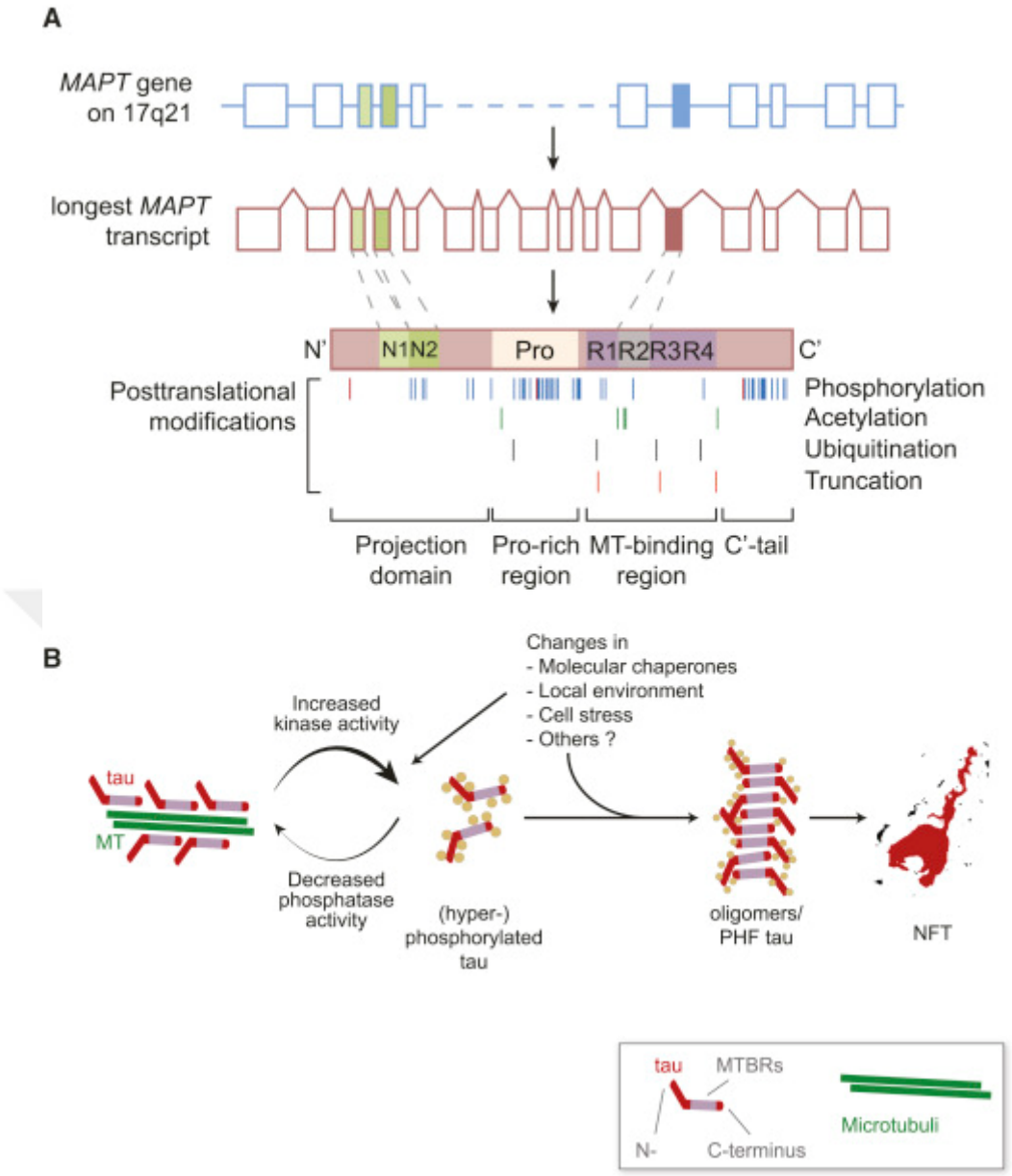

Figure 1 *The MAPT gene, tau transcript, and potential post-translational modifications (A) and pathological changes (B), obtained from Ittner & Ittner, 2018.*

This family of proteins provide stability by associating with tubulin, inducing formation of microtubules (Iqbal, Liu, Gong & Grundke-Iqbal, 2010). In AD, tau is phosphorylated and form tangles inside neurons (Sottejeau et al., 2015). Studies have documented that phosphorylated tau affects microtubule assembly in brain cytosol of rats, whereas recombinant tau stimulated microtubule bundling (Li et al., 2007) Tau disassembles from microtubules, possibly as a result of altered phosphorylation, causing impaired stability, releasing soluble tau, which are consequently altered by PTMs leading to dimerisation,

followed by oligomerisation and finally two promoters cause these to assemble into NFTs (Martin, Latypova & Terro, 2011). Tau protein is vulnerable to phosphorylation at a number of sites (Guo, Noble & Hanger, 2017).

1.2.2 Tau localisation

As tau associates with microtubules to provide cellular support, it is mostly localised around axons (Guo et al., 2017). Al-Bassam et al. (2002) state that tau and MAP2 collaborate to support axons by stabilising microtubules, finding that they bind at protofilaments. Hoover et al. (2010) highlight that phosphorylation of tau causes mis-localisation and tau accumulation in dendritic spines mediates synaptic dysfunction in the early stages of the disease. It is important to understand the location of tau, as it may give an indication of how tau pathology develops over time, allowing researchers to map the disease course and would also enable them to see whether it has any interactions with other components in that particular location.

1.2.3 Tau & Synapses

The synapses have a lot to tell regarding the nature of the disease. Researchers have shown that phosphorylated forms of tau is present both presynaptic and postsynaptically, however there appears to be a higher amount in the latter (Tai et al., 2012). On the other hand, by increasing expression of hTauP301L, Harris et al (2012) found that phosphorylated tau levels were higher in the presynaptic regions. However, evidence on the mechanisms that causes increase in pathological tau in critical regions, falls behind. In the past, studies have identified that tau pathology spreads from one neuron to another in a “prion-like” manner, which could explain Braak staging (DeVos et al., 2018). Braak staging has been adopted since 1991 to identify phases in those with AD (Braak et al., 2006). The stages originate from hippocampal areas, and are spread by neurons that are impaired by neurofibrillary tangles

through the temporal, neocortical and occipital regions (MacDonald, 2007). In their model of Alzheimer's researchers found that axon related deficits in the subiculum impacts presynaptic terminals, possibly a characterization of early synaptic loss (Trujillo-Estrada et al., 2014).

1.2.4 Tau Secretion

Unfortunately, there is limited knowledge on the properties of extracellular tau in the disease model (Dubal, 2018). In AD, extracellular tau mediates the spread of disease in a trans-cellular fashion (Yamada, 2017). Aggregates are taken in by cells and propagate fibrillization of intracellular tau, the newly aggregated tau protein then travels amongst other cells in the culture (Frost et al., 2009).

1.2.5 Tau & Dendritic spines

Post-synapses are found on dendritic spines of the receiving neurone (Ittner & Ittner, 2018) Studies have found that impairments are caused by synaptic dysfunction as a result of accumulating hyperphosphorylated forms of tau inside dendritic spines (Hoover et al., 2010). Merino-Serrais et al., (2013) have identified that tau aggregates that form NFTs within pyramidal cells caused development of defects in dendritic spines, including a decrease in the amounts and structural changes. Dendrites are heavily implicated in disease pathology, thus researchers should continue to investigate how pathological tau reaches this component.

1.3 Genetics

For decades, three genes had been known to be implicated, the amyloid precursor protein (APP), presenilin (1 and 2) and as a risk factor, APOE4 (Bekris et al., 2011). For a

number of years, research centred on APOE4, as the most frequent risk factor for LOAD (Tang & Gershon, 2003). Recently, many more susceptibility genes have been identified in AD through GWAS analysis, one of which has been BIN1, giving reason to investigate its pathological role in neurodegeneration (Tan, Yu, Tan, 2013). Only in the last decade, genome-wide association studies ranked BIN1 as the second prominent susceptibility locus (Chapuis et al., 2013).

1.3.1 BIN1, amyloid beta and tau

A review of literature reveals that the emerging papers on BIN1 are often related to how the protein associates with tau. A number of papers find correlation between the expression of the protein and neurofibrillary tangles, but there are some that report the contrary (De Rossi et al., 2017). It appears that when researchers began investigating the gene they believed that increased expression of BIN1 induces tau pathology, and in the absence of the BIN1 ortholog, tau related toxicity is reversed (Chapuis et al., 2013). There are fewer papers that report a link between BIN1 and amyloid-beta, and those that do, report that knockdown of BIN1 leads to a build up of BACE1, and thus amyloid beta, because the recycling mechanism is impeded (Miyagawa et al., 2016; Ubelmann et al., 2017). Gathering information from multiple sources, Tan et al. (2013) indicate that BIN1 is involved in a number of processes, importantly in endocytosis and cellular trafficking,

1.3.2 BIN1

BIN1 (also referred to as amphiphysin 2) is found on chromosome 2q14.3 and has 10

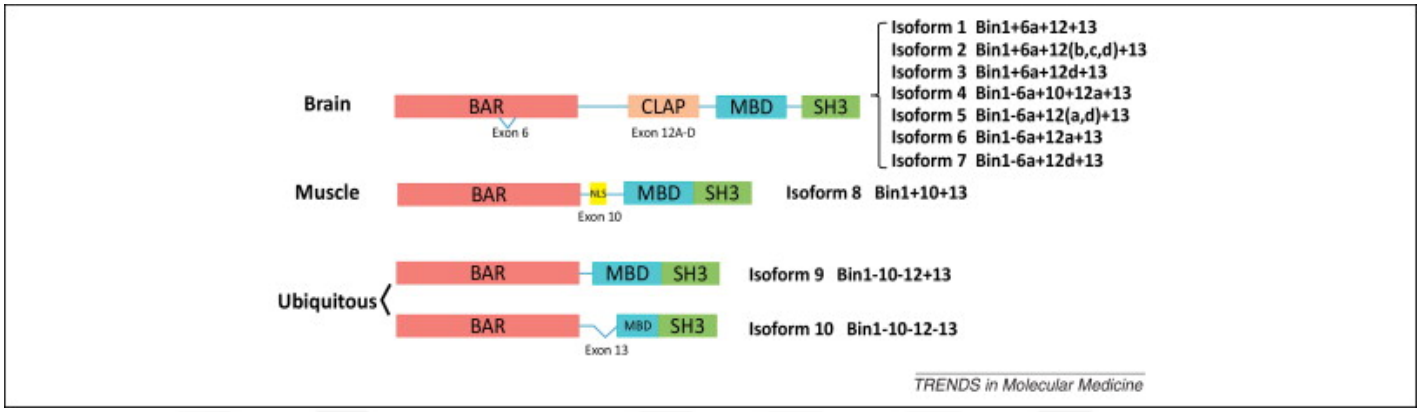

Figure 2: *Brain, muscle and ubiquitous isoforms of BIN1, obtained from Tan, Yu & Tan (2013)*

isoforms (see figure 2.), encoding for muscle, brain and ubiquitous tissue (Tan, Yu, & Tan, 2013). BIN1's role in the nervous system remains unclear, but since the 1990s researchers had speculated that it is a tumour suppressor (Wechsler-Reya et al., 1997).

Papers have identified that BIN1 is reduced in sporadic cases compared to individuals without dementia (Glennon et al., 2013). Studies have reported absence of BIN1 to cause pathological changes in a variety of diseases. De Rossi et al. (2016) found that BIN1 loss is in correlation with demyelinated MS lesions. Researchers reported structural flaws (as those reported in biopsies of humans with centronuclear myopathy) when they knocked down BIN1 in a CNM2 model of Zebrafish, and that the organisation of myofibres is affected (Smith et al., 2014)

1.3.3 BIN1 & Endocytosis

Tau is an intracellular protein, but in diseased brains it is known that aggregates are present extracellularly (Frost et al., 2009). These aggregates enter neurons by endocytosis in AD and FTD-tau (Wu et al., 2013). Wu et al. (2013) documented that extracellular, low molecular weight tau is taken inside neurones, co-localising with dextran, indicative of bulk-

tosis. Decades ago, it was established that the amphiphysin family which BIN1 is a member of, is populated around nerve terminals and associates and co-localises with dynamin indicating that it has a role in endocytosis (David et al., 1996). Endocytosis is a mechanism, which internalises molecules on the outside of cells with the support of dynamin in the clarithin-mediated pathway (Singh & Jadhav, 2016). Now, we know are certain that several genes associated with AD are known to have functions in endocytosis (Giri et al., 2016) some of which are APOE, SORL1, CD33, CD2AP, PICALM and importantly BIN1, the gene of interest in this study. A phenomenal paper was published with many findings that shed light on the association between tau and BIN1. Calafate et al., 2016 identified that i) reduction in levels of BIN1 induces tau pathology because of increased endocytosis (as BIN1 represses the endocytic flux) and ii) that tau pathology began to decline when dynamin was inhibited, and more importantly iii) that aggregates damage and leak from the membrane to the cytoplasm. Dynamin has a prominent role in endocytic fission, is essential for vesicles to form, and is also believed to be involved in recycling of synaptic vesicles and trafficking through the Golgi body (Hinshaw, 2000).

1.3.4 BIN1 & Synaptic Vesicle Recycling

A prominent stage of the synaptic vesicle cycle is recycling, which is carried out by one of three mechanisms, kiss and run, kiss and stay or clathrin-mediated (Südhof, 2004). Di Paolo et al. (2002) recorded deficiency in synaptic vesicle recycling and cognitive impairments in mice where the amphiphysin 1, another member of the BAR family, was knocked out. It is important to investigate (if there are effects of BIN1 on tau) whether these are related to synaptic vesicle recycling. Studying tau intracellularly may give an indication of whether mechanisms inside the cell are working efficiently.

1.3.5 BIN1 & Structure

BIN1 appears to have a number of roles in cell structure. Over a decade ago, researchers recognised the ability of BIN1 in tubulation, suggested that BIN1 is implicated in cellular processes by providing support, binding CLIP-170 (a protein implicated in microtubule stabilisation) to microtubules (Meunier et al., 2009). It has also been established that it modulates the curving of membranes, across endocytic pits (Tan et al, 2013). D'Alessandro et al. (2015) found that BIN1 modulates the position and shape of the nucleus which is different in patients with centronuclear myopathy. Studies have recently identified that BIN1 and tau bind together by their SH3-PRD domains, but this regulation begins to decline when tau is phosphorylated at the Thr231 residues, and interestingly the co-localization of these domains with the actin cytoskeleton could not be seen in the presence of Thr231-p-tau (Sottejeau et al., 2015).

1.3.6 The use of technology to analyse microscopy images

Researchers claim that statistically, data collected through fluorescent staining are susceptible to false positives (Moser et al., 2017). Nevertheless, these tools allow researchers to capture fluorescent tags to visually see molecules, and to analyse interactions between them in situ or in other terms, their co-localization (Pompey et al., nd.) There are hardly any studies that have measured co-localization between tau and other neuronal components in the absence of BIN1. Thus, we have used two applications that are utilised in co-localization analysis, i) Image J and ii) Nikon Elements. This paper will criticise the utility of these two methods in detail.

1.4 Aims of research

The volume of research on BIN1 is limited in literature, this study aimed to contribute, by analysing whether BIN1 has an effect on intracellular tau and various subcellular regions, by measuring the effect of BIN1 on the total amount of tau, phosphorylated and dephosphorylated tau, pre-synapse (synaptophysin) and post-synapse (PSD95) through western blot analyses.

Next, we aimed to capture microscopic images to visualise the amount of tau when BIN1 is knocked down, at different subcellular locations including synaptophisin (pre-synapse), psd95 (post- synapse), the dendrites (MAP2) and active synapses.

Lastly, we aimed to observe whether BIN1 knockdown has any effect on extracellular tau release/secretion, by ELISA and western blotting.

It is important to continue to research BIN1 as most suggest that it has therapeutic implications, BIN1 could be specifically targeted to enhance neuroprotection (Tan et al., 2013) as researchers have shown altering levels of BIN1 can effect tau levels, perhaps in the future methods to increase/decrease the expression of this gene could be developed. Before devising any treatments, the precise mechanisms should be understood, hence why we have conducted this research, with the hope to contribute to literature in acknowledging the nature of the gene.

Materials and Methods

2.1 Antibodies

For staining

Primary antibodies	
	1/250 total tau (from Dako)
	1/250 Syn C (from santa cruz)
	1/100 MAP2 (from GenTex)
	1/100 PSD95 (from Cell signalling)
Secondary Antibodies	1/250 anti-mouse 647 (PSD95) (from Invitrogen)
	1/250 anti-rabbit 546 (synaptophysin) (from Invitrogen)
	1/250 anti-goat 488 (PSD95) (from Invitrogen)
	1/100 anti-chicken 350 (MAP2) (from Invitrogen)
For the Western-blots	PHF 1/2,000 (gift from Peter Davies)
	Tau1 1/2,000 (from Millipore)
	1/2,000 ABT21 (from Millipore)
	1/10,000 total tau (from Dako)
	1/2000 synaptophysin (from Santa Cruz)
	1/2000 psd95 (from Millipore)
	1/10,000 Dako (from Dako)
	1/500 99D (from Millipore)
	1/5000 actin and tubulin (from AbCam)

For Tau ELISA	BT2 (aa 194-198) mouse monoclonal 1:100 (from Cell Signal) DAKO (aa 243-441) rabbit polyclonal 1:1000 (from Cell Signal) Goat anti-rabbit IgG HRP-linked whole antibody 1:500 (from Cell Signal)

2.2 Methods

BIN1 Knockdown

The knockdown was carried out 5 days in vitro, using a 6-well plate. Firstly, media was removed, then 700 ul media was added in each well. 7 ul of BIN1 shRNA lentivirus was added to half of the wells and scrambled shRNA lentivirus was added to the other half. The media was removed after one day and 3mls of conditioned media was added to each of the wells, followed by incubation of the neurones at 37 degrees 5 % CO2, until 21 days in vitro.

Animals, harvesting and plating

Primary neurones were harvested from neurons in the cortical region of E18 rat pups. The cortex was transferred to a 50 ml falcon tube, and washed with 5mls of HBSS -/- (Life technologies). Then, HBSS was added till the 5ml line was reached on the tube. 100 ul 2.5% trypsin was also added. This was then left to incubate for half an hour at room temperature (the tube was swirled every 10minutes).

5ml 10ugml-1 DNAse, 1ml-triturating solution (1% albumax, 25mg trypsin inhibitor, 10 ugml-1 DNAse in HBSS, all from Sigma) were added consecutively and then, triturated. 1ml neurobasal media was added per pup. A 70 um cell strainer was placed on a 50ml falcon and the cell suspension was pipetted into the top of the cell strainer.

The cells were then counted, and it was ensured that they were suspended decently. 10 ul cell suspension was added to 90 ul trypan blue (Sigma); which was mixed by pipetting.

10ul was put on one end of a heomocytometer. The cells were counted in each corner and averaged, this number was then multiplied by 100,000.

Coverslips from Marienfeld/Shcott were sterilised and transferred to plates via forceps. The plates/coverslips were coated by incubating overnight at room temperature with 20ugml-1-poly-D-lysine (from Sigma) in borate buffer. The PDL was aspirated and washed twice with water. It was ensured the neurones were suspended decently, and were then plated (100,000 per well for 12 well plate).

Immunocytochemistry and staining

Methanol Fixation

Cells were fixed in 4% paraformaldehyde FA and 4% sucrose in PBS and incubated for 10 minutes at room temperature. They were then fixed in -20ºC of methanol, and incubated at 10 minutes at room temperature. They were then washed in PBS, twice, for 10 minutes. They were then permealised and blocked in 2% FBS 0.1% triton in PBS, for 1 hour at room temperature. Then we incubated with primary antibody, which was diluted in 2%FBS, for 1 hour at room temperature. Then they were washed in PBS for three times 5 minutes. They were then incubated with secondary antibody, again diluted in 2%FBS, for 1 hour at room temperature. They were then washed in PBS three times, 5 minutes. Finally, they were mounted with ProLong antifade reagent (from Invitrogen), and left to dry in the dark room overnight. Slides were stored in 4ºC.

Cells were then incubated overnight with primary antibodies (see antibodies). These were then diluted in 2% FBS. The next day, coverslips were washed in PBS for 3 cycles (10 mins each), then incubated for 1 hour with the secondary antibodies (see antibodies)

Again, the coverslips were washed as previously described, then dried and mounted on the slides via pro-long diamond mounting media.

Harvesting cells for Western blot

The protein samples Tau 1-4 were prepared following a general harvesting protocol, which will be outlined below. Media was removed and placed in eppendorfs. The wells were then washed with PBS, and this was then removed by pipetting and disposed. Then, 500µl of PBS was added again. The wells were scraped and the contents were then added to eppendorfs and centrifuged at 13 000 g. Following this, they were placed in the centrifuge at 13,000 (4ºC for 10mins). Next, the supernatant was removed, and pellet left at the bottom of the eppendorfs. Then, 75µl of RIPA buffer (50mM Tris, 150 mM NaCl, 0.5% (w/v) Sodium deoxycholate, 1% (v/v) NP-40, pH 8.0) was used to re-suspend the pellet, then incubated for 30 minutes at 4ºc. Finally, the lysates were centrifuged for 10 minutes at 13,000 g at 4ºc, the supernatant was removed and the pellet was discarded.

Electrophoresis, Transfer and Antibodies

A general electrophoresis procedure was carried out, where 10% running and stacking gels were prepared.

First, the running gel was made and poured inside the pre-assembled electrophoresis plates (1.5mm). dH2O was then added to the top quarter. After 10 minutes, the water was poured out, and the stacking gel was added. A comb was placed inside for the wells.

In the meantime, water and lamlii buffer was mixed inside eppendorfs and protein was added. These were placed on an incubator for 5 minutes at 95ºc. They were then placed into the centrifuge for 2-3 seconds to ensure the liquid settles. Then, the same amount of protein was filled in each well after the combs placed inside the gels were removed.

The proteins were then run for 2.5 hours at 100V. Following this, the proteins on the gels have been carried from the gels to the nitrocellulose membrane by means of wet

sandwich transfer, which ran for 80 minutes at 100V in transfer buffer, then to be blocked with 1% dried skimmed milk (0.75g dried skimmed milk in 15ml TBST) for 1 hour.

Antibody solutions were made and poured on the membranes (antibodies and dilutions can be found in section 2.1). This was then left inside the cold room overnight. The next day, the membranes were washed with TBST. They were washed quickly for three times, then 3x 10 minutes. Secondary antibodies were poured onto the membrane and left for 1 hour on the shaker at room temperature. Then, the membrane was given 3 quick washes, followed by 3x 10 minute washes in TBST. The membranes were then taken to be imaged. The membranes were loaded into a chemi-doc imager from bio-rad and the software Image Lab was set up. Next, the housekeeping protein actin/tubulin was poured onto the membrane and left overnight. The same procedure was followed.

Experiment 1

5 sets of samples were prepared by harvesting and BCA calculations, to measure total tau, psd95, synaptophysin, p-tau and dephos-tau. The same amount of protein was loaded into each well for each blot. The synaptophysin-PSD-95 blot was cut into halves.

Experiment 1.1 BIN & Tau

A western blot was run and two membranes were produced to examine the total amount of tau across bin1 knockdown/ control cells. The primary antibodies 1/2,000 AST21 2% BSA was used to identify BIN1 and 1/10,000 DAKO was used to identify tau.

The next day cells have been probed with anti-mouse secondary antibodies. The membranes were then imaged through ImageLab software. Tubulin (10ml) was added, and imaged within the next few days.

Experiment 1.2 P-tau & Dephos-tau

The anti-mouse primary antibody PHF1 1/2,000 was used to identify phosphorylated and tau1 was used to identify dephosphorylated tau. The cells have been probed with anti-mouse secondary antibodies.

Then, tubulin has been poured overnight, to be imaged the next day.

Experiment 1.3 Synaptophysin & PSD95

1/2000 synaptophy was used as primary antibody and 1/2000 PSD95 for the identification of PSD95. Again, these were imaged and tubulin poured overnight for imaging.

Analysis of data (1.1-1.3)

The western blots were first quantified using Image J. These values were then carried to excel. First, the background was subtracted, and then the figures were converted into percentages, then to a relative amount in decimals, as the numbers were too large. Un-paired samples t-tests were run on the data in Prism.

Experiment 2

A total of 20 BIN1/Scrambled images were analysed via the Nikon Elements software. 10 images were analysed by Image J software.

Particle Analysis for Co-localization

Experiment 2.1 Nikon

Neurones have been transfected with BIN1 siRNA or control siRNA to be imaged for total tau, MAP2, PSD95 or synaptophysin. The two analyses software Nikon Elements and Image J have been utilised to analyse the images. Settings have been designed to run a general co-localization analysis on tau and PSD-95, tau and MAP2, tau and active synapses and tau and synaptophysin (see Appendix 1.)

Images were aligned and then deconvolved and analysed to extract ratio of co-localization.

A co-localization analysis was run, and data was exported to excel. The ratios from each image have been averaged. The final data are averages relative to controls.

Ratio: Tau and MAP2

Ratio 1: Tau and PSD-95

Ratio 2: Tau and Synaptophysin

Ratio 3: Tau and active synapses

Experiment 2.2 Image J

The deconvoluted images were then carried over to Image J. Channels were split and adjusted for brightness/contrast. A plug-in that was downloaded earlier, “Colocalization” was applied on the first set of channels of interest (i.e. channel 1-2), then a Z-project was applied onto this image (with maximum intensity). The image was inverted, then a z-project was applied to the channels. A circle was then drawn around the area of interest (nucleus) (84mm-

86mm), then, the number of particles was analysed. The same process was repeated for the rest of the channels.

Channel 1: PSD-95

Channel 2: Tau

Channel 3: Synaptophysin

Channel 4: MAP2

Co-localization of c1/2, c2/3, c2/4 and c1/3 were tested.

Analysis of data (2.1-2.2)

Data was then carried over to excel. The average size columns for each co-localization (i.e. channels 1-2 or channels 1-4) were averaged. Then percentages of these values were calculated. The numbers were converted to a relative amount to controls (in decimals). This was then carried over to Prism to run un-paired t-tests.

Experiment 3

There were 4 types of cells, BIN1 knockdown and control (no knockdown), to examine the effects of bin1 knockdown on tau release and stimulated (+) (with NaCl) and unstimulated (-) cells to examine the effect of stimulating BIN1 on tau release. There was three of each condition *(n=3)*, totalling to 12 samples per run. The same amount of protein was loaded into each well for each set of samples. Western blots were run to measure intracellular tau. Then a tau ELISA was conducted to normalise this against the blot, in measuring extracellular tau. The data was then entered into Prism to run unpaired t-tests and draw graphs.

Tau ELISA

Nunc MaxiSorp plates were coated with the capture antibody BT2 by incubation of BT2 diluted 1/x in coating buffer (2 µg/mL BT2 antibody), then 50 ul was added to each well of a 96-well plate and incubated for 8 days at 4ºC. Tau Elisa was carried out over 3 days. On the first day 96-well Nunc™ MaxiSorp™ plates were washed with TBS-T, 3 times for 5 minutes. Then 200µL of StartingBlock™ (ThermoScientific) that was diluted in 1:1 ratio in TBS was added to each well, and left for 4 hours at room temperature. Following this, another round of 3x5 minute washes in TBS-T was carried out. Next, 50 µL of sample was added to each well, in duplicate.

The plates were then sealed and placed to incubate at 37º c overnight. The following day, they were washed 3x 5mins with TBS-T. Capture antibody, DAKO (1:1000) was prepared by diluting Superblock 1:4 in 1XTBS. Followed by this, 50 µL was added to each well and incubated overnight at room temperature on a shaker.

On the third day, again, 3 rounds of washes were made with TBS-T. Then 50 µL of goat anti-rabbit (IgG HRP-conjugated secondary antibody (1/500) diluted in 5% milk/TBS) was added for one hour RT.

Next, another 3 washes (5mins) were carried out, and 100 µL of TMB chromagen substrate solution (from ThermoScientific) was added in each well, and after the blue reaction substrate appeared, the reaction was stopped adding 100 µL of 1N HCl in each well. The absorbance was read at 450nm.

The effects of BIN1 knockdown on intracellular tau, phosphorylated & dephosphorylated tau, synaptophysin and PSD-95

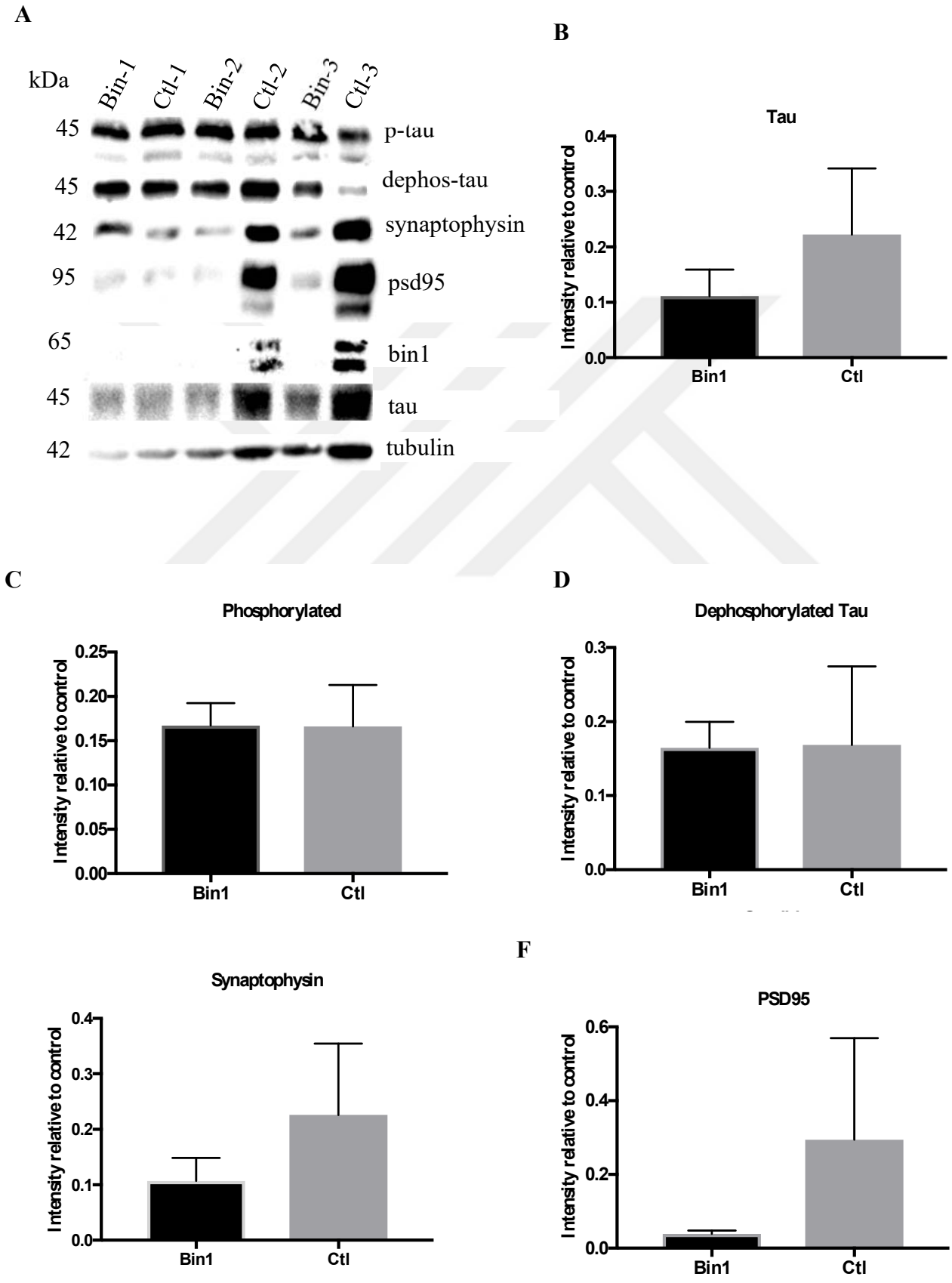

Figure 3 The effect of BIN1 on total intracellular tau, p-tau, dephosphorylated tau, PSD-95 and synaptophysin (see legend below)

Figure 3 BIN1, total tau, phosphorylated/dephosphorylated tau, synaptophysin and PSD95 expression in E18 primary neurones

BIN1 was probed with 1/2,000 ABT2 2% BSA primary antibody, and tau was probed with 1//10,000 DAKO. Tau and BIN1 were detected using anti-rabbit IgG HRP linked whole secondary antibodies (1/10,000, 2% BSA). The antimouse primary antibodies, PHF 1/2,000 (for phosphorylated) and Tau 1 (for dephosphorylated) were used.

Finally, 1/2,000 synaptophy was used as primary antibody to detect synaptophysin, and 1/2,000 PSD95 was used to detect PSD95.

These images were then quantified through Fiji.

The effect of BIN1 on TOTAL tau

We sought out to understand the effect of knocking down BIN1 on the total amount of tau (Figure 3 b). A number of papers in the past had shown that increase in BIN1 exacerbates tau pathology (Chapuis et al., 2013), but later studies had identified the opposite, finding that a decline in BIN1 causes increase in tau (Smith et al., 2014; Calafate et al., 2016). This experiment aimed to contribute to literature as findings on the effect of BIN1 on intracellular levels of tau is contradicting and limited. We dissected primary neurones from E18 mice pups, and treated half of these with BIN1 shRNA and the other half with scrambled control lentivirus, and measured the total level of intracellular tau, phosphorylated and dephosphorylated tau, synaptophysin, PSD95 and BIN1 by Western blotting cell lysates.

This experiment aimed to observe the effect of knocking down BIN1 on the level of intracellular tau. It was conducted because growing literature had suggested that loss of BIN1 induces tau pathology. Knocking down half of the cells with BIN1 shRNA and the other half with scrambled shRNA, we conducted a western blot analysis to detect the level of tau and BIN1. We found no significant difference between cells that have been knocked down and controls *(p=0.209).* In the knock-down condition, the level of total intracellular tau was half (*m=0.111*) of what was observed in the control group (*m=0.222*). Also, no overlap between the error bars was observable, supporting the strength of this significance. To conclude from this experiment, BIN1 knockdown does not significantly alter tau levels in primary neurones.

The effect of BIN1 on phosphorylated and dephosphorylated tau

Phosphorylated tau is a pathological characteristic of many tauopathies, and relatively, AD (Noble et al., 2013). It is clear that in AD, there are changes relative to tau phosphorylation. Drosophila studies show that human tau attachment to microtubules is dependent on the level of phosphorylation and accumulation is in correlation to neurodegeneration in these flies (Feuillette et al., 2010). In the previous experiment we had shown that knocking down BIN1 had an effect on the level of intracellular tau. What we next sought to understand was whether BIN1 had an effect on the level of tau phosphorylation (Figure 3 c & d). Not only does BIN1 modulate cell structure by binding the protein CLIP-170 to microtubules for stabilization (Meunier et al., 2009), more recently it has also been identified that it binds to tau through the SH3-PRD domains in the pathological state (Sottejeau et al., 2015). There are hardly any papers in literature that studies the association of BIN1 to tau phosphorylation, hence why we set out to investigate this. This time, we detected the level of phosphorylated and dephosphorylated tau in E18 primary neurones in cells transfected with BIN1/Scrambled shRNA by western blotting.

We found no significant difference between BIN1 knockdown (m=0.167) and control (m=0.166) for phosphorylated tau, $p=0.98$. We also found a non-significant difference between BIN1 knockdown (m=0.165) and control (m=0.169) for dephosphorylated tau, $p=0.95$ (see figures 3c & d) This suggests that BIN1 does not alter the level of phosphorylated tau in primary neurones.

The effect of BIN 1 on synaptophysin and PSD95

The detrimental effects of AD on the synapses are clear from patient studies (Koffie et al., 2011). Mutated forms of tau is toxic for neurones, in particular causes NFTs, synaptic deficits, death of neurones and behavioural dysfunctions (Sheng et al., 2012). Clearly, past studies have seen BIN1 related changes in tau (Chapuis et al., 2013; Calafate et al., 2016) and although non-significant we too have seen different levels of intracellular tau across our two conditions (see Figure 3b). We next aimed to understand the effect of BIN1 on synapses as measured by the post-synaptic density protein PSD95 and the presynaptic synaptophysin (Figure 3 e & f). Studying the synapses may give an indication of how BIN1 affects tau pathology, and literature again, falls short in investigating this. Studies have documented that post-synaptic neurones degenerate later on in the disease course as indicative by less PSD-95 (Shao et al., 2011). Thus, it is important to observe whether a knockdown has direct effects on the pre/post synapses, and if there is, it would make BIN1 a therapeutic target, because it appears to be synapse loss that causes fatal damage in those diagnosed by Alzheimer's disease. By using knocked-down and scrambled cell lines we have run western blots to measure the level of synaptophysin and PSD-95 in E18 mice pup primary neurones. Interestingly, we found no significant difference between BIN1 knockdown (m=0.107) and control (m=0.226) for synaptophsyin, $p=0.202$ or BIN1 knockdown (m=0.04) or control (m=0.29) for PSD95, $p=0.18$. Interestingly the level of synaptophysin is over twice more in the control condition. In the control condition measuring PSD-95, there is 7.25x more PSD-95 than when BIN1 is knocked down. The error bars appear far apart, suggesting a trend towards near significance. Also, there is over double the amount of synaptophysin in the control condition, and although closer, the error bars still do not overlap.

Although non-significant, BIN1 knockdown appears to impact the level of synaptophysin and PSD-95.

The effect of BIN1 knockdown on co-localization of tau with synaptophysin, PSD-95, MAP2 and active synapses

The next experiment aimed to visualise whether there is a co-localization between tau and the presynaptic, postsynaptic and dendritic regions, and active synapses, when BIN1 is knocked out. Also, it gives an indication of which neuronal components tau is located on when BIN1 is knocked down. To do this, again we transfected half of our cells with BIN1 shRNA and the other half with scrambled shRNA, and then stained the cells for PSD-95, MAP2, and synaptophysin, and captured images using the microscope. We then used two software programmes to analyse the data, Nikon Elements and Image J.

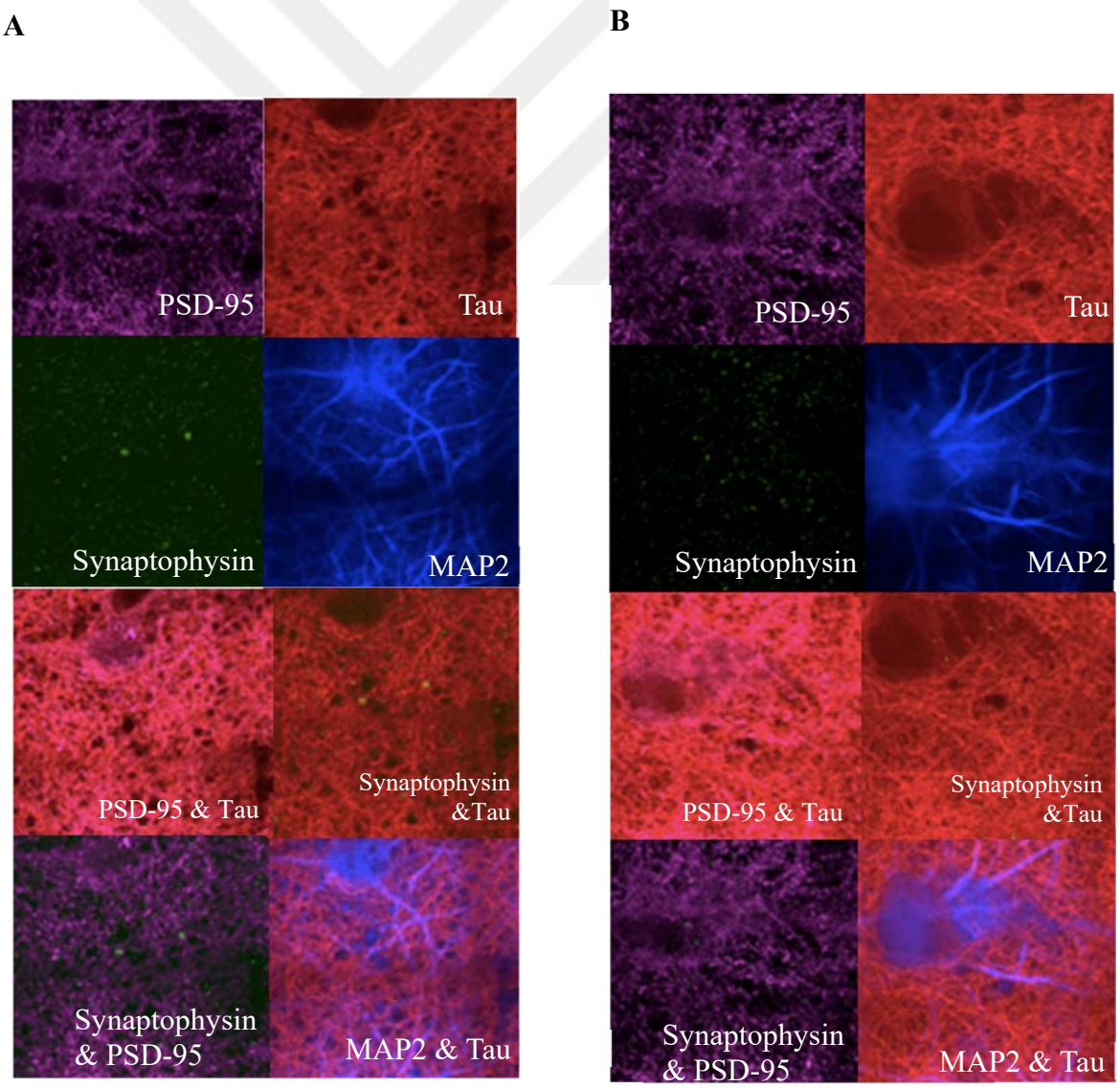

Figure 4 Images captured by microscopy, measuring PSD-95, synaptophysin, active synapses and MAP2 and their co-localization with tau (see legend below)

Figure 4 Microscopy images of co-localization between tau and synaptophysin, tau and PSD-95, tau and active synapses and tau and MAP2 in the absence of BIN1.

(A) Ctl shRNA was used across half of the cells that were stained with antibodies (see 2.1) and imaged using the microscope. This image has been aligned, and then de-convoluted using Nikon Elements and then co-localization analysis was carried out in Image J. (B) This time BIN1 shRNA was used to knock down BIN1 across the other half of the cells. The same procedure as (A) was carried out to measure co-localization. The channels have been merged to visualise the co-localization. The images in figure 4 consist of channels on their own and channels co-localized.

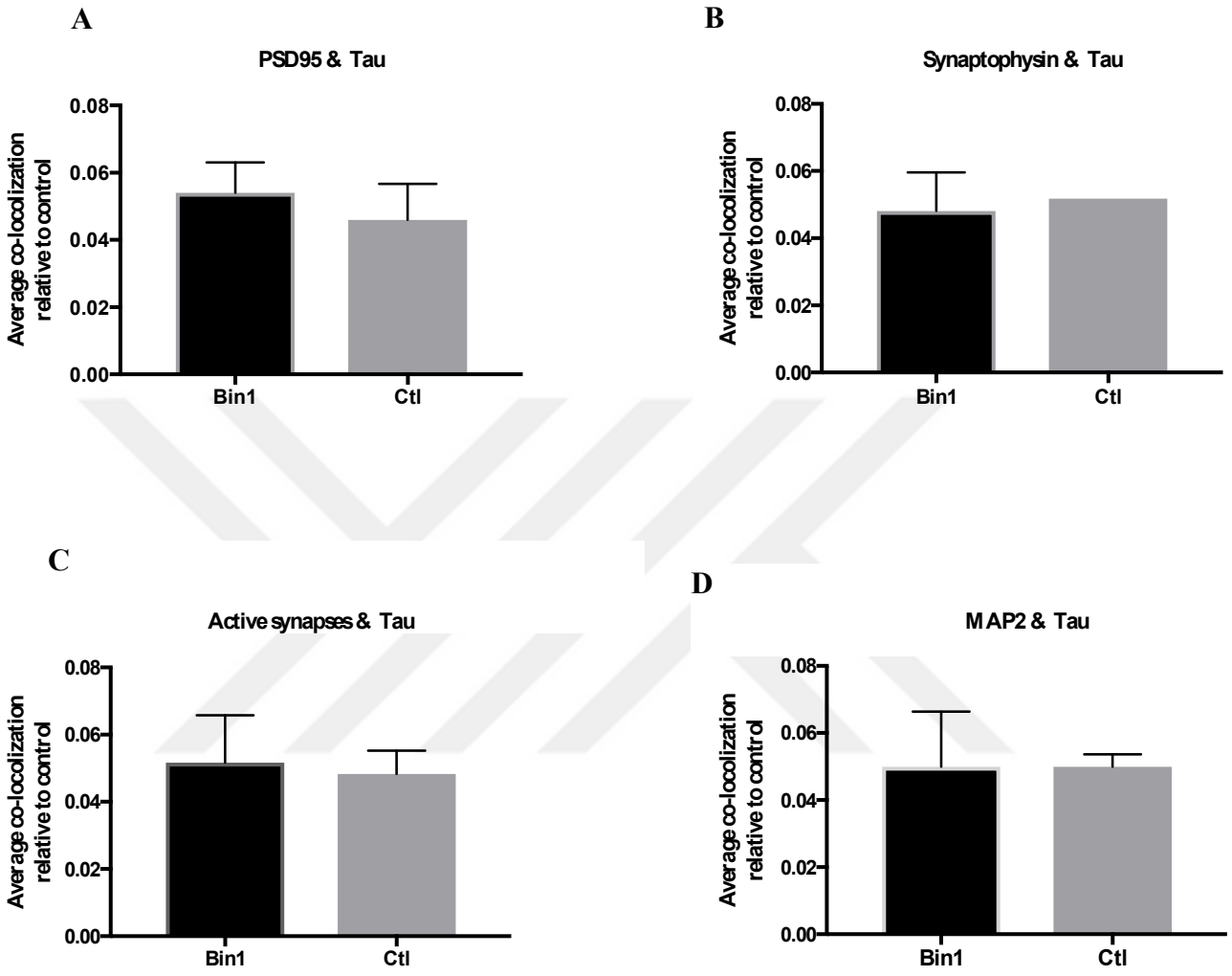

Figure 5 Co-localization of tau with PSD-95, synaptophysin, active synapses and MAP2 as measured by Nikon Elements (A) Co-localization of tau and PSD-95 (B) Co-localization of tau and synaptophysin (C) Co-localization of tau and active synapses (D) Co-localization of tau and MAP2.

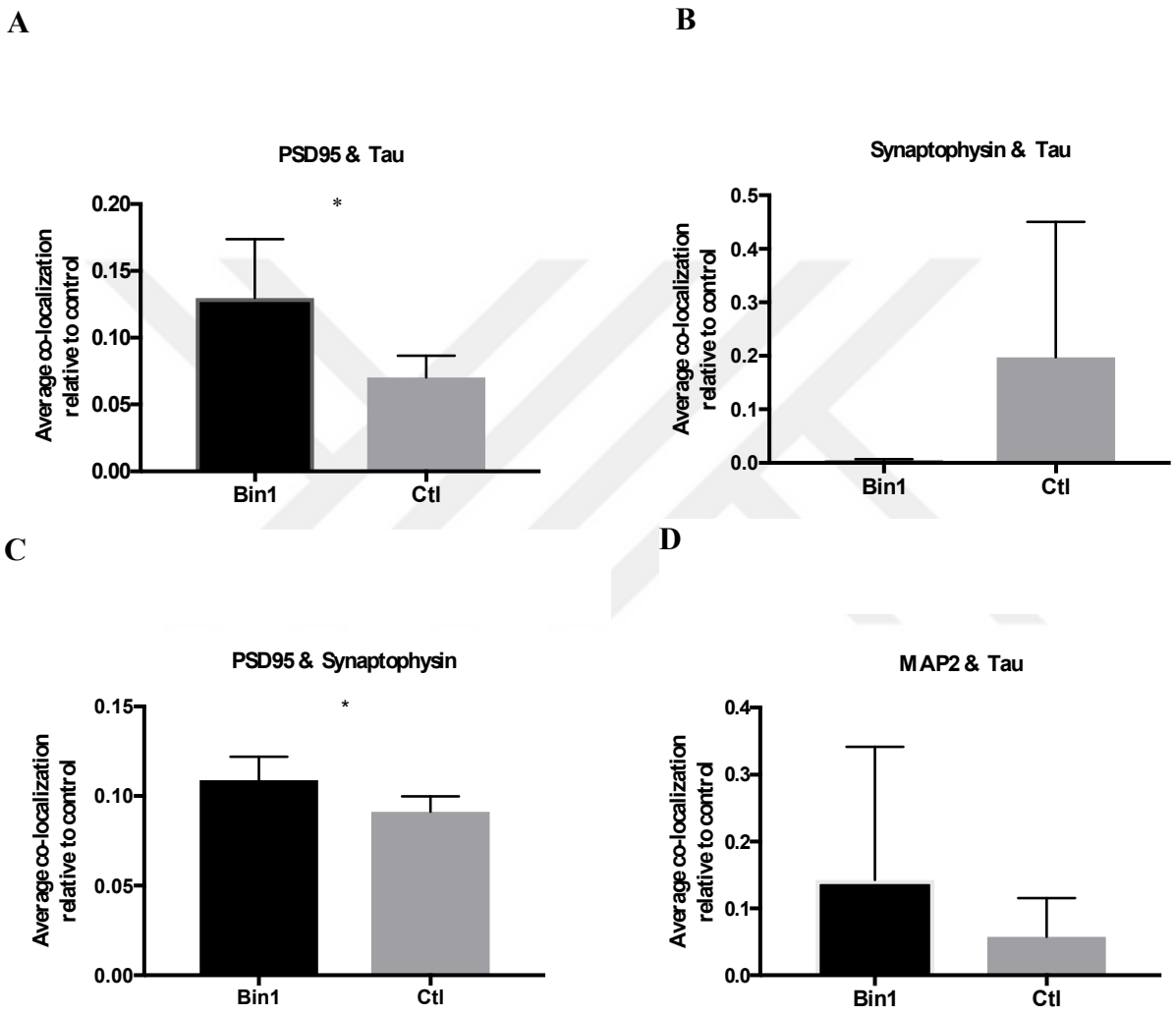

Figure 6 Co-localization of tau with PSD-95, synaptophysin, active synapses and MAP2 as measured by Image J (A) Co-localization of tau and PSD-95 (B) Co-localization of tau and synaptophysin (C) Co-localization of PSD-95 and synaptophysin (D) Co-localization of tau and MAP2.

Colocalization of tau & PSD-95 & active synapses

This experiment aimed to measure the average level of co-localization between tau and the post-synaptic PSD-95. In our previous experiment we showed that BIN1 knockdown affected the level of PSD-95 to a certain extent, but the difference was non-significant. In this instance, we wanted to visualise the effect of this knockdown on localization of tau, in particular post-synaptically, as studies had shown that there is serious synaptic dysfunction when there is pathological tau because tau can damage the membrane and leak into the cytoplasm (Calafate et al., 2016), possibly en route to post-synaptic damage.

Although there are hardly any studies that have documented the effect of BIN1 on tau subcellular location. We set out to understand whether BIN1 knockdown has an effect on tau co-localization with PSD-95. In the images analysed through Image J, the difference between the average co-localization of BIN1 knockdown cells (m= 0.129) and control cells (m=0.07) was significant, *p=0.02.* There was no overlap between the error bars. The level of co-localization was higher when BIN1 was knocked down.

This suggests that tau is localized at the post-synaptic regions nearly two times more in the absence of BIN1. Although, this effect was not observable when the images were analysed through Nikon Elements. There was a non-significant difference between bin1 knockdown cells (m=0.054) and control cells (m=0.045), p=0.08.

Again, the level of co-localization was slightly higher in the BIN1 knockdown condition.

Active synapses

Interestingly, analysis by Nikon elements revealed that the difference between average co-localisation of tau and active synapses across BIN1 knockdown *(m=0.052)* and control samples *(m=0.048)* was non-significant, *p=0.507.* Although, there was a significant

difference between average co-localisation across BIN1 knockdown *(m=0.108)* and scrambled samples *(m=0.091)*, *p=0.037* when measuring co-localization between PSD-95 and synaptophysin through Image J.

Co-localization of tau and Synaptophysin

In one study, Sottejeau et al., (2015) were not able to show that BIN1 and tau complexes co-localize with markers of pre-synapses. Although, as one of the most important functions of BIN1, synaptic vesicle recycling, is impaired in AD, leading to accumulation of tau inside the cell (Di Paolo, 2002) it is likely to cause pre-synaptic damage. However, as research on BIN1 began fairly recently, there are also a lack of papers on whether altered levels of BIN1 causes pre-synaptic deficits. In our previous experiment, the level of synaptophysin was less than half in the BIN1 knockdown condition, suggesting that BIN1 may affect the pre-synaptic region. Sottejeau's (2015) paper had focused on the domain between BIN1, while there may be other mechanisms that BIN1 mediates toxicity to presynaptic regions, as BIN1 is a multi-functional protein. Thus, we investigated the level of co-localization between tau and synaptophysin when BIN1 is knocked down. The difference between the average co-localisation across BIN1 knockdown *(m=0.048)* and control samples *(m=0.052),* was non-significant *p=0.331,* when measured with Nikon Elements. The difference between average co-localisation across BIN1 knockdown *(m=0.003)* and control samples *(m=0.197),* was also non-significant *p=0.124* when measured with Image J.

Tau and MAP2 Co-localisation

Destruction of dendritic spines is the leading cause of impaired synaptic function in AD (Dorostkar et al., 2015). In early stages, the disease manifests deficits in memory (Selkoe, 2002). Hyperphosphorylated tau accumulates in the dendritic spines and causes synaptic dysfunction (Hoover et al., 2010). Knowing that BIN1 is somehow implicated in the level of tau (see. figure 3b) the next aim was to understand whether knockdown of BIN1 has an effect on the localization of tau with MAP2, a dendritic marker. Again primary cortical

neurones that were stained for MAP2 were imaged with microscopy and analysed through the two computer programs, Image J and Nikon Elements. Half of the captured images were of cells that had BIN1 knockdown and the others were controls.

Firstly, the difference between the average co-localisation across BIN1 knockdown *(m=0.142)* and control samples *(m=0.057)* was non-significant, *p=0.389* when measured with Image J. The difference between these two groups was also non-significant, *p>0.99*, when analysed with Nikon Elements, where the mean of the two groups were equal *(m=0.05)*.

The effect of BIN1 on extracellular tau in treated and untreated cells

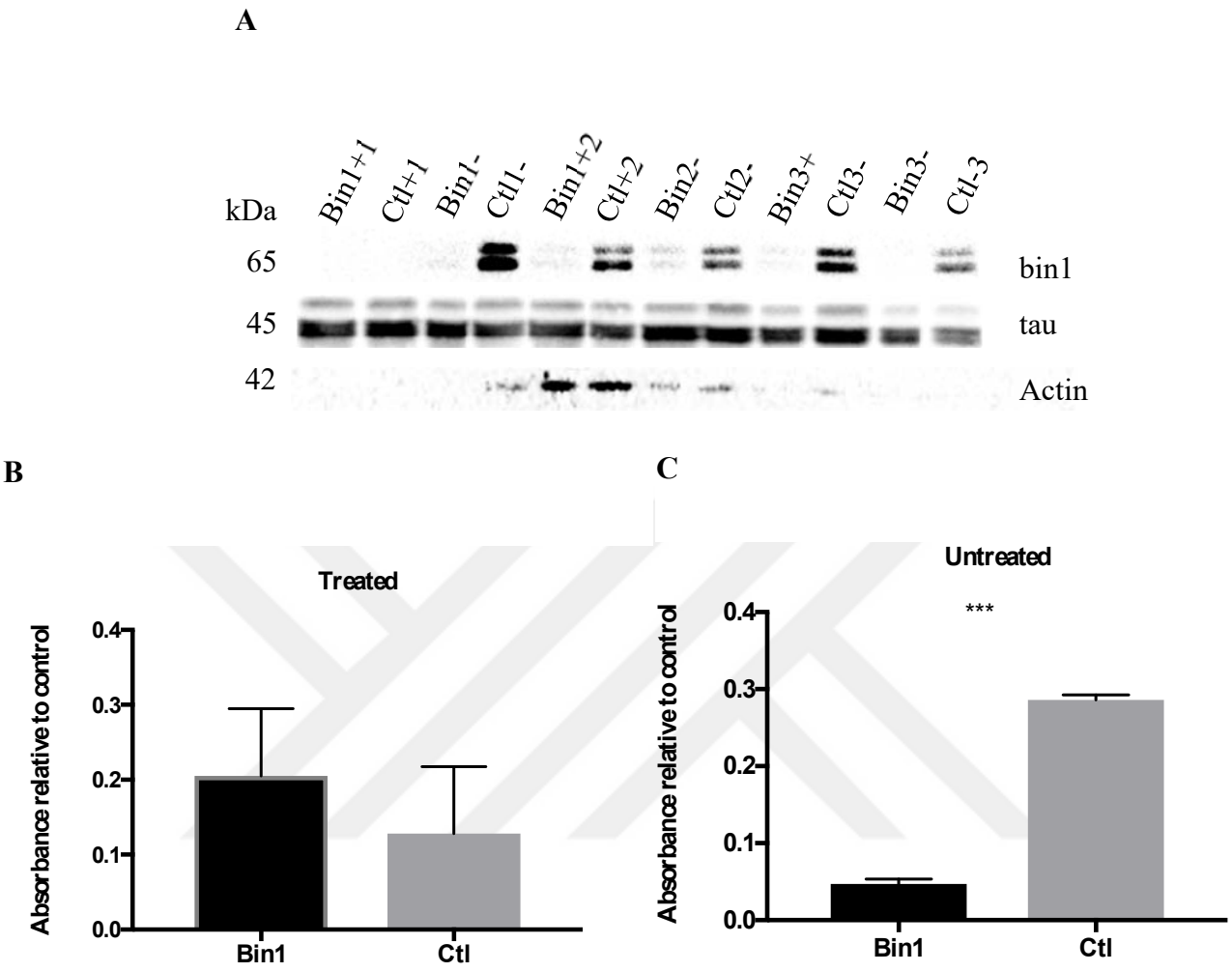

Figure 7 BIN1 and DAKO expression in rodent brain cells *(n=3).*
Tau was detected using anti-rabbit IgG HRP linked whole antibodies.. Bin1 was detected using anti-mouse IgG HRP linked whole antibodies. These images were then quantified through Fiji.

Tau is known to be present extracellularly in AD brains (Frost et al., 2009). Studies have shown that internal tau aggregates are released from the cell into extracellular space, the cytoplasm (Calafate et al., 2016) It seems as if once triggered, this is an endless cycle that causes spread of the disease, through trans-cellular propagation of extracellular tau (Yamada, 2017). Again, knowing that BIN1 knockdown induces tau related changes (Calafate et al.,

2016) we set out to investigate whether it affects tau release in primary neurones of E18 mice pups. BIN1 shRNA was used to knockdown half of the cells. Another condition of the experiment was whether the cells were treated or not. This is because studies had suggested that treatment can increase endogenous tau release (Pooler et al., 2013). Immunoblots have been taken over a number of weeks. They consist of Tau samples (1-3) to ensure replicability *(n=3).* Following quantification, tau (DAKO) values were exported to excel, to calculate the percentage of the protein. In calculating the level of protein, values from treated and untreated TAU ELISA were used.

We found a significant difference between BIN1 knockdown cells (m=0.047) and the control cells *(m=0.286)* from the untreated samples, *p=0.0007* ***. However, the difference between BIN1 cells (m=0,205) and control cells (0.128) was non-significant *p=0.350* for the treatment group.

Discussion

Reduction in the level of tau when BIN1 is knocked down

This experiment aimed to understand the effect of BIN1 on total intracellular tau. We identified that there was less tau when we knocked down BIN1. We expected there to be an increased amount of tau across our BIN1 knockdown samples because i) BIN1 is a well-known risk factor for LOAD and studies documented that it is reduced in sporadic cases of AD (Glennon et al., 2013), ii) because BIN1 knock-downs have been documented to induce pathological changes (Smith et al., 2014; De Rossi et al., 2016, Calafate et al., 2016). Although the level of tau is higher in the control condition, the difference between BIN1 knockdown and control samples (Experiment 1.1) is statistically non-significant. The levels of tau may have been higher in the BIN1 knockdown condition, in line with literature, if the experiment was repeated two more times and with more samples to ensure that the findings were not a result of experimental error or low power size due to the low number of samples. Lastly, in human patients, the development of disease is gradual and measured by Braak stages (Braak et al., 2006), thus it is possible to speculate that the lower levels of tau absorbance in knockdown samples are due to the limited time frame in the laboratory, which does not simulate the disease course- perhaps tau did not increase in amount, as it did not have sufficient time. Calafate et al. (2016) have identified that aggregated tau impairs the membrane of the endosome, leaking into the cytoplasm, exacerbating the situation. Another suggestion relative to the age of the mice pups could be the involvement of clearance mechanisms in early stages of the disease. There are many known examples of the body's natural tendency to maintain homeostasis. Relative to AD is the NLRP3 inflammasome, which is activated as an inflammatory response to protect (Oliveira BCL, 2017), however promotes further pathological changes by overexpressing cytokines. Perhaps, in the

beginning (in the case of the E18 mice pups, early stages of the disease) protective mechanisms are involved to battle against deteriorating tau pathology, which could be why an increase in tau is not evident. Researchers suggest that the same tau oligomers that activate degradation mechanisms could damage the proteasome, and could lead to further accumulation and hindered autophagy (Chesser et al., 2013), which is again a difference that could be seen over time.

BIN1 did not have an effect on tau phosphorylation

We aimed to understand the effect of a BIN1 knockdown on the level of phosphorylated and dephosphorylated tau. It appears that BIN1 knockdown has no effect on phosphorylated tau, but a minor difference in dephosphorylated tau between BIN1 and control samples. Knowing that i) hyperphosphorylated tau is a hallmark feature of AD (Grundke-Iqbal et al., 1986) and that ii) GWAS analysis demonstrated that BIN1 is a major risk factor for late onset AD (Hu et al., 2011), one may expect to see increase in hyperphosphorylated and decreased dephosphorylated tau associated to BIN1, dephosphorylated tau representing healthy and phosphorylated tau representing toxic tau. However, due to the lack of studies in literature, it has hardly been possible to make predictions. Importantly, research shows that there is a greater amount of phosphorylated tau in AD in contrast to controls (Hampel et al., 2010). However, the non-significant effect was on the contrary (figure 4c).

Interestingly, Siegel & Rajendran (2018) also had not observed a significant effect on phosphorylated (thr231) tau when they down-regulated BIN1, proposing one limitation to be primary neurons obtained at the embryonic stage, under representing late-onset AD. Again, our primary neurons from E18 pups do not represent LOAD, nor the phosphorylation state of individuals with AD at later stages of their lives.

BIN1 caused loss of synapses

Koffie, Hyman & Spires-Jones (2011) note that Alzheimer's is a disease of the "synapses gone cold", highlighting synaptic dysfunction as the most prominent characterisation. It has been important to study the synapses as researchers had identified that recycling of synaptic vesicles is impaired when BIN1 was knocked out across mice, correlating with increases in death and cognitive deficits (Di Paolo et al., 2002).

From our study, it is clear that there are more pre and post synapses across control samples, and the loss of these synapses with the absence of BIN1 appears to be in line with mouse models which exhibit "early synapse loss" (Hong et al., 2016) and remarkable reduction of synapses as observable across post-mortem brains by synaptic staining (Hamos, DeGennaro & Drachman, 1989). The lack of significant difference may be attributable again to the age of the neurons obtained from mice pups, as synaptic loss is in line with cognitive decline over time (Scheff et al., 2006)

BIN1 knockdown did not affect the dendrites

A number of studies had emphasised that the dendritic spines are heavily affected by tau accumulation (Hoover et al., 2010) and NFTs (Merino-Serrais et al., 2013). However the effect of BIN1 knockdown on the dendrites was on the contrary, as there was a non-significant co-localization between tau and MAP2. If dendrites are the receivers in the neurone (Ittner & Ittner, 2018), then perhaps at this stage tau has not been taken up to fill in dendrites as yet, and our method would fall short in measuring these changes that occur over time.

Co-localisation of Tau/PSD95, Tau/Synaptophysin, Tau/MAP2 and PSD95/Synaptophysin in BIN1 knockdown cells vs. Scrambled analysed by Image J & Nikon Elements

Aiming, to understand the effect of BIN1 on pre and post-synaptic regions, we investigated the effect of BIN1 knockdown on co-localisation of tau with PSD-95, synaptophysin, MAP2, and pairs of PSD95 and synaptophysin (considered active synapses). We set out to investigate whether BIN1 knockdown has an effect on tau accumulation in post-synaptic, presynaptic, dendritic locations. A study conducted by Zhou et al. (2017) found that FTDP-17 mutant tau causes presynaptic damage to fly neurons. They find greater localisation of pathogenic tau in presynaptic terminals compared to wild-types and that this is in line to their findings in human patients, which they suggest could be explained by reduced affinity to bind to microtubules. By increasing expression of mutant human tau in mice entorhinal cortex, Harris et al. (2012) identified that phosphorylated tau levels were higher in the presynaptic regions. Interestingly, we observed that the average size of co-localisation between PSD95-tau was greater when BIN1 was knocked down. However, this effect could not be observed when analysed with Nikon Elements. A difference could be seen although fewer images were analysed on Image J. Although, there were non-significant differences in co-localisation between MAP2-tau or synaptophysin-tau in both software analyses. The only other significant difference was observable in the image J analysis between BIN1 knockdown and control samples, finding greater co-localisation between, PSD-95 and synaptophysin in the absence of BIN1. The significant size of co-localisation of PSD-95 and tau is in contrast to synaptophysin and tau. Studies have highlighted that BIN1 is a pre-synaptic protein as it is well- established that it serves functions in endocytosis. In line with the studies that found that bin1 knockout induces tau related pathological changes (Calafate et al., 2016), it would be expected that localisation of tau and synaptophysin is to a larger extent than psd-95. However, the fact that in the absence of BIN1 tau co-localises more with psd-95 could be

indicative of trans-cellular propagation. Kfoury et al. (2012) found that i) tau misfolding can enhance as a result of release and uptake (transcellular propagation). Interestingly, in our untreated cells in (experiment 3) we had aimed to measure the release of extracellular tau, which was enhanced by BIN1 knockdown. Perhaps, this is in line to Kfoury et al's (2012) observation, and what could be visualised through identification of colocalisation between PSD95 and tau here is re-uptake by the post-synaptic neurone, suggesting propagation of tau pathology between cells, and as we had observed co-localisation between psd-95 and synaptophysin there is a possibility that these regions are active and interacting.

BIN1 has a greater effect on post-synapses. Also, there was a greater co-localization between PSD95 and tau when BIN1 was knocked out.

The effect of BIN1 on tau release/secretion across untreated/treated cells

This experiment was carried out to understand the level of extracellular tau and whether treatment of cells with KCl would stimulate neurones in the absence of BIN1. Interestingly, across our untreated cells, there was less extracellular tau across our samples with BIN1 knockdown in contrast to our control samples. This difference appeared to be greater across untreated cells in contrast to treated cells. This is very important, as studies conducted in the past had identified that treatment of cells increases endogenous tau release (Pooler et al., 2013). We see that there is relatively more tau when BIN1 is knocked down in the treated condition, although non-significant, suggesting an inclination towards the trend reported in literature. Perhaps a significant difference may be seen in the treated condition if minor experimental changes are introduced in future studies. We recommend that this experiment is repeated with stimulation and by adjusting the time that stimulants are administered i) to wait for accumulating tau to break through the membrane (as Calafate et al., 2016 had suggested is happening) and ii) Pooler et al. (2013) had suggested that there is potential to mediate mechanisms implicated in tau release by regulating stimulation of neurons, to see if the level of extracellular tau across BIN1 knockouts will exceed that of control samples. It could also be that the treatment induces other physiological mechanisms, for example a series of different proteins regulating different functions, thus creating a difference when cells are treated/untreated.

Criticisms of Image J & Nikon Elements

As part of this study, the two computer programs Image J and Nikon Elements have been utilised to co-localise images. Improving technology has allowed users to visually see sub-cellular regions in higher resolution, becoming an excellent method in investigating structure and function (Abdulreda et al., 2011). These programs have built co-localization functions, and for Image J, is easily downloadable as an additional plug-in.

Time: Of the two programs, it took longer to utilise Image J to analyse co-localization. This is due to a number of factors i) images have to be loaded on to Image J, which could take several seconds to several minutes, due to their large size as they are taken with the microscope, ii) the images have to be split into their channels, and co-localization of of interest have to be performed one by one i.e. channel 1 (PSD-95) and channel 2 (tau) then, channel 1 (PSD-95) and channel 3 (synaptophysin) iii) in our study, we took a two-step approach, in which we co-localised first, then performed Z-projects and apart from these, other settings had to be adjusted per co-localization, i.e. brightness/contrast, inversion etc., iv) the particles in each co-localization had to be analysed manually, which means the user has to identify regions of interest, draw circles around these regions for measuring. At times, there could be a large number of regions of interest, for example in one image their could be 10-15, which takes a long time to process, particularly in this study, this would have had to be repeated 4 times per image.

Although some time is spent writing settings in Nikon Elements, these could be saved to use in other experimental procedures. It is possible to refer to this method as semi-automatic as, once the settings are applied, the images could be analysed in batches or one by one by the computer itself. Aligning and de-convoluting the images prior to analysis lasts approx. 5 minutes, which is possibly as long as setting up the analysis on Image J, however, the computer again does this by itself, once the settings are selected.

Accuracy: One of the most important issues in using Image J to analyse images is that the user has to draw circles around the ROIs. These circles are not consistent when performing co-localization on alternate channels, or in new images. The number of particles may be higher/lower, dependent on the size of the circle rather than cellular changes. On the other hand, the greatest advantage of utilising Nikon Elements is that the computer is instructed to apply the same pre-defined settings across all of the images, allowing consistency.

Quality of the images: The are abundant settings that could be applied to the images on Nikon Elements, allowing the user to smoothen, clean, denoise, remove dark/light backgrounds or sharpen, allowing users to visualise sub-cellular locations and the localization of proteins of interest more clearly in Nikon Elements.

Software Interface: At first, it is highly difficult to use Nikon Elements because of the complicated interface. More importantly, there are hardly any documents available to help the user learn how to utilise the program and to understand certain features. For example, when the general analysis was run, data was extracted from the images, ready to be copied into excel. However, it was very difficult to understand the data, as there was too much information which was difficult to interpret, and there were no guides that defined the terms that was present in the results. For Image J, there are abundant documents online that allow the user to understand how to use the software.

In conclusion, researchers must continue to investigate BIN1's association to Alzheimer's disease, because differences could clearly be seen in experiments. This and consequent studies that show the effect of BIN1 on tau may be translational to medicine in the future, when the mechanisms that are involved are understood clearly, researchers may be able to alter the expression of BIN1 to treat Alzheimer's. Although it must be remembered that the picture is more complicated, and there are a number of mechanisms involved. Nevertheless, these findings contribute to literature by finding that BIN1 levels affect the synapses, which are essential for our communications.

References

Abdulreda, M. H., Rodriguez-Diaz, R., Berggren, P. O., & Caicedo, A. (2011). Three-dimensional colocalization analysis in immunostained sections of human pancreas. *Protocol Exchange*.

Al-Bassam, J., Ozer, R. S., Safer, D., Halpain, S., & Milligan, R. A. (2002). MAP2 and tau bind longitudinally along the outer ridges of microtubule protofilaments. *The Journal of cell biology*, *157*(7), 1187-1196.

Barber, R. C. (2012). The genetics of Alzheimer's disease. *Scientifica*, *2012*.

Bekris, L. M., Galloway, N. M., Millard, S., Lockhart, D., Li, G., Galasko, D. R., ... & Schellenberg, G. D. (2011). Amyloid precursor protein (APP) processing genes and cerebrospinal fluid APP cleavage product levels in Alzheimer's disease. *Neurobiology of aging*, *32*(3), 556-e13.

Braak, H., Alafuzoff, I., Arzberger, T., Kretzschmar, H., & Del Tredici, K. (2006). Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta neuropathologica*, *112*(4), 389-404.

Calafate, S., Flavin, W., Verstreken, P., & Moechars, D. (2016). Loss of Bin1 promotes the propagation of tau pathology. *Cell reports*, *17*(4), 931-940.

Chapuis, J., Hansmannel, F., Gistelinck, M., Mounier, A., Van Cauwenberghe, C., Kolen, K. V., ... & Grenier-Boley, B. (2013). Increased expression of BIN1 mediates Alzheimer genetic risk by modulating tau pathology. *Molecular psychiatry*, *18*(11), 1225.

Chesser, A., Pritchard, S., & Johnson, G. V. (2013). Tau clearance mechanisms and their possible role in the pathogenesis of Alzheimer disease. *Frontiers in neurology*, *4*, 122.

D'Alessandro, M., Hnia, K., Gache, V., Koch, C., Gavriilidis, C., Rodriguez, D., ... & Labouesse, M. (2015). Amphiphysin 2 orchestrates nucleus positioning and shape by linking the nuclear envelope to the actin and microtubule cytoskeleton. *Developmental cell*, *35*(2), 186-198.

Dahm, R. (2006). Alzheimer's discovery. *Current Biology*, *16*(21), R906-R910.

David, C., McPherson, P. S., Mundigl, O., & De Camilli, P. (1996). A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proceedings of the National Academy of Sciences*, *93*(1), 331-335.

De Rossi, P., Buggia-Prévot, V., Clayton, B. L., Vasquez, J. B., van Sanford, C., Andrew, R. J., ... & Rao, E. (2016). Predominant expression of Alzheimer's disease-associated BIN1 in mature oligodendrocytes and localization to white matter tracts. *Molecular neurodegeneration*, *11*(1), 59.

De Rossi, P., Buggia-Prevot, V., Andrew, R. J., Krause, S. V., Woo, E., Nelson, P. T., ... & Thinakaran, G. (2017). BIN1 localization is distinct from Tau tangles in Alzheimer's disease. *Matters*, *2017*.

DeVos, S. L., Corjuc, B. T., Oakley, D. H., Nobuhara, C. K., Bannon, R. N., Chase, A., ... & Hyman, B. T. (2018). Synaptic tau seeding precedes tau pathology in human Alzheimer's Disease brain. *Frontiers in neuroscience*, *12*, 267.

Di Paolo, G., Sankaranarayanan, S., Wenk, M. R., Daniell, L., Perucco, E., Caldarone, B. J., ... & De Camilli, P. (2002). Decreased synaptic vesicle recycling efficiency and cognitive deficits in amphiphysin 1 knockout mice. *Neuron*, *33*(5), 789-804.

Dorostkar, M. M., Zou, C., Blazquez-Llorca, L., & Herms, J. (2015). Analyzing dendritic spine pathology in Alzheimer's disease: problems and opportunities. *Acta neuropathologica*, *130*(1), 1-19.

Dubal, D. B. (2018). The Way of Tau: Secretion and Synaptic Dysfunction. *Trends in molecular medicine*.

Feuillette, S., Miguel, L., Frébourg, T., Campion, D., & Lecourtois, M. (2010). Drosophila models of human tauopathies indicate that Tau protein toxicity in vivo is mediated by soluble cytosolic phosphorylated forms of the protein. *Journal of neurochemistry*, *113*(4), 895-903.

Frost, B., Jacks, R. L., & Diamond, M. I. (2009). Propagation of tau misfolding from the outside to the inside of a cell. *Journal of Biological Chemistry*, *284*(19), 12845-12852.

Giri, M., Zhang, M., & Lü, Y. (2016). Genes associated with Alzheimer's disease: an overview and current status. *Clinical interventions in aging*, *11*, 665.

Glennon, E. B., Whitehouse, I. J., Miners, J. S., Kehoe, P. G., Love, S., Kellett, K. A., & Hooper, N. M. (2013). BIN1 is decreased in sporadic but not familial Alzheimer's disease or in aging. *PLoS One*, *8*(10), e78806.

Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., & Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences*, *83*(13), 4913-4917.

Guo, T., Noble, W., & Hanger, D. P. (2017). Roles of tau protein in health and disease. *Acta neuropathologica*, *133*(5), 665-704.

Hamos, J. E., DeGennaro, L. J., & Drachman, D. A. (1989). Synaptic loss in Alzheimer's disease and other dementias. *Neurology*, *39*(3), 355-355.

Hampel, H., Blennow, K., Shaw, L. M., Hoessler, Y. C., Zetterberg, H., & Trojanowski, J. Q. (2010). Total and phosphorylated tau protein as biological markers of Alzheimer's disease. *Experimental gerontology*, *45*(1), 30-40.

Harris, J. A., Koyama, A., Maeda, S., Ho, K., Devidze, N., Dubal, D. B., ... & Mucke, L. (2012). Human P301L-mutant tau expression in mouse entorhinal-hippocampal network causes tau aggregation and presynaptic pathology but no cognitive deficits. *PloS one*, *7*(9), e45881.

Hinshaw, J. E. (2000). Dynamin and its role in membrane fission. *Annual review of cell and developmental biology*, *16*(1), 483-519.

Hong, S., Beja-Glasser, V. F., Nfonoyim, B. M., Frouin, A., Li, S., Ramakrishnan, S., ... & Lemere, C. A. (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science*, *352*(6286), 712-716.

Hoover, B. R., Reed, M. N., Su, J., Penrod, R. D., Kotilinek, L. A., Grant, M. K., ... & Ashe, K. H. (2010). Tau mislocalization to dendritic spines mediates synaptic dysfunction independently of neurodegeneration. *Neuron*, *68*(6), 1067-1081.

Hu, X., Pickering, E., Liu, Y. C., Hall, S., Fournier, H., Katz, E., ... & Alzheimer's Disease Neuroimaging Initiative. (2011). Meta-analysis for genome-wide association study identifies multiple variants at the BIN1 locus associated with late-onset Alzheimer's disease. *PloS one*, *6*(2), e16616.

Isik, A. T. (2010). Late onset Alzheimer's disease in older people. *Clinical interventions in aging*, *5*, 307.

Iqbal, K., Liu, F., Gong, C. X., & Grundke-Iqbal, I. (2010). Tau in Alzheimer disease and related tauopathies. *Current Alzheimer Research*, *7*(8), 656-664.

Ittner, A., & Ittner, L. M. (2018). Dendritic Tau in Alzheimer's Disease. *Neuron*, *99*(1), 13-27.

Kfoury, N., Holmes, B. B., Jiang, H., Holtzman, D. M., & Diamond, M. I. (2012). Trans-cellular propagation of Tau aggregation by fibrillar species. *Journal of Biological Chemistry*, *287*(23), 19440-19451.

Koffie, R. M., Hyman, B. T., & Spires-Jones, T. L. (2011). Alzheimer's disease: synapses gone cold. *Molecular neurodegeneration*, *6*(1), 63.

Kolarova, M., García-Sierra, F., Bartos, A., Ricny, J., & Ripova, D. (2012). Structure and pathology of tau protein in Alzheimer disease. *International journal of Alzheimer's disease*, *2012*.

Kamboh, M. I., Demirci, F. Y., Wang, X., Minster, R. L., Carrasquillo, M. M., Pankratz, V. S., ... & Logue, M. W. (2012). Genome-wide association study of Alzheimer's disease. *Translational psychiatry*, *2*(5), e117.

Korolev, I. O. (2014). Alzheimer's disease: a clinical and basic science review. *Medical Student Research Journal*, *4*, 24-33.

Li, B., Chohan, M. O., Grundke-Iqbal, I., & Iqbal, K. (2007). Disruption of microtubule network by Alzheimer abnormally hyperphosphorylated tau. *Acta neuropathologica*, *113*(5), 501-511.

Li, H. L., Yang, P., Liu, Z. J., Sun, Y. M., Lu, S. J., Tao, Q. Q., ... & Wu, Z. Y. (2015). Common variants at Bin1 are associated with sporadic Alzheimer's disease in the Han Chinese population. *Psychiatric genetics*, *25*(1), 21-25.

MacDonald, A. B. (2007). Alzheimer's disease Braak Stage progressions: reexamined and redefined as Borrelia infection transmission through neural circuits. *Medical hypotheses*, *68*(5), 1059-1064.

Martin, L., Latypova, X., & Terro, F. (2011). Post-translational modifications of tau protein: implications for Alzheimer's disease. *Neurochemistry international*, *58*(4), 458-471.

Merino-Serrais, P., Benavides-Piccione, R., Blazquez-Llorca, L., Kastanauskaite, A., Rabano, A., Avila, J., & DeFelipe, J. (2013). The influence of phospho-tau on dendritic spines of cortical pyramidal neurons in patients with Alzheimer's disease. *Brain*, *136*(6), 1913-1928.

Meunier, B., Quaranta, M., Daviet, L., Hatzoglou, A., & Leprince, C. (2009). The membrane-tubulating potential of amphiphysin 2/BIN1 is dependent on the microtubule-binding cytoplasmic linker protein 170 (CLIP-170). *European journal of cell biology*, *88*(2), 91-102.

Miyagawa, T., Ebinuma, I., Morohashi, Y., Hori, Y., Young Chang, M., Hattori, H., ... & Iwatsubo, T. (2016). BIN1 regulates BACE1 intracellular trafficking and amyloid-β production. *Human molecular genetics*, *25*(14), 2948-2958.

Moser, B., Hochreiter, B., Herbst, R., & Schmid, J. A. (2017). Fluorescence colocalization microscopy analysis can be improved by combining object-recognition with pixel-intensity-correlation. *Biotechnology journal*, *12*(1), 1600332.

Noble, W., Hanger, D. P., Miller, C. C., & Lovestone, S. (2013). The importance of tau phosphorylation for neurodegenerative diseases. *Frontiers in neurology*, *4*, 83.

Oliveira BCL (2017) Microglial NLRP3 Activity in Alzheimer's Disease. Int J Brain Disord Treat 3:019. doi.org/10.23937/2469-5866/1410019

Pompey, S. N., Michaely, P., & Luby-Phelps, K. Quantitative Fluorescence Co-localization of Lipoproteins with the LDL Receptor.

Pooler, A. M., Phillips, E. C., Lau, D. H., Noble, W., & Hanger, D. P. (2013). Physiological release of endogenous tau is stimulated by neuronal activity. *EMBO reports*, *14*(4), 389-394.

Reitz, C., Brayne, C., & Mayeux, R. (2011). Epidemiology of Alzheimer disease. *Nature Reviews Neurology*, *7*(3), 137.

Scheff, S. W., Price, D. A., Schmitt, F. A., & Mufson, E. J. (2006). Hippocampal synaptic loss in early Alzheimer's disease and mild cognitive impairment. *Neurobiology of aging*, *27*(10), 1372-1384.

Selkoe, D. J. (2002). Alzheimer's disease is a synaptic failure. *Science*, *298*(5594), 789-791.

Shao, C. Y., Mirra, S. S., Sait, H. B., Sacktor, T. C., & Sigurdsson, E. M. (2011). Postsynaptic degeneration as revealed by PSD-95 reduction occurs after advanced Aβ and tau pathology in transgenic mouse models of Alzheimer's disease. *Acta neuropathologica*, *122*(3), 285-292.

Sheng, M., Sabatini, B. L., & Südhof, T. C. (2012). Synapses and Alzheimer's disease. *Cold Spring Harbor perspectives in biology*, a005777.

Siegel, G., & Rajendran, L. (2018). Analysis of Aβ x-42/Aβ x-40 and Tau upon siRNA-mediated downregulation of APOE, BIN1, PICALM, CLU, PRNP and CST3 in wildtype mouse primary neurons. *Matters*, *4*(5), e201803000014.

Singh, M., & Jadhav, H. (2016). Dynamin Functions and Ligands: Classical Mechanism Behind. *Molecular pharmacology*, mol-116.

Smith, L. L., Gupta, V. A., & Beggs, A. H. (2014). Bridging integrator 1 (Bin1) deficiency in zebrafish results in centronuclear myopathy. *Human molecular genetics*, *23*(13), 3566-3578.

Sottejeau, Y., Bretteville, A., Cantrelle, F. X., Malmanche, N., Demiaute, F., Mendes, T., ... & Dourlen, P. (2015). Tau phosphorylation regulates the interaction between BIN1's SH3 domain and Tau's proline-rich domain. *Acta neuropathologica communications*, *3*(1), 1.

Südhof, T. C. (2004). The synaptic vesicle cycle. *Annual review of neuroscience*, *27*, 509.

Tai, H. C., Serrano-Pozo, A., Hashimoto, T., Frosch, M. P., Spires-Jones, T. L., & Hyman, B. T. (2012). The synaptic accumulation of hyperphosphorylated tau oligomers in Alzheimer disease is associated with dysfunction of the ubiquitin-proteasome system. *The American journal of pathology*, *181*(4), 1426-1435.

Tan, M. S., Yu, J. T., & Tan, L. (2013). Bridging integrator 1 (BIN1): form, function, and Alzheimer's disease. *Trends in molecular medicine*, *19*(10), 594-603.

Tang, Y. P., & Gershon, E. S. (2003). Genetic studies in Alzheimer's disease. *Dialogues in clinical neuroscience*, *5*(1), 17.

Trujillo-Estrada, L., Davila, J. C., Sanchez-Mejias, E., Sánchez-Varo, R., Gomez-Arboledas, A., Vizuete, M., ... & Gutiérrez, A. (2014). Early neuronal loss and axonal/presynaptic damage is associated with accelerated amyloid-β accumulation in AβPP/PS1 Alzheimer's disease mice subiculum. *Journal of Alzheimer's Disease*, *42*(2), 521-541.

Ubelmann, F., Burrinha, T., Salavessa, L., Gomes, R., Ferreira, C., Moreno, N., & Almeida, C. G. (2017). Bin1 and CD2AP polarise the endocytic generation of beta‐amyloid. *EMBO reports*, *18*(1), 102-122.

Wechsler-Reya, R., Elliott, K., Herlyn, M., & Prendergast, G. C. (1997). The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein, the localization of which is altered in malignant cells. *Cancer research*, *57*(15), 3258-3263.

Wu, J. W., Herman, M., Liu, L., Simoes, S., Acker, C. M., Figueroa, H., ... & Di Paolo, G. (2013). Small misfolded Tau species are internalized via bulk endocytosis and anterogradely and retrogradely transported in neurons. *Journal of Biological Chemistry*, *288*(3), 1856-1870.

Yamada, K. (2017). Extracellular Tau and Its Potential Role in the Propagation of Tau Pathology. *Frontiers in neuroscience*, *11*, 667.

Zhou, L., McInnes, J., Wierda, K., Holt, M., Herrmann, A. G., Jackson, R. J., ... & Vilain, S. (2017). Tau association with synaptic vesicles causes presynaptic dysfunction. *Nature communications*, *8*, 15295.

Appendix 1

Colocalisation Protocol for Nikon Elements

Channel no. on fiji	Channel	Default Settings
C4	MAP2/myDAPI, dendritic marker	**Pre-processing** Local contrast [size 50] Advanced denoising **Threshold** 135-26607 0.25 size Smooth 1x Clean 3x Fill holes OFF SPR OFF **Binary processing** Smooth 4x
C1	PSD95/myCy5, postsynaptic marker	**Pre-processing** Advanced denoising- Power 1 Sharpen Sharpen slightly Sharpen slightly **Threshold** 3800- 14024 Smooth OFF Clean OFF Fill holes OFF SPR OFF
C2	Total Tau/myRFP	**Threshold** 190-4000 Smooth OFF Clean 8x Fill holes OFF Separate OFF **Binary Processing** Contour Vanish small items on processing
C3	Synaptophysin/myGFP	**Pre-processing** Rolling ball correction Smooth 1x Remove dark background Rolling ball correction **Threshold** 786-65535 Smooth 1X Clean 1X Fill holes OFF

		Separate OFF Size 0-1.48 **Binary Processing** Filter on Object Area min 0 max 10