

School of Life Sciences

The development of a reverse phase protein array (RPPA) method, to form an Alzheimer's disease panel using brain samples from hTau mice.

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by

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1. Literature Review

1.a. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which has the ability to become increasingly debilitating and to cause dementia.

In the 2015 WHO report, there were recorded to be 48.2 million people living with dementia around the world. This is predicted to rise considerably to approximately 131.5 million within the short time span of 33 years (Prince et al., 2017). AD not only affects the individual diagnosed with it, but also family and friends as well as the society in general. Recent studies performed in the UK, have shown that the economic burden for health and social costs for dementia are actually more than those for cancer, heart disease and stroke combined (Alzheimer's research UK, 2017). This goes to show how much of an impact dementia has on the economy on a worldwide scale.

The symptoms of Alzheimer's include, but are not limited to episodic memory loss, disturbance in language (Soukup, 1996), anger and irritability are also common. Patients with Alzheimer's disease initially present with episodes of memory loss, which have begun to affect the day to day life of the patient. This will normally be detected by a family member rather than the patient themselves. After this, serial assessments will be carried out to assess the gradual decline in cognition, in order to fully diagnose the person with dementia of the Alzheimer's type (Mulligan, Van der Linden and Juillerat, 2003).

There is a need for early diagnosis of Alzheimer's disease, due to its progressive nature with the passage of time. Presently, screening by use of quick cognitive testing such as the mini mental state examination are not proving effective enough to yield reliable information.

1.b. Amyloid hypothesis

The most widely accepted hypothesis of Alzheimer's disease is the amyloid hypothesis. This suggests the presence of amyloid plaques, which are aggregated fibres of the misfolded protein in the brains of patients with Alzheimer's disease (Chan, 2008). This is then followed by the deposition of neurofibrillary tangles, which are composed of hyper phosphorylated tau aggregates. The result is dysfunction in synaptic and neuronal abilities, and subsequent loss of structures (Frisoni et al., 2010).

The extracellular amyloid beta deposits cause inflammation, which may in turn upregulate the production of tau tangles (McGeer and McGeer, 2013). This inflammation causes neurotoxicity, a feature which is unaccounted for in the amyloid cascade hypothesis. Hence an extension to the hypothesis is required.

1.b.i Amyloid beta precursor protein (APP) processing

Amyloid beta (Aβ) is formed by cleavage of the amyloid beta precursor protein (APP). The cleavage of APP involves the use of three different enzymes; α, β and γ secretases. The non-amyloidogenic pathway which involves cleavage of APP using α- secretase cleaves at the glutamine-lysine site within the Aβ, which then prevents the formation of amyloid beta. Instead, sAPPα is formed. sAPPα has been seen to have beneficial effects on neurones (Chow et al., 2010), by preventing glucose and oxygen deprivation, and has brain developing abilities.

The amyloidogenic pathway however cleaves APP at the N-terminal of Aβ with the use of β- secretase to form sAPPβ, a further cleavage then takes place at the C-terminal using γ-secretase, forming Aβ. The enzyme γ-secretase uses four different proteins, including presinilin, nicastrin, Aph-1 and Pen-2 (Mattson, 2004). In order to form an excess of amyloid beta, the latter pathway must be preferential over the former. The process is seen in figure 1. There are two highly toxic forms of Aβ, Aβ-40 and Aβ-42. In AD patients, Aβ-42 is seen more prominently in the form of aggregates (Roher et al., 1993)

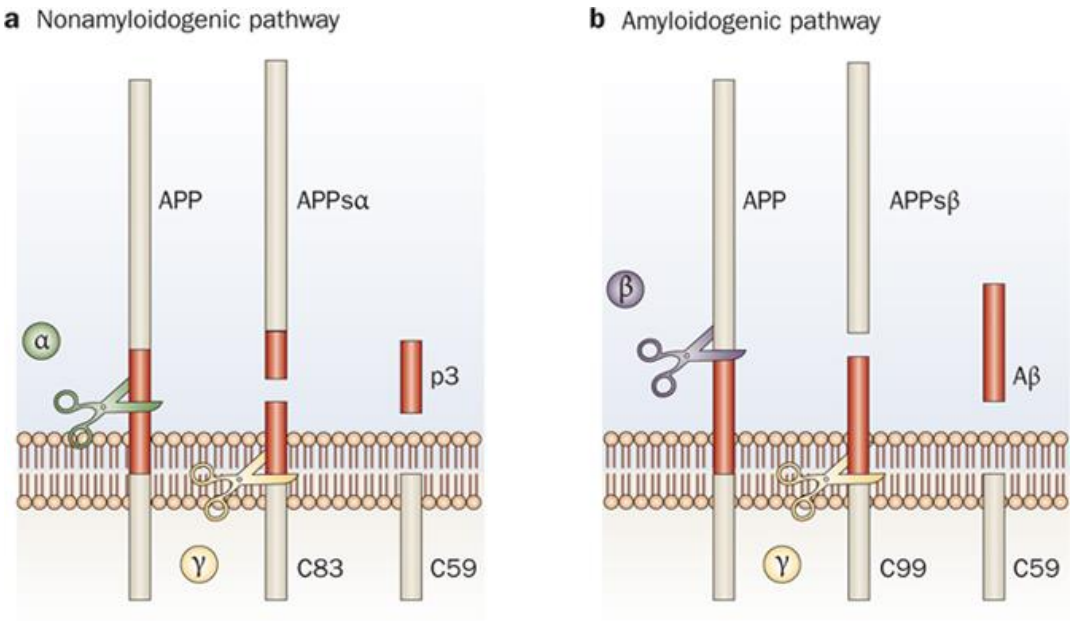


Figure 1. APP Processing- this figure shows the two different pathways of APP processing; a. the non-amyloidogenic and b. the amyloidogenic. a. α-secretase cleaves the APP protein within amyloid beta, thereby preventing formation of Aβ. b. β-secretase and γ-secretase cleave APP to form Aβ. (De Strooper, Vassar and Golde, 2010).

Murine models have been used to study the abnormalities in amyloid beta processing. A prominent model for this purpose is the APPswe/PS1dE9 model. This model mouse is formed by the breeding of mice with mutant APP and those with PSEN1 mutations. They develop amyloid beta plaques by around six months of age (Jankowsky et al., 2004), with extensive pathology observed at around nine months of age. This model was used for the optimisation purposes of this project.

1. c. Pathology in Tau

Tau is a microtubule associated protein, which can be found in mature neurones. In total, there are three major microtubule associated proteins (MAPs) found in neurones. These include MAP tau, MAP1 and MAP2 (Iqbal et al., 2005). The neuron has the ability to compensate for the loss of one of these MAPs, due to the presence of the other two MAPs. Studies have shown that no developmental deficiencies are seen when one of the MAPs are knocked out, however double-knockout mice show deficiencies in the development of the neuron.

In a normal human brain, tau can be found in six different isomeric forms, due to alternative splicing of the pre-mRNA gene. The difference in these six isoforms is seen in the C-terminal half, by the presence of either three or four microtubule binding repeats. This is formed by the alternative splicing of exon 10 in the gene (Liu and Gong, 2008). In a normal neuron, this then means that tau is expressed as either a 3R- tau or a 4R- tau, and is found in equal amounts normally. The N-terminal part may have zero, one or two amino acid inserts, again providing differences between the six isoforms. The six isoforms can be seen in figure 2.

The function of tau is to form microtubules, by interacting with tubulin in assembly and stabilization (Iqbal et al., 2010). In Alzheimer's disease, tau becomes abnormally hyper phosphorylated. This disrupts the normal functioning of tau, which was to interact and stabilize microtubules. Tau loses the affinity to bind with the microtubules, and transforms from being highly soluble, to soluble and insoluble tau aggregates. This hyper phosphorylation occurs in all six isoforms in an AD brain. The levels of tau are also increased from four to eight-fold in AD brains, as compared to age matched control groups (Khatoon, Grundke-Iqbal and Iqbal, 1992), and this increase is seen in phosphorylated tau.

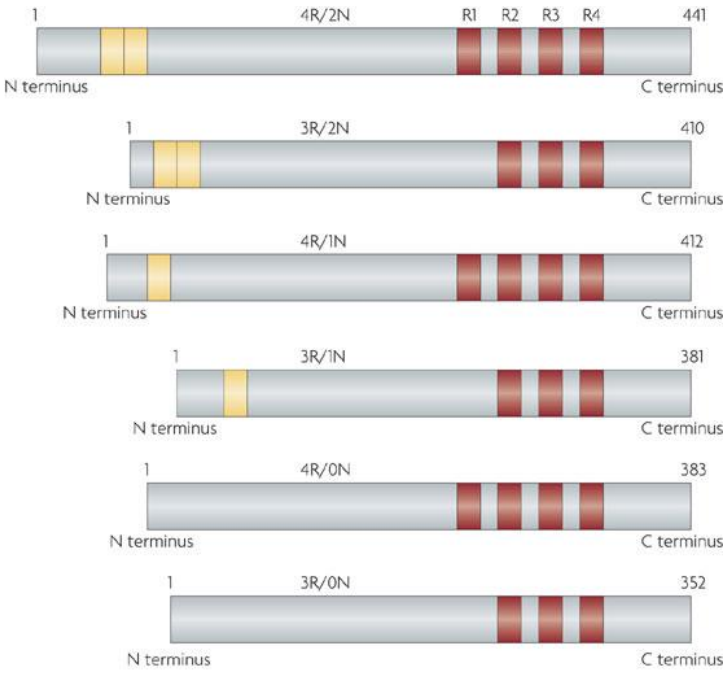


Figure 2: shows the six human isomers found in human neurones. The yellow part shows the presence of either zero, one or two amino acid inserts at the N-terminal. The red part shows the presence of either three or four tubulin binding domains seen at the C-terminal. (Ballatore, Lee and Trojanowski, 2007).

Different mouse models are available to study the pathologies in the tau protein. One of these include the hTau mouse, which includes the nonmutated human form of the tau gene, with the mouse tau gene knocked out. This mouse line was created by crossing two previous tau mouse lines; the 8c, which contained all six human tau isoforms, and the KO mouse, which was a tau knockout mouse. The resulting hTau mouse included all six human isoforms of tau, in the absence of murine tau (Andorfer et al., 2003). This mouse line shows similarities to cases of people with AD, in that it is dependant of age in the development of the disease. It also shows pathology in the same brain regions as those seen in AD cases. Not only does it have similar features with distribution of tau, a decrease in cortical thickness is also observed in both cases (Polydoro et al., 2009).

Mouse brains differ in tau content from human brains, in that they almost exclusively contain the 4R-tau. Hence, rather than the six isoforms found in human brains, only three are present in murine brain (Liu and Götz, 2013).

1.d. Genetic abnormalities

There are certain genetic abnormalities which may either cause, or increase the risk of certain individuals from attaining Alzheimer's disease. Significant evidence suggests that chromosome 14 harbours the locus for abnormality in Alzheimer's disease with the PSEN1 gene as the abnormal gene (Sherrington et al., 1995). This abnormality identifies a gene which showed to produce AD in many different pedigrees, with more than 150 mutations causing the familial form of AD. The mutations observed are primarily missense mutations occurring in the gene, although deletions, insertions and splicing has also been seen. However, the common factor among all mutations within this gene, is that it causes the protein to gain toxicity in its function (Strooper, 2007).

Mutations were also noticed in chromosome 1, in which less cases of AD were seen. The increase in susceptibility of AD was noticed in the presence of a particular isoform of the apolipoprotein E, the E4 isoform (Roses, 1997). It has been suggested that this isoform is involved in the increased accumulation of amyloid beta, and also the decrease of its clearance.

1.e. Disease progression

According to McGeer and McGeer (2013), there are six stages of disease progression for Alzheimer's. The first stage is characterised by disease onset, and takes place around 10 years prior to clinical symptoms. During this time, amyloid beta deposits itself in the brain, and consequently levels reduce in the CSF. Around 5 years later, phase two starts. This is shown by continued decreasing amyloid beta

levels in the CSF, and an increase in CSF tau levels. The third phase is characterised by a decrease in the metabolic rate, and more tangles of tau. The fourth phase is typically another 5 years later, and at this point irreversible brain damage has occurred, and hence total disease prevention is now not an option. Clinical diagnosis can be done in phase 5, and an increase in amyloid beta deposits in the brain are seen. There is a subsequent loss of brain volume. The sixth and last phase of disease progression is the point where the patient now requires full time supervision and care. Every aspect of the brain functions is now in decline.

1.f. Current treatments

With regards to therapeutics, the highest chances of success are early on, in the first phase. This is before any aggregation of tau in the brain. However presently, the most number of clinical trials with regards to Alzheimer's disease are taking place in the sixth and last phase, the point where any therapeutic capabilities are at the lowest. Treatments are being aimed at blocking the activity of the β and γ secretase activity, in order to prevent over-production of Aβ.

Acetylcholinesterase inhibitors, in particular donepezil are the leading treatments for AD (Cacabelos, 2007). Other acetylcholinesterase inhibitors include galantamine and rivastigmine. These are used to treat those patients with early to mid-stage AD. These act by inhibiting the activity of the acetylcholinesterase enzyme, which has been seen to increase the accumulation of amyloid beta peptide (León, G. Garcia and Marco-Contelles, 2011). Donepezil has been seen to be highly specific to the enzyme in the CNS, with activity in other tissues being non-existent or very limited (Becker et al., 1991). The same selectivity is observed in rivastigmine, which imitates the behaviour and action of the enzyme thus allowing its activity to falter (McGleenon, Dynan and Passmore, 1999). For the more severe stages of AD, memantine is the administered drug of choice. This acts by blocking the NMDA receptors, and was found to prevent neuronal cell death as well as the prevention of disruption of neuronal signalling (Zhang et al., 2016). However, these treatment options have been seen to show limited benefits in AD, and only show symptomatic benefits rather than anything disease modifying (Drachman, 2014).

1.g. Synapses in Alzheimer's disease

Synaptic dysfunction is an important characteristic of AD, which has been depicted in numerous studies, which show a significant loss of synaptic activity early on in the disease (Masliah et al., 2001). Loss of synapses is widespread in the hippocampus as well as the cortex, and this may contribute to the different degrees of cognitive decline as the disease progresses (Scheff, Neltner and Nelson, 2014). There is also a loss of these synapses prominently seen surrounding the senile plaques, and hence soluble oligomeric amyloid beta is being seen as an important factor of synaptic loss in AD (Koffie, Hyman and Spires-Jones, 2011).

Important receptors which are involved in synaptic transmission include the N-methyl-D-aspartate receptors (NMDARs) (Zorumski and Izumi, 2012). They are involved in the glutamate-mediated excitatory signalling, and are believed to be involved in synaptic transmissions which underlie the functioning of learning and memory building. NMDARs play a key role in brain functioning, and help in processing information at a higher speed. Changes in NMDARs activation or processing could effectively result in AD pathology, in that they disrupt the synaptic activity of neurones (Zhang et al., 2016). It has been suggested that a mild increase in the NMDAR activation, could have an effect on the accumulation of amyloid beta (Parameshwaran, Dhanasekaran and Suppiramaniam, 2008). Aβ is able to increase the excitotoxicity in neurones via the NMDA receptor, as well as mediate an affinity for an increase in the levels of intracellular $Ca^{2}+$. These NMDA receptors can be found in both presynaptic and postsynaptic terminals.

Quantitative studies have been performed on mouse as well as human brain samples, to analyse synaptic density in control and AD subjects, and within these studies, a decrease of 25-50% in the density of synapses in AD can be seen in the frontal cortex (Scheff and Price, 2001). This is compared with the 10-15% loss seen in control during the normal ageing process. Predominant loss is seen in the early stages of the disease in glutamatergic and cholinergic synapses, although as the disease develops, further loss is observed in other neurotransmitters (J. Selkoe, 2002). The loss of synapses is directly linked to the level of cognitive decline in AD patients.

Synaptic activity is most commonly measured using the protein markers Synaptophysin and PSD95. Synaptophysin is a vesicle protein, found abundantly spanning the transmembrane in all nervous tissue (Kwon and Chapman, 2011). Post-synaptic density 95 (PSD95) protein, is found on the postsynaptic synapses. The function of this protein is to attach and cluster synaptic proteins as well as the NMDA receptor in the postsynaptic density (Kim and Sheng, 2004). Decreases in level of these two proteins are observed in AD. During confocal microscopy on AD mouse brain, significant reduction was observed in synaptophysin in the barrel cortices. Loss was also seen in PSD95 levels in the barrel cortices when comparing with control samples (Tampellini et al., 2011).

1.h. Targeting multiple Alzheimer's disease biomarkers simultaneously

The definitive diagnosis of AD can only be done by examination of the brain post-mortem, by conducting immunohistochemical analysis to identify the presence and severity of amyloid beta and tau tangles. Considering just the brain samples from human's post-mortem, these can come in a number of different forms, including frozen, fixed etc. In clinic, CSF is extracted by lumbar puncture, and most extensively, the levels of Amyloid beta and tau are measured to determine whether or not AD has developed. This is highly invasive, and often uncomfortable or painful for the patient.

Also, levels of some biomarkers in the CSF could fluctuate under certain circumstances, and so it is important to simultaneously be able to target the various biomarkers to portray an accurate picture of AD presence/severity.

A system is therefore necessary in which several types of samples can be used in diagnostics simultaneously.

1.i. Reverse phase protein array

Reverse phase protein arrays (RPPA) provide a new technology in which the levels of minute amounts of proteins can be detected in numerous samples simultaneously. They are generated by printing small amounts of protein onto nitrocellulose coated microscope slides, which are then probed by relevant antibodies (Charboneau et al., 2002). These antibodies must however be highly specific to the protein in question, in order to get an accurate representation of protein level.

In RPPAs, the protein intensity is not measured by the molecular weight of the protein, as is done in other methods such as western blotting, rather only the presence of the protein is measured. Hence it is very important to first determine antibody specificity using western blotting techniques, before it can be determined whether or not the antibody is reliable for use in RPPA. This is to avoid false positive results. An RPPA can be constructed on various different types of slide, including single slides or multiple pad slides. A multiple pad slide is one which has numerous 'pads' of nitrocellulose on a single glass slide. These can be separated from each other using a gasket, when different antibodies are probed (Espina et al., 2004). The process by which protein arrays are printed and probed can be seen in Figure 3.

The main markers of biological changes, diagnosis and functional markers are predominantly proteins. Therefore in order to fully understand the biological pathways and cellular functioning in disease progressions, it is these proteins which must be targeted. This is where RPPA plays a part, as this is a high through-put method detecting the interactions between protein-antigen (Nishizuka et al., 2003).

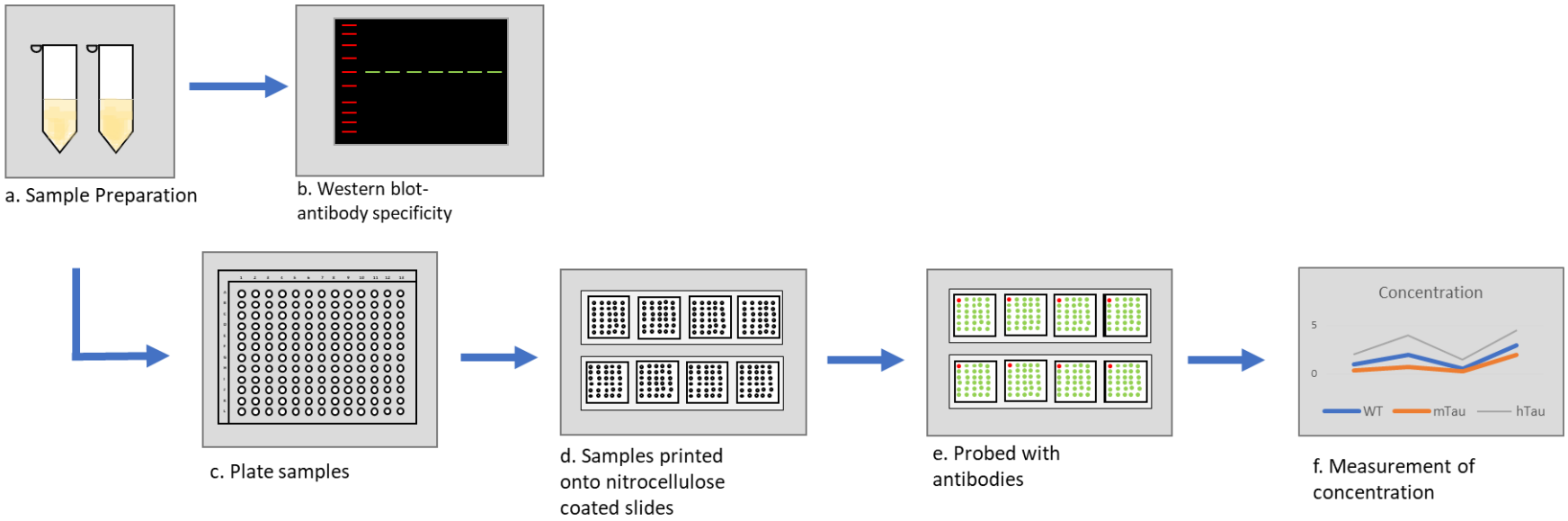


Figure 3. RPPA method flowchart. a. The preparation of the sample which includes homogenisation of the brain tissue in RIPA buffer, and an addition of RLT buffer. b. Checking for the specificity of the antibody through western botting. Prior to RPPA commencement. c. Samples are plated on a 364 well plate. d. then plate is then inserted into the printer, which prints samples onto nitrocellulose coted slides I replicates. e. once printed, the slides are probed with primary and secondary antibodies. f. the concentration of sample and control are measured using different software packages. Image produced by author.

2. Aims and Objectives

Current technology enables researchers and clinicians to be able to identify the presence of AD definitively in only post-mortem brain samples. Limitations occur when several biomarkers need to be identified simultaneously. The aims of this study were to develop a system in which several AD biomarkers can be targeted at the same time, on a number of different sample types. As a method, reverse phase protein micro-arrays (RPPAs) have been used extensively with cultured cell samples, however there is a deficiency with regards to using RPPA with brain tissue. Hence the specific aim of this study was to use RPPA in developing the detection method of a panel of AD biomarkers using brain samples from AD pathology mice.

The objectives of this project were to optimise the protein extraction methods, to concentrate the required proteins from other components found in brain samples. Validation of the biomarkers was to be done by western blotting techniques, prior to use in RPPA. The final objective was to then validate this method on tau pathology mice of hTau background.

By carrying out these experiments, I can hypothesise that the micro-arraying method will give high throughput results and allow important proteins found in the AD brain to be seen simultaneously.

3. Validation of biomarkers using Western Blot

3.a. Principle:

Western blotting is a technique which separates different proteins from a sample, based on the molecular weight of the protein. The sample is loaded onto a gel, which is run at a constant voltage. The gel is then transferred onto a nitrocellulose membrane, and probed for specific primary antibodies. A secondary antibody is then added for visualization purposes (Mahmood and Yang, 2012). The western blot system used in these experiments was the poly-acrylamide system with the use of tris-glycine buffer. Several western blots were carried out in this project, in order to validate the specificity of several antibodies, to be used in an Alzheimer's disease panel. The solutions mentioned below indicate the recipe used for each western blot, unless otherwise stated in each example.

3.b. Solutions used:

RIPA lysis buffer was made up of, 50mM Tris HCl, 0.1% Triton-X-100, 1mM EDTA, 150mM NaCl, 0.25% N-Deoxycholate, 1mM NaF, 1mM Na_3VO_4, 80mM β-Glycerophosphate. These were diluted in dH_2O, and made up to 500ml. 50ml of solution was taken and a single protease inhibitor cocktail tablet was added (Complete EDTA-free; Roche Diagnostics).

Electrophoresis running buffer: Tris base 30.3g, Glycine 144g, SDS 10g, made up to 1 litre in dH_2O.

Transfer Buffer: Tris base 30.3g, Glycine 144g, dH_2O 8 litres, methanol 2 litres. Kept cold in refrigerator.

All primary and secondary antibodies were made up to the respective concentrations mentioned in the same medium as the blocking buffer, which was 5% milk TBST 0.1% Tween 20.

Optimisation

3.c. Animal Tissue:

The mice used in this project were mostly of a tau pathology background. hTau mice from Jackson laboratories (Bar Harbor, USA) were purchased and kept for breeding and experimental purposes at the University of Nottingham, according to the Animals Scientific Procedures Act (1986). Tissue collection was done by a colleague with a full home office license.

hTau+/- mice were used which were mice over-expressing human tau pathology, including all six non-mutated isoforms of human tau, which is the same as is seen in AD. In order to fairly compare, mice with human tau knocked out (hTau-/-) were used as a negative control. Murine tau mice were also used here. The mTau+/- mice overexpressed murine tau, and as a negative control, murine tau knockout mice (mTau-/-) were used. The use of murine tau mice is important in order to

understand the AD pathology as seen in mice and as a comparison to that seen in the hTau mice which primarily express the human form of tau pathology. The wild type littermates (WT) were also utilised.

3.d. Sample preparation:

The general experimental procedure is described below, and any changes which were made for specific antibodies are described in the relevant sections. The first step in all experiments was to homogenise the samples in 5x cold RIPA lysis buffer. A bicinchoninic acid assay (BCA) was then performed using a protein assay kit (Novagen). Samples were then made up to 2 mg/ml with RIPA buffer. Laemmli buffer was added to dilute samples to 1mg/ml. Samples were then kept at -80˚C until needed.

3.e. Western blot procedure

Samples were defrosted and required amounts heated at 100˚C for 5 minutes, to denature the proteins. These were immediately centrifuged at 13,000xg for 1 minute, in order to collect any condensation of sample which may be present in the cap of the Eppendorf during the heating stage. The samples were loaded onto polyacrylamide gels, amounts of each solution were altered to accommodate for the percentage of gel being used in that experiment. These were run at 200V for 45 minutes, using the Mini-Protean tetra system (Bio-Rad). Meanwhile, transfer cassettes with filter paper and nitrocellulose membrane were soaked in cold transfer buffer. The gels were then transferred at 100V for 1 hour on ice, as a wet transfer. A ponceau stain was carried out to observe presence of protein bands. The membrane was then blocked for 1 hour in 5% milk TBST 0.1% tween 20 on a see-saw rocker at room temperature. Next, the membrane was incubated at 4˚C in one of the primary antibodies overnight on a see-saw rocker. The next day, the membrane was washed in TBST 0.1% Tween, for 3x5 minutes followed by 3x10 minutes. IRDye 800CW Goat anti-mouse IgG (1.15,000; Li-Cor), and IRDye 680RD Goat anti-Rabbit IgG (1.15,000; Li-Cor) were used as the secondary antibodies, at 1.15,000 concentrations. The membrane was then washed again for 3x5 minutes followed by 3x10 minutes.

3.f. Determination of gel percentage:

Trial western blots were done initially, using a 20% polyacrylamide gel. Results for this showed incomplete separation of the molecular weight marker ladder (Li-Cor), and also some samples missing from the membrane. This was put down to the very high percentage of gel used, causing low resolution of images.

Consequently, a lower gel was used in the next experiment, to counter the negative effects seen in the 20% gel. A 15% polyacrylamide gel was used. There was a better separation of molecular weight marker in this experiment, which showed that a lower percent gel was giving better resolution images. 10% gels were also used in some experiments, as suggested in literature to observe specific protein bands. The percentage of polyacrylamide gel used is specified in each experiment further.

3.g. Synaptophysin and PSD95:

Western blots were now done using synaptophysin and PSD95 antibodies, to measure the pre- and post-synaptic activity respectively in the brain samples.

The samples used for this western blot were brain extracts from mice of both tau and Aβ background. These samples were used in order to study levels of biomarkers in both pathology type mice, as both Aβ and tau are characteristic of AD. For this purpose, two hTau, two mTau and two of their wild type littermates were taken. As well as this, two APPswe/PS1dE9 mice, and two of their littermate wild type mice were used.

Samples were made up to 1mg/ml, after determination of concentration by BCA. 10ul of each sample was loaded onto a 10% polyacrylamide gel. Primary antibodies were monoclonal anti-synaptophysin (1.10,000; Sigma Aldrich), monoclonal anti PSD95 (1.10,000; Sigma Aldrich) and anti-rabbit GAPDH (1.20,000; Sigma Aldrich). Probing with secondary antibodies was done as stated in the above section. Concentration of antibody was done according to the concentrations used by Scullion, Hewitt and Pardon (2013).

Results: Bands here were very clear as can be seen in figure 4. As these western blots had previously been done in the lab, it had been pre-determined that both antibodies could be probed on the same gel. Synaptophysin is seen at 40kD, and PSD95 at 97kD.

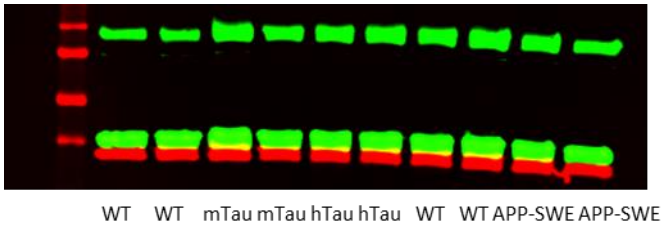


Figure 4: western blot representation of synaptophysin and PSD95 (green) with GAPDH as the house-keeping protein (red).

3.h. Tau Western blots:

The same samples were used as in the synaptophysin and PSD95 western blots. The concentration of each sample was made up to 4mg/ml, and 10ul of sample was loaded onto 10% polyacrylamide gels. Primary antibodies used were Tau46 (1.250; Sigma Aldrich), to detect the total tau, PHF1 (1.250; Dr. Peter Davies, Feinstein Institute for Medical Research, Manhasset, NY, USA) to detect the phosphorylated tau, and GAPDH (1.10,000; Sigma-Aldrich).

Results:

As expected, the hTau samples are shown as the strongest bands, whereas mTau is almost non-visible. WT from the hTau littermate background is showing stronger bands as compared to the samples from the amyloid beta mouse background. All

results were found to be as expected from such samples and are further discussed in the results section.

Tau46

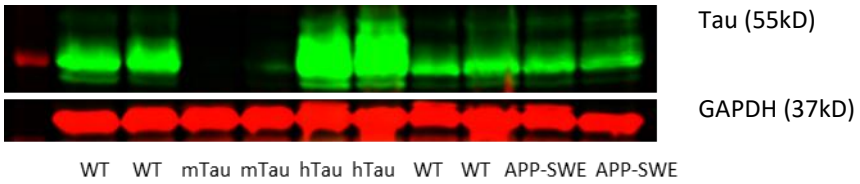


Figure 5: western blot using Tau46 as the primary antibody

PHF1

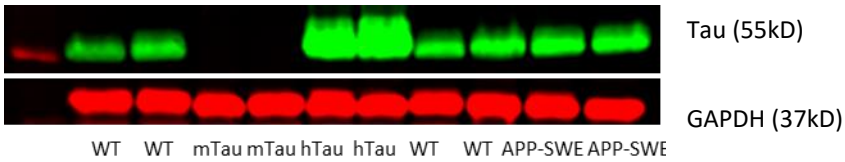


Figure 6: western blot representation using PHF1 as the primary antibody

3.i. Amyloid beta western blots:

A set of western blots were then carried out in order to identify Aβ protein. Aβ is a characteristic hallmark of Alzheimer's disease, and an important marker for disease presence and progression.

Aβ-i:

For these western blots, pre-made 10-20% tricine gradient gels (Criterion TGX-Pre-cast Gels; Bio-Rad) were used. The samples were at a concentration of 4mg/ml. Primary antibodies include Anti-Beta Amyloid 1-42 (abcam, ab180956; 1.1000) and GAPDH (1.20,000; Sigma Aldrich).

Results:

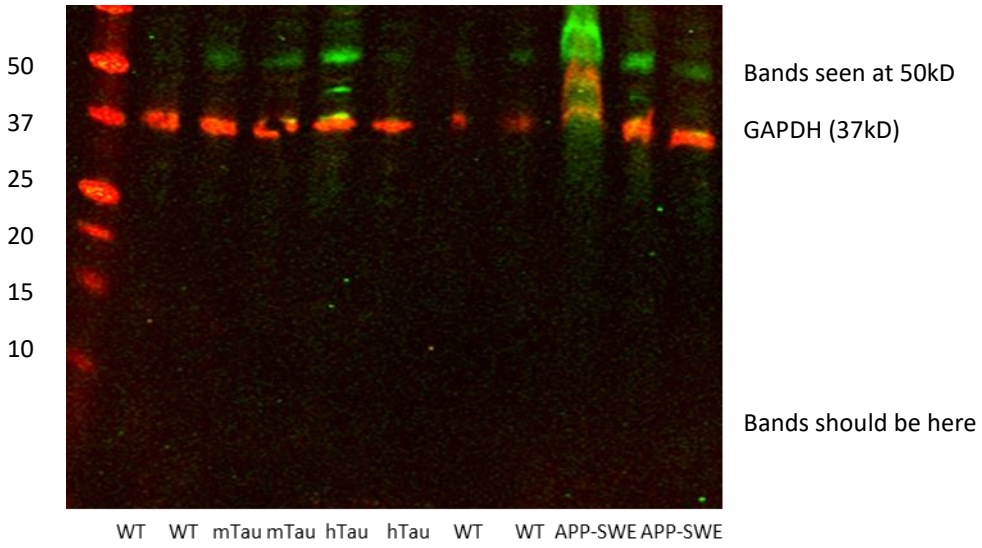


Figure 7: the western blot representation of the initial amyloid beta experiment. Bands should be seen at the 5kD mark, however are seen at 50kD.

Bands are seen here again in the wrong place at around 50kD, whereas Aβ should be seen at around 5kD. There is non-specificity of the bands, with spreading of the protein noted in some of the samples.

<u>Aβ-ii:</u>

Conditions for this blot were changed slightly to accommodate for the use of tricine gels. 15ul of each 4mg/ml sample were loaded onto 10-20% tricine gradient gels. Concentrations were used according to Rosen et al., (2010), in which 50-60ug of total protein is advised to be optimal for detection. The paper also suggested running the gels for 90 minutes, and a constant voltage of 125V, to get better separation of bands. The running buffer was also modified, with concentrations as follows; 100mM Tris, 100mM Tricine 0.1% SDS (Haider, Reid and Sharp, 2012).

Transfer times were kept the same as in earlier experiments, at 60mins at a voltage of 100V. Antibody concentrations were the same as before.

Results: Bands should have been seen at around 5kD, but they were seen in the wrong place in this case, as can be seen in figure 8. Bands are seen at around 50kD again.

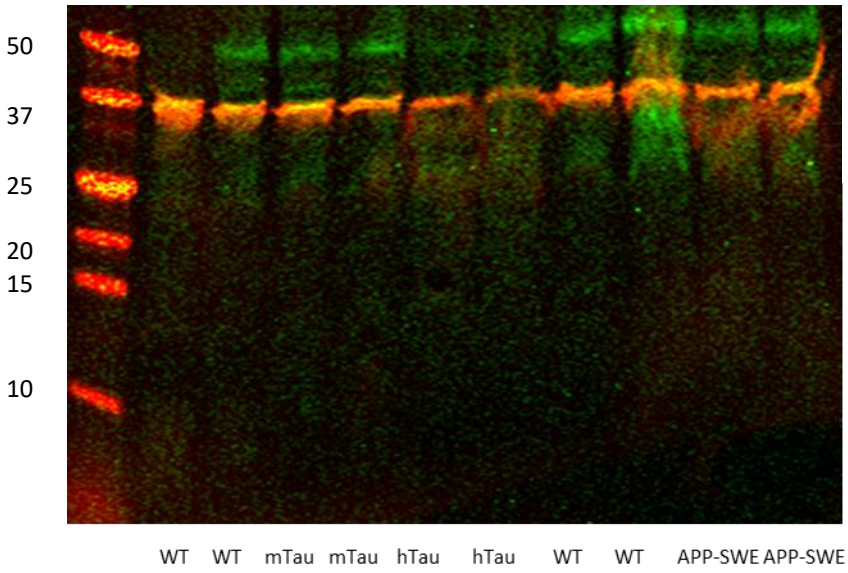


Figure 8: western blot representation of experiment using amyloid beta for the second time. Bands should be seen below the 10kD mark on the ladder, at around 5kD. Instead are noted at 50Kd. Amyloid beta is seen in green, and GAPDH in red.

Aβ-iii:

To accommodate for the very small nature of amyloid beta, a smaller pore sized nitrocellulose membrane was used. Other conditions were kept the same as before.

Results: the results from this scan showed no bands, not even the ones at the wrong place as last time. To try and resolve the problem, a new Aβ-42 antibody was tested.

Aβ-iv:

A self-made 15% polyacrylamide gel was used to load 10ul of 4mg/ml samples. A new amyloid beta-42 antibody was tested in this experiment. A 1.1000 concentration of anti-beta Amyloid 1-42 Conformation- specific (Abcam; ab201060) was used as per instructions on data sheet provided. Anti-GAPDH (1.40,000; Sigma Aldrich) was also used as a detection method for a house-keeping protein.

Results: bands were again not seen at relevant places. The resulting scan can be seen below. In this case GAPDH was also missing.

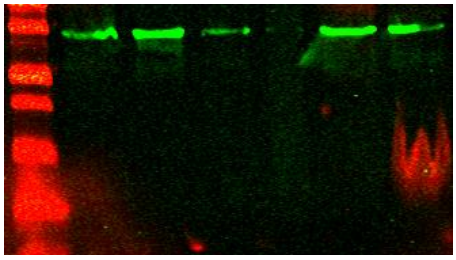


Figure 9: western blot of amyloid beta using a different antibody. Bands are seen at around 50kD again instead of the 5kD it should have been. The amyloid beta is green, and the GAPDH should have been seen in red.

3.j. Result analysis:

All western blots were analysed in the same way; scanned using a Li-Cor infrared scanner, using Odyssey image studio software. Intensities were measured by drawing squares around each band of both antibody and GAPDH. The antibody intensities were normalized to GAPDH and expressed as a ratio to GAPDH.

3.k. Statistical Analysis:

All statistical analysis was done using Invivostat. One or two-way ANOVA tests were performed as appropriate, followed by Post-hoc (Unadjusted LSD) pairwise comparisons. Graphs were made with standard error of the mean (SEM) displayed as error bars, and any statistical significance displayed as stars on the graphs.

3.l. Discussion

Western blots performed over the five Alzheimer's disease specific biomarkers, showed four antibodies to have clean results and good enough to use further in this project. Those were Synaptophysin, PSD95, Tau 46 and PHF1. Aβ was not able to be verified within this project and hence was not used further in the RPPA experiments. Reasons for why Aβ may not have proved successful are discussed later on in the discussion section.

4. RPPA Optimisation

4.a. Principle

Reverse phase protein array as a technique has many advantages over western blotting. A single microscope slide which is coated with nitrocellulose can be printed with thousands of different samples and probed with multiple antibodies simultaneously. Hence proving to be more time saving and economic in the long run as compared to the standard western blot. It can also be more accurate than western blotting as the gel to gel differences often experienced in westerns blots is negated. The amount of sample required is also significantly lower in RPPA, as is the amount of antibody used, again decreasing costs of antibody purchasing.

The RPPA technique was used in this project in order to develop a panel of Alzheimer's disease samples, which can be probed with several AD antibodies concurrently.

4.b. Development of technique

Pilot:

In the initial trial at the RPPA method, 10 different samples were used. These included two hTau, two mTau, two wild type littermates from these mice, it also included two mice from the APP/SWE background, with two littermate wild types.

Abbreviation	Full
APPswe/PS1dE9	Mutant APP and mutant PSEN1(more information in section 1.b.i)
hTau	Human tau
mTau	Murine tau

Table 1- table showing the full and abbreviated versions of the mouse strains used.

BCA was previously done on the samples using a protein assay kit (Novagen), in order to determine the concentration.

To prepare for RPPA, the samples were homogenised in RIPA buffer as was done with the samples used for western blotting. These were then adjusted to 5mg/ml with water. To this ¼ of sample buffer was added, making the final concentration to 4mg/ml. The 4x sample buffer was made up of 3.3ml of 1.5 Tris HCl at pH 6.8, 6ml Glycerol, 6.4ml 20% SDS and 2.3ml dH_2O. This was then diluted in a 1:3 dilution with RIPA buffer. 5ul of each sample was added to a 364 well plate, to which 5ul of the RIPA-SDS buffer was also added. The plate was heated to 95˚C for 5mins, and immediately centrifuged for 1 min.

A 16-pad microscope slide coated in nitrocellulose was printed and frozen immediately, ready for analysis of protein levels. Once defrosted, the slide was

washed in water for 5mins. 100ul of a Revert total protein stain (Li-Cor) was then added to each well for a total of 5mins, and consequently washed off with a washing buffer (30% methanol, 6.7% acetic acid). The Revert stain was added to visualize the amount of total protein in the samples. The slide was scanned using a LiCor scanner, and the Odyssey image studio programme.

The slide was then blocked in 5% milk TBST 0.1% tween for 1 hour on a rocker at room temperature. 100ul of each primary antibody was added to individual squares on the slide, and incubated overnight at 4˚C. The antibodies used can be seen in table 2.

The concentrations used were those which had been determined in western blots.

Antibody	**Concentration**
Monoclonal Anti-Synaptophysin (Sigma-Aldrich)	1/10,000
Monoclonal Anti-PSD95 (Sigma-Aldrich)	1/10,000
Monoclonal Anti-Tau (Sigma-Aldrich)	1/250
PHF1	1/250

Table 2- table showing the antibodies used in the experiment, and at which concentration. PHF1 was gifted generously by Professor Peter Davies (NY, USA).

Scanning was performed as previously. However, very limited results were seen in this scan and hence the slide was not further analysed. An explanation for this could be not having an antibody which was strong enough to give a good signal in this technique, and hence this is the reason why further experiments were done using more concentrated antibodies.

<u>Blocking buffer experiment:</u>

In this, the differences between different blocking buffers was determined. Three LiCor Blocking buffers were used, namely Odyssey blocking buffer (PBS), Odyssey blocking buffer(TBS) and Casein blocking buffer, all from the blocking buffer optimisation kit (Li-cor). A 3% fish skin gelatine was used as the fourth buffer. The duration of blocking was also increased to 3 hours, from the 1 hour in the previous attempt. This was done in order to minimise the background seen previously. IRDye-800CW Goat anti-mouse IgG was used as the secondary antibody (1.15,000).

Results: the Licor TBS blocking buffer showed the strongest results and was the buffer used in the rest of the experiments.

Primary antibody concentration experiment:

Previous work using concentrations of antibodies determined using western blots showed weak to no signals in protein intensities. Hence an experiment was carried out which would determine the optimal concentration of antibody to use in RPPA.

Primary antibody concentrations were altered in this experiment, and can be seen in table 3.

	Concentration of antibody used			
Antibodies	As in WB	Modification 1	Modification 2	Modification 3
Synaptophysin	1/10,000	1/1000	1/1000	1/1000
PSD95	1/10,000	1/1000	1/1000	1/1000
Tau46	1/250	1/200	1/150	1/500
PHF1	1/250	1/200	1/150	1/500

Table 3. The name and concentration of antibodies used throughout this experiment

Results: synaptophysin and PSD95: 1.1000. Tau 46 and PHF1: 1.150. These were the concentrations showing optimal results, and the concentrations of antibody used in the rest of the experiments.

Test for optimal housekeeping protein and sample buffer

The frontal cortex of six different samples were used in this experiment. After homogenisation in 5 times RIPA lysis buffer, benzonase enzyme (5units per ml) in dilution buffer (1.1000) was added to each sample. This was in order to break down and digest any RNA and DNA material within the samples, to reduce viscosity, and hence to validate ease in printing of sample on nitrocellulose on the slide. As before, the samples were added to a 364 well plate, to which RIPA-SDS buffer was added in a 1.1 ratio. Duplicates of samples were added in this case, and to test for buffer effectiveness, a second sample buffer was also used, which was 2M betaine. Previous experiments performed in the lab had shown the effectiveness of 2M betaine as a sample buffer using isolated cell samples. Hence this is why the efficacy was tested using brain samples in this case.

Samples were printed on a 16 pad-slide, and frozen as before.

After defrosting, 1 square was dedicated to an immunogold stain (100ul), whilst another to a pure Total protein stain (100ul). Primary antibody incubation was done as before, however Anti- GAPDH (Sigma Aldrich) was also added to duplicates of the other primary antibodies. This can be seen in figure 10 below.

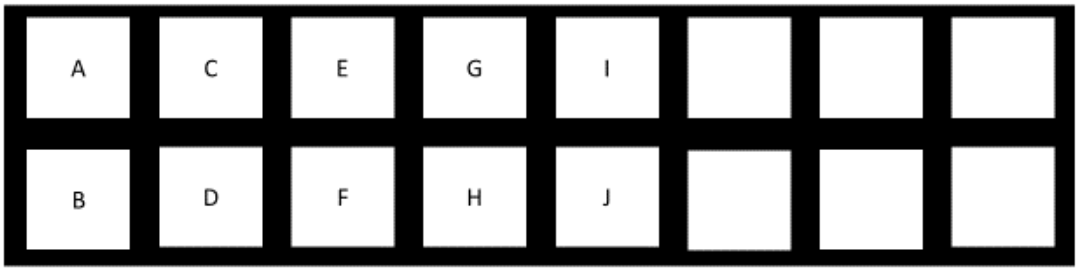


Figure 10. 16 pad slide layout. A-Immunogold. B- Total protein stain. C- Synaptophysin and GAPDH. D- Synaptophysin. E- PSD95 and GAPDH. F- PSD95. G- Tau46 and GAPDH. H- Tau46. I- PHF1 and GAPDH. J- PHF1

Results: the results were too weak to analyse; however, it was noted that the RIPA-SDS buffer results showed slightly stronger intensities as compared to the 2M betaine buffer results. This could be because betaine does not show the same effects on brain samples as it does on cell samples. In any case, 2M betaine was not used in the following experiments, rather RIPA-SDS was the sample buffer of choice.

Protein amplification:

This experiment was performed in order to try and amplify the protein level signals, as weakness in levels had been observed previously. A 16-pad slide was printed and probed with primary antibodies as described earlier. This was then washed in PBST 0.1% Tween. It was then incubated in a Biotinylated anti-mouse antibody (1/1000) for 3 hours. After further washes in PBST 0.1% Tween, an incubation of streptavidin Cy-5 (1/2000) was executed for 5mins. The slide was washed 3 times for 3mins in PBST 0.1% Tween. A further incubation involved addition of biotinylated anti-streptavidin (1 ng/ml) for 30mins. Streptavidin Cy5 was added again after another round of washes. The whole process was repeated, providing two rounds of amplification to the slide. This was then scanned. This particular system was used as it had shown positive amplification results in previous experiments in our lab and so provided a good first step in this amplification process.

Results: Results were overall amplified. In future experiments, this step was used when amplification of sample was required.

4.c. Data analysis

All slides were processed and analysed in the same way. After secondary antibody incubation, the slides were scanned using a Li-Cor scanner and Odyssey image studio. This was to obtain infrared images. Scanning was done at 3.0 intensity for the 700 channel (red), and 8.5 intensity for the 800 channel (green).

This is then followed by analysis on Genepix Pro. All the circles are placed on the printed dots as closely as possible. Results are obtained through the software.

4.d. Result analysis

Abbreviation	Meaning
F800	The forward or local intensity for the 800 channel
B800	Background intensity for the 800 channel
F700	The forward or local intensity for the 700 channel
B700	Background intensity for the 700 channel

Table 4- abbreviations used in this section and their meaning

Results were analysed using Microsoft Excel. Data obtained from Genepix pro was viewed in Excel, and results for the F800 median-B800, and F700 median-B700 were the ones used. The median number for all F700 results was obtained. The F800 was normalised by multiplying the F800 value, by the median of F700 divided by each F700 value. The formula used was:

$$\text{Normalised F800= F800 median -B800 x } \frac{\text{Median of all F700}}{\text{F700 median}-\text{B700}}$$

4.e. Statistical analysis

All statistical analysis was done using Invivostat. One or two-way ANOVA tests were performed as appropriate, followed by Post-hoc (Unadjusted LSD) comparisons. Graphs were made with standard error of the mean (SEM) displayed as error bars, and any statistical significance (defined as $p<0.05$) displayed as stars on the graphs.

4.f. Discussion

A gradual increase in protein intensity was being achieved throughout the optimisation steps for RPPA. However as of yet, without streptavidin amplification the intensities were still too low. Hence another approach was taken in order to maximise protein levels, and to minimise the presence of other parts of the sample which may be contributing to the low signals seen.

5. Optimisation of extraction methods

5.a. Technique

After 5 attempts at the RPPA method, it was clear that a method to eliminate lipids and concentrate proteins on the membrane was needed. In order to do this, three rest of the brain (ROB) samples were taken from different mice, and homogenised in RIPA buffer. These were then mixed together to form one sample. The sample was spun at 14,000xg for 15mins at 4˚C, and the supernatant and pellet were separated. The supernatant was then spun again in the same conditions for 15mins. The step was repeated twice. After each centrifugation step, it was observed that three layers were forming; the clear layer at the top, a cloudy layer in the middle, and a solid pellet at the bottom of the tube. These were all separated on each centrifugation. This can be seen in figure 11.

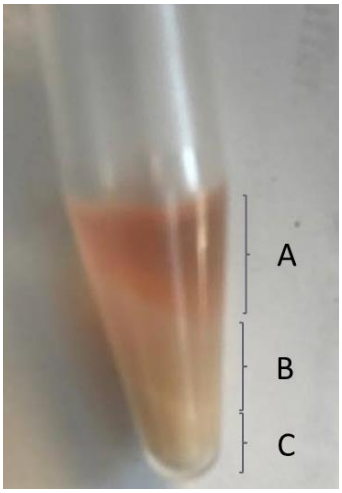


Figure 11: shows the sample between centrifugation steps. A: the top clear layer. B: cloudier layer in the middle. C: a solid pellet at the bottom of the tube which was very viscous and slimy.

An acetone precipitation of proteins was also performed. Acetone was cooled down to -20˚C prior to use. 40ul of this was then added to 10ul of each of the three supernatants taken from the lipid extraction experiment. The tube was vortexedand incubated at -20˚C for 60 minutes. The sample was then centrifuged at 15,000xg for 10 minutes. After centrifugation, the supernatant was disposed of, and the pellet left at the bottom of the tube, this was then allowed to be left uncapped in order to evaporate the acetone in the sample, at room temperature and for 30 minutes. The pellet was then re-homogenised in RIPA buffer.

The slide was printed as described previously, including all pellet and supernatant fractions obtained from the lipid extraction as well as acetone precipitation.

RLT Buffer:

Pellets from the previous experiment were too viscous to print, so RLT buffer (1.1; Qiagen) was added to these in order to minimise the viscosity, and to break down any RNA and DNA present. This was then put through spin columns (Qiagen) and

centrifuged at 15,000xg for 1 minute. As the RLT buffer would have increased the salts in the samples, a de-salting process was carried out. In 50ul of the sample in RLT, 950ul of RIPA-SDS buffer was added, and this was centrifuged for 3mins at 4000xg. The slide was re-printed and probed with antibodies as described previously.

Results:

As can be seen in figure 12 below, the pellet samples seem to show the strongest intensities. The Acetone is almost non-existent. The 'A' sample is the clear sample from the top of the tube, and the 'B' samples are the middle more viscous layer. 'C' is again a top layer from the second centrifugation. The Pellet is the bottom layer seen in figure 11. As can be seen, passing thorough the shredder tube has some benefits in one half of the samples. The top half of the shredder tube samples can be seen to have negligible amounts of protein. The salt removal had no visible protein. This could be due to the tau and Aβ aggregates having dissolved in RLT buffer, but then precipitating in the RIPA buffer.

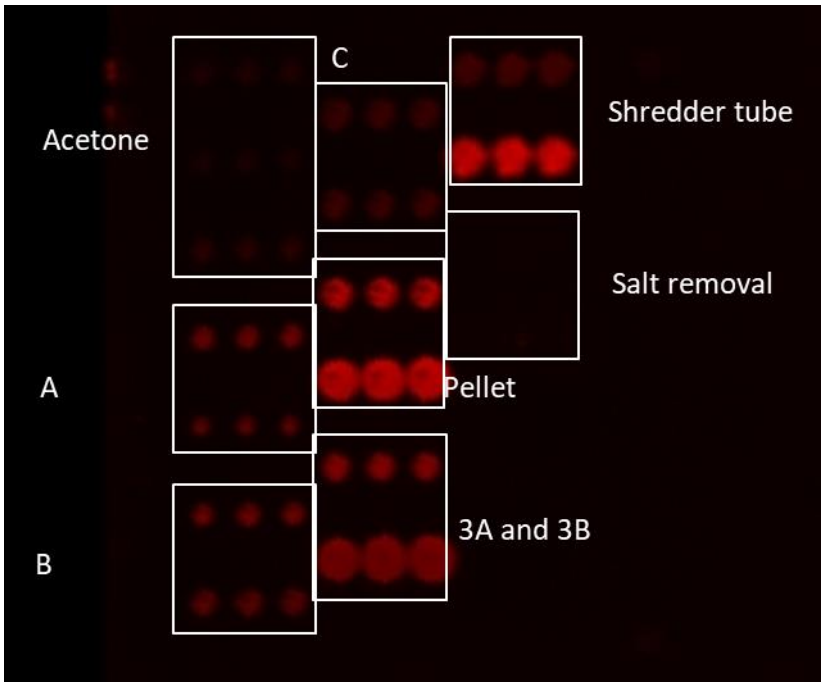


Figure 12: the RPPA images for the protein concentration optimisation experiment. Shown here with total protein stain probing (red). Acetone- shows the samples after an acetone precipitation. 'A'-the top clear layer as seen in image 11. 'B'-the middle more viscous layer as seen in image 11. 'C'- the cloudy layer seen at the bottom of the tube in image 11. Pellet- the solid pellet collected from the bottom of the tube. 3A and 3B- samples gone through further centrifugation steps. Shredder tube- samples put through the shredder tube before printing. Salt removal- samples after a salt removal process.

The conclusion from this experiment was that all layers and parts of the sample needed to be added into the experiment, as all had presence of protein in them.

All fragments and RLT buffer addition:

A new ROB sample was taken and weighed. This was homogenised in double the amount of RLT buffer. A print was prepared for the sample as it is, and also with an added excess of RLT buffer, passed through a shredder tube. The slide was processed and probed as before.

When the RPPA experiment was carried out using all fragments of the brain and homogenized with RLT buffer (Qiagen), it was observed that clean and strong prints of protein could be seen (Image 13). However, the samples with excess buffer were actually fainter than those with a 1.1 ratio. Those put through a shredder tube were of equal intensities to those which had not been put through the tube.

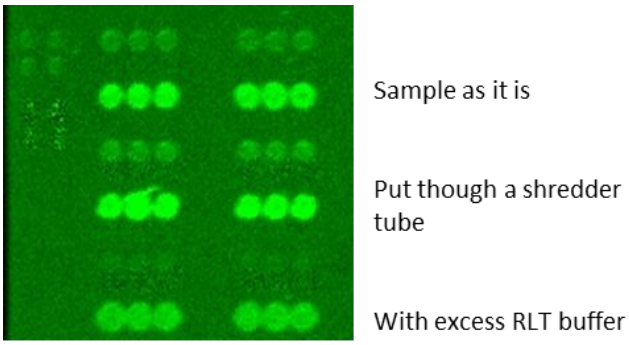


Figure 13: shows the RPPA print with Tau46 antibody (green).

5.b. RLT buffer; use in protein isolation:

It was seen that adding this buffer in a 1.1 ratio during the homogenization stage of the experiments, gave stronger dots seen on the nitrocellulose slides. RLT is a lysis buffer, which is used for protein isolation (Qiagen.com, 2017). Previously in the optimisation experiments, a lot of viscous material was being seen, which potentially could have been DNA. This buffer was able to successfully homogenise that material, and provide a clear sample to make printing onto the glass slide easier. Excess buffer was noted to decrease protein intensity which could have been due to the fact that RLT buffer when added in excess will potentially form crystal structures on the nitrocellulose coated slides during the drying stages. This binding then causes an interference of proteins binding to the nitrocellulose, and the signals seen are weak. Hence a balance in the use of this buffer for this purpose is important.

Putting the sample through a shredder tube after homogenisation with RLT buffer also did not increase intensities of protein. This could be because the buffer was able to homogenise the sample in such a way that no viscosity remained to be filtered through the fine tube. Hence results were almost identical to those seen in samples which had not been put through the tube.

6. Application of method to hTau mice

6.a. Rest of the brain samples

Once the method had been validated using RPPA, and a panel of antibodies had been successfully validated with western blotting, the method was applied on tau pathology mice. For this purpose, the rest of the brain sections of hTau pathology mice were initially taken. These were the mouse brain with the cortex, frontal cortex, hippocampus and cerebellum removed.

Western blot:

Five of each hTau+/-, hTau-/-, mTau+/-, mTau-/- and WT littermates rest of the brain samples were taken, and homogenised in RIPA buffer. These were then loaded onto pre-made 4-20%, 26 well gels. Primary antibodies were synaptophysin (1.10,000; Sigma Aldrich), PSD95 (1.10,000; Sigma Aldrich) and GAPDH (1.20,000; Sigma Aldrich). The gels for synaptophysin and PSD95 contained 10ug of protein. The same process on the same gels was carried out for Tau46 and PHF1 antibodies. 40ug of protein was loaded for each gel in these antibodies.

RPPA:

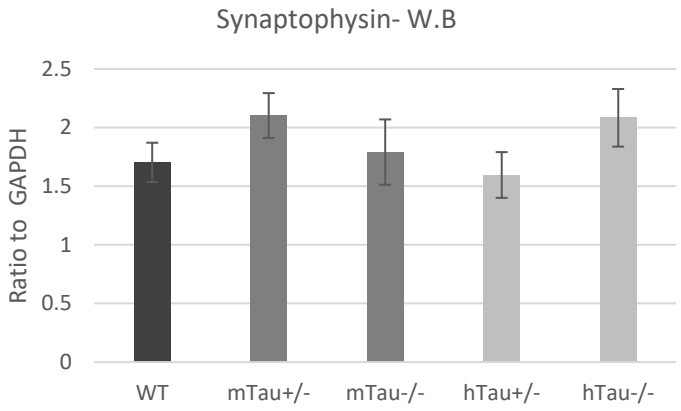
The corresponding experiment of RPPA for these samples was also done. 25ul of each sample was aliquoted into separate Eppendorf tubes. To this, 50ul of RLT buffer was added. The slide was printed and probed as earlier, as a 4-pad slide. The squares for synaptophysin and PSD95 showed weak signals, and hence a single streptavidin amplification cycle as described earlier was performed.

Results:

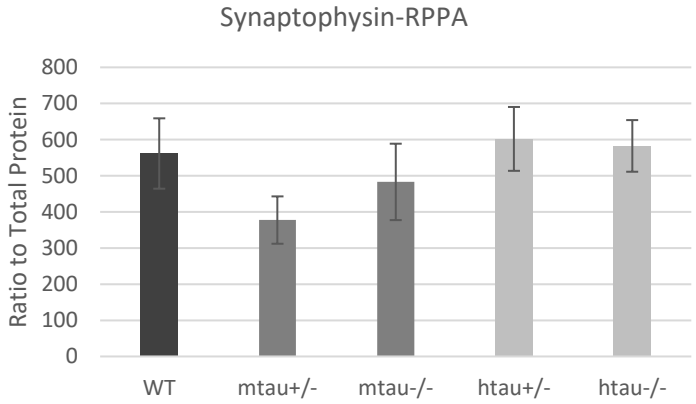
The results are given here as a comparison between western blot and RPPA for each antibody. Statistical significance is defined as p=0.05 (*), p=0.01 (**) and p=0.001 (***). Significance is noted on the graphs in the form of stars. When mentioning overall significance, p=0.05.

6.a.i. Synaptophysin:

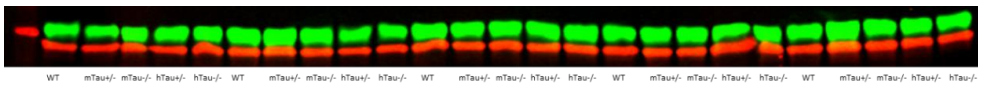
A:



B:



C:



Synaptophysin (40kD)
GAPDH (37kD)

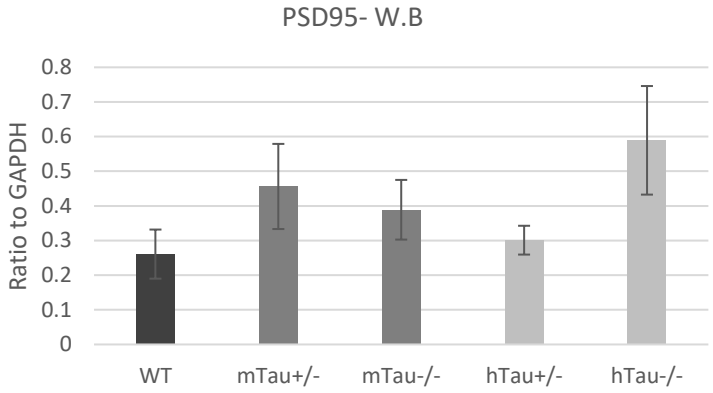
Graph 1: A: shows the protein intensities measured during western blotting experiments using synaptophysin as the primary antibody, and represented as a ratio to GAPDH. B: the intensities of protein measured using the RPPA method, as a ratio to the total protein in each of the samples. C: the western blot representation of the experiment, showing synaptophysin at 40Kd and GAPDH at 37Kd. The samples were run through the gel in a sequential order of WT, mTau+/-, mTau-/-, hTau+/- and hTau -/-.

No statistically significance difference between the sample types was seen in either of the experiments using synaptophysin as the primary antibody in a one-way ANOVA test. Some differences could be seen between the two graphs. When comparing both murine tau samples (mTau+/- with mTau-/-) between WB and RPPA, it can be seen that mTau-/- decreased in WB, it increased in the RPPA samples, however. The opposite was in effect for the hTau samples (hTau+/- with hTau-/-), in that hTau-/- increased in WB, and a decrease was seen for it in RPPA.

Overall, results between both western blot and RPPA methods showed some similarities in that the differences between the genotypes were not statistically significant.

6.a.ii. PSD95:

A:



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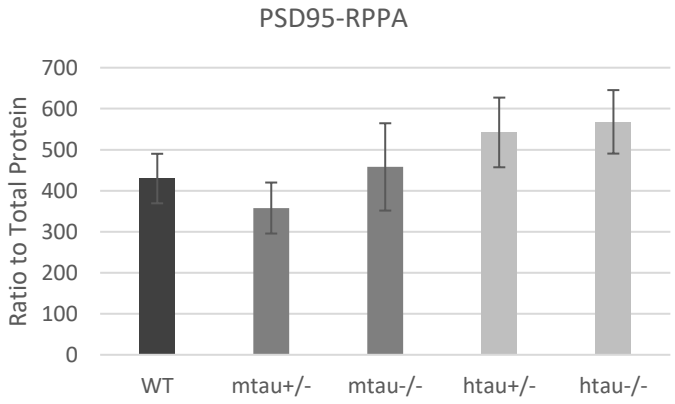

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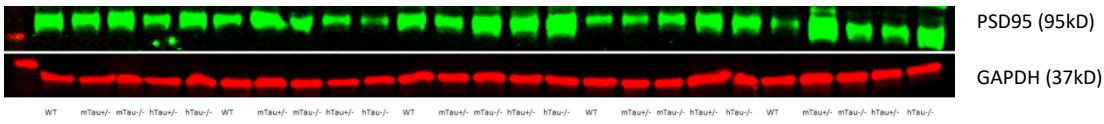


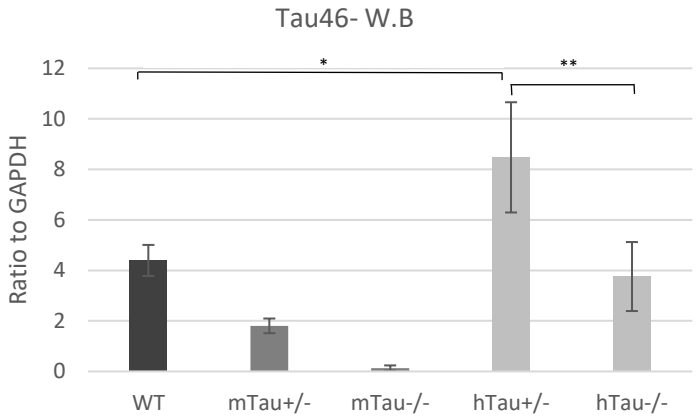
Graph 2: A: protein intensity measured using western blotting, and normalised to GAPDH, presented as a ratio to GAPDH. B: Intensity of protein measured using RPPA, as a ratio to total protein. C: the western blot representation including all five genotypes, in the order WT, mTau+/-, mTau-/-, hTau+/- and hTau-/-.

No statistical significance was seen in a one-way ANOVA test in either of the experiments. Looking at the two graphs, both the hTau samples showed similarresults between both experiments. The mTau ones, however, showed opposing results, with hTau-/- decreasing in WB and increasing in RPPA, when compared with the mTau+/-. A higher level of variability is noted in the WB graphs.

As seen in the synaptophysin experiments, the overall results were similar when comparing both techniques, however some opposition wa noted. Both were not significantly different between genotypes.

6.a.iii. Tau46:

A:



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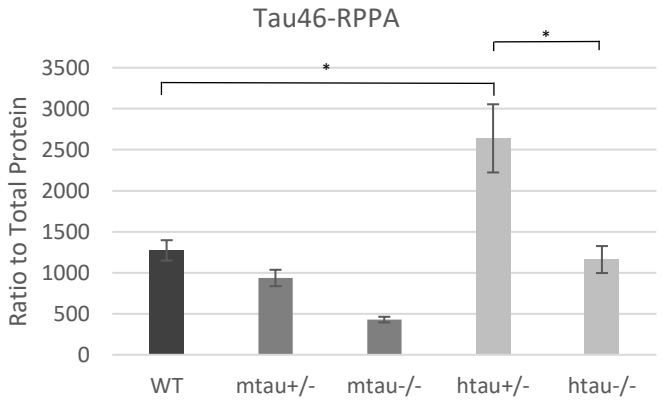
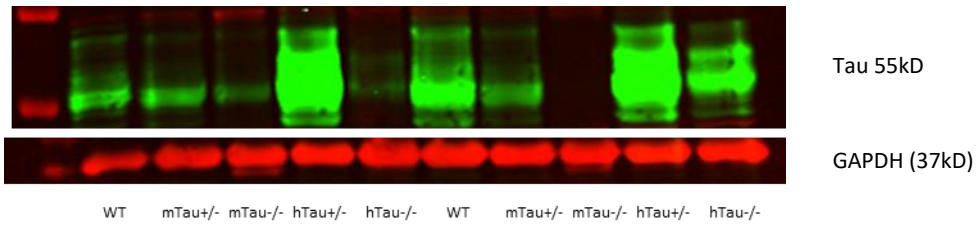


C:



Graph 3: A: shows the western blot results for protein levels represented as a ratio to GAPDH. B: the RPPA results for protein levels as a ratio to total protein. C: the western blot representation using Tau 46 as the primary antibody, represented as WT, mTau+/-, mTau-/-, hTau+/- and hTau-/-.

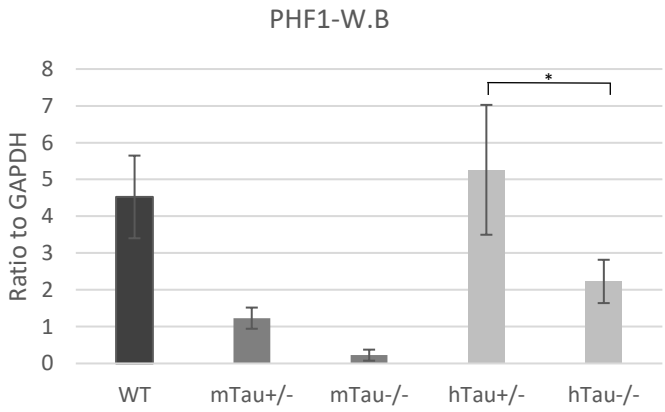
There was an overall statistical significance in the western blotting results with genotype as the factor ($p<0.05$) Pairwise comparisons showed significant differences between hTau+/- vs hTau-/- ($p<0.05$) and between WT vs hTau+/- ($p<0.05$). There was also an overall difference observed in the RPPA experiments

(p<0.05). Pairwise comparisons show significant differences between hTau+/- vs hTau-/- (p<0.05).

The similarity between the two techniques seen here was observed in all trends. There was higher variability in the results for western blotting, however, overall the two techniques were alike in results.

6.a.iv. PHF1:

A:



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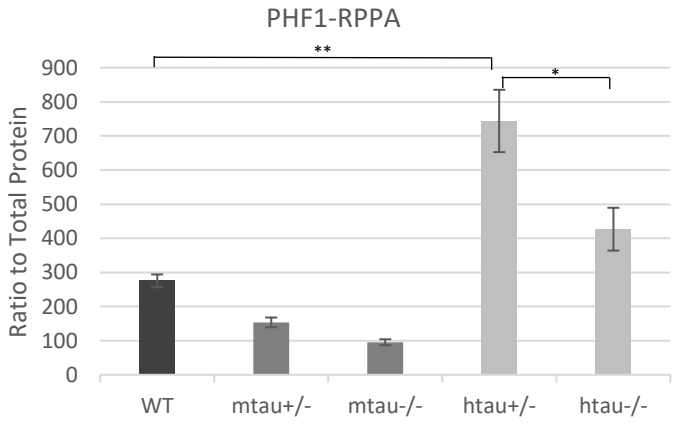
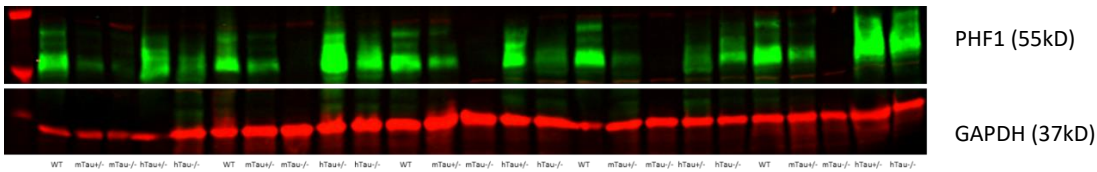


C:



Graph 4: A: the western blot results showing the protein concentration normalised to GAPDH statistical significance is seen between hTau+/- vs hTau-/- (p=0.0411) B: RPPA result with normalisation to total protein. Statistical significance is seen between hTau+/- vs hTau-/- (p=0.0271) and between WT vs hTau +/- (p= 0.0021). C: western blot representation blot showing tau (green) and GAPDH (red).

An overall significant difference was seen in the western blot results (p<0.05) between the genotypes. This was also the case for the RPPA results (p<0.001). The

same overall trends were observed in both experiments. Wild type samples showed higher levels in western blots, however, and no statistically significant difference was seen between the wild type and hTau samples in the western blots. This difference could be down to the limitations of gel to gel differences which are observed in western blots.

6.b. hTau samples:

Once results had been verified using the rest of the brain samples, a panel of hTau pathology mice was used for further validity of the method. The frontal cortex was used for each genotype

Samples used in this experiment are as follows:

Genotype-Treatment	Number of samples
WT- PBS	10
WT- LPS	10
mTau- PBS	10
mTau- LPS	10
hTau- PBS	10
hTau- LPS	9

Table 5: The samples and number of each genotype used. LPS: Lipopolysaccharides, used to induce and inflammatory state

Samples were taken from mice used in other experiments in relation to inflammatory responses seen in Alzheimer's disease. These were kindly given as troubleshooting samples by a colleague. Here LPS treatment is the inflammatory stimuli, and PBS is the control.

Western blot:

10% self-made polyacrylamide gels were used in this case. Concentrations of proteins and antibodies were as described on page 28 (6.a. Rest of the brain samples).

RPPA:

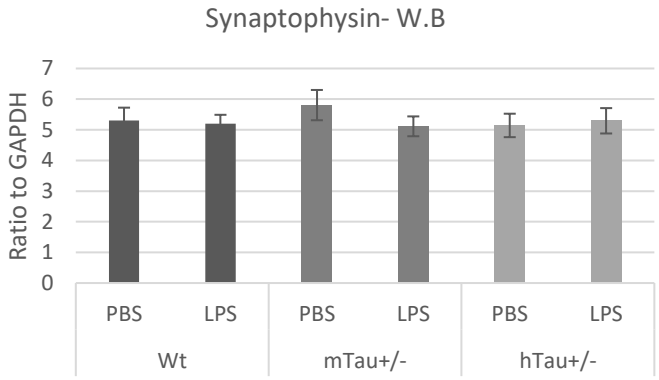
This experiment was done the same as the ROB (Rest of the brain) sample experiment. The synaptophysin and PSD95 was amplified again with one round of streptavidin amplification.

Results:

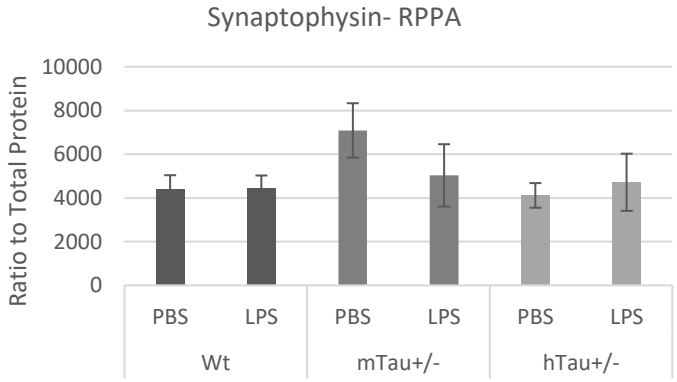
Results here are displayed as a comparison between the two methods, for each antibody.

6.b.i. Synaptophysin:

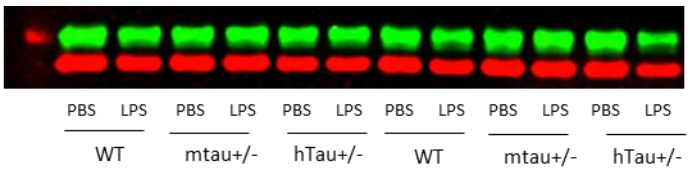
A:



B:



C:



Synaptophysin (40kD)
GAPDH (37kD)

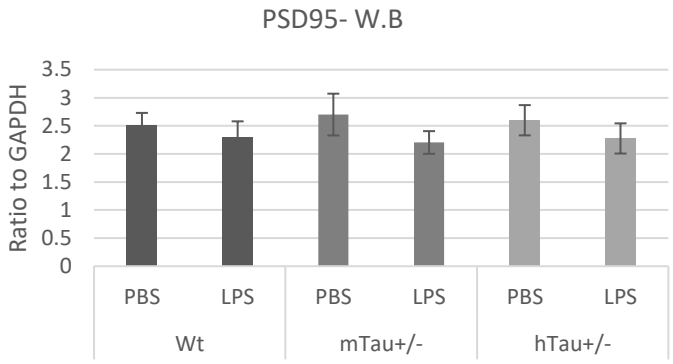
Graph 5. A: Intensity of protein as a ratio to GAPDH in WT, mTau and hTau brain samples. B: Intensity of protein as a ratio to the total protein in each of the samples in WT, mTau and hTau brain samples. C: the representative western blot, with Synaptophysin at 40kD and GAPDH at 37kD. Each genotype and treatment effect is represendted.

Similar trends could be seen between the two methods, with the wild type PBS and LPS samples generating almost equal signals, mTau+/- PBS was slightly higher than the mTau+/- LPS levels. Whereas hTau+/- LPSwais slightly higher in levels compared to the corresponding PBS samples. A slightly higher level of mTau PBS was noted in the RPPA.

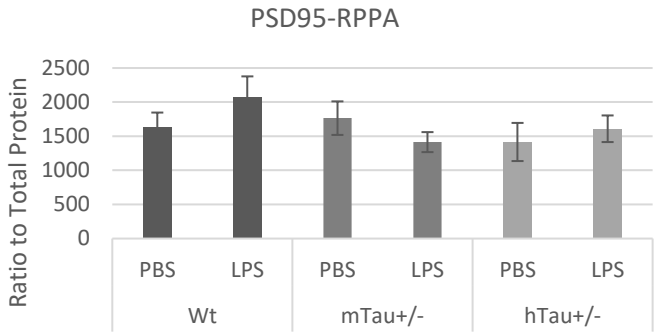
Results from the statistical analysis of western blots and RPPA done using synaptophysin as the antibody showed that there were no statistical differences between either genotype or treatment factors in either of the experiments. These results coincided with those seen in the previous set of experiments on rest of the brain samples from tau pathology mice.

6.b.ii. PSD95:

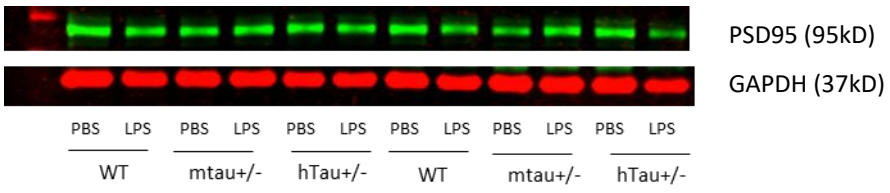
A:



B:



C:



Graph 6: A- shows the intensity measured thorough western blotting as a ratio to GAPDH. B: shows the intensity of protein measured as a ratio to total protein stain using the reverse phase protein array method. C: the western blot representation of the experiment, showing protein intensities for WT, mTau+/- and hTau+/- with both treatment factors of LPS and PBS.

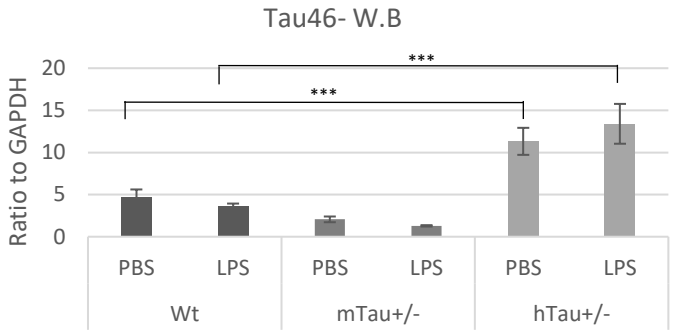
Results for the mTau for both experiments were similar in nature, with PBS samples at a higher protein level than the LPS corresponding samples. However, for WT and hTau +/- in western blotting and RPPA, results showed opposite effects, with a higher intensity being seen in LPS samples for RPPA, and a higher PBS sample intensity seen in the western blot samples.

The two-way ANOVA statistical test showed no significant difference in western blotting or in RPPA signals between either genotype or treatment factors. This parallelled the results seen in the previous experiments for rest of the brain.

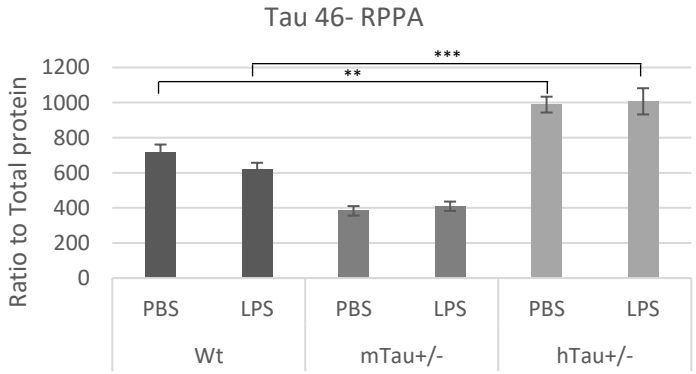
6.b.iii. Tau 46:

The western blotting showed a significant overall difference between genotypes, when tested with a two-way ANOVA test. The same results were seen in the RPPA. These can be seen in the graphs below.

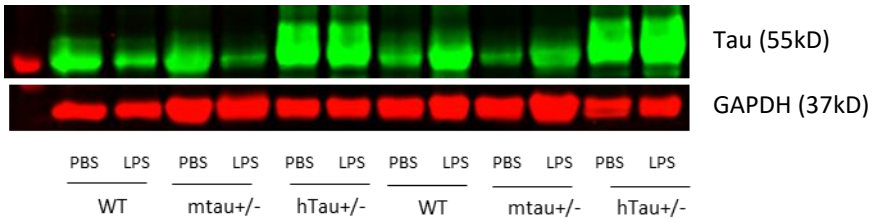
A:



B:



C:



Graph 7: A: shows the graph for the results seen in western blotting using Tau46 as the antibody and normalised to GAPDH and presented as a ratio to GAPDH. B: the graph shows the results obtained from the experiment of RPPA using Tau 46 as the antibody. The results are presented as a ratio to the total protein. Error bars are added showing the standard error of the mean (SEM). Statistical significance is depicted on the graph by adding stars whereby **=p<0.01 and ***=p<0.001.

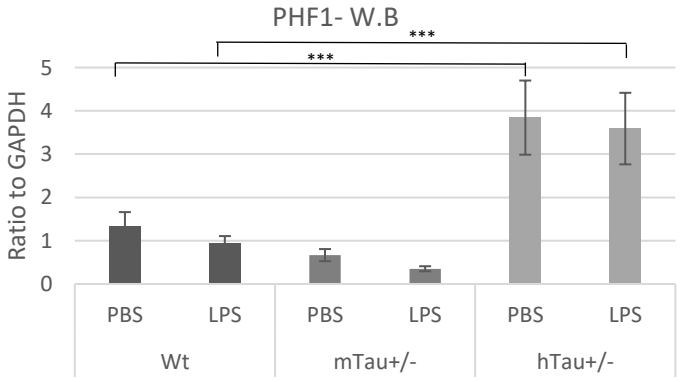
The trends on both graphs overall are similar. A higher variability is observed in western blotting as compared with the RPPA.

Pairwise comparisons were seen for results from the western blots. Statistical significance was seen between WT LPS vs hTau LPS ($p<0.001$), and is depicted in the graph. Significant differences ($p<0.001$) were also seen between WT PBS vs hTau PBS. Other pairwise comparisons which showed statistical significance included mTau LPS vs hTau LPS ($p<0.001$) and mTau PBS vs hTau PBS ($p<0.001$). No statistical significance was seen in treatment groups.

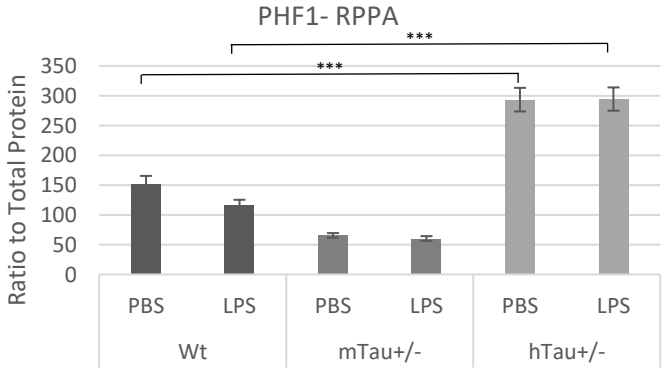
Pairwise comparisons seen for the RPPA set of results showed statistical significance between mTau+/- LPS vs hTau+/- LPS ($p<0.001$) and mTau PBS vs hTau PBS (p0.001). No significant differences were seen in pairwise comparisons for differences in treatments.

6.b.iv. PHF1:

A:



B:



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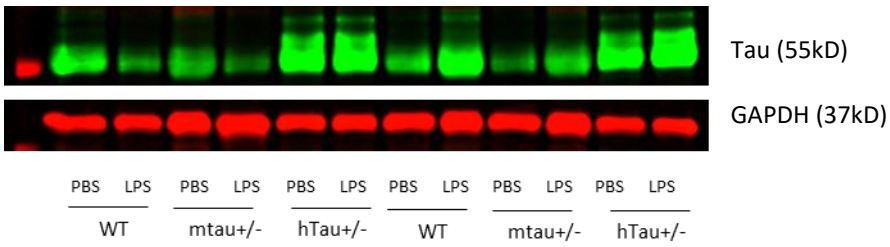

Graph 8: A: shows the results obtained from western blotting using PHF1 as the primary antibody. Samples have been normalised to GAPDH and represented as a ratio to GAPDH. Statistical significance is seen between WT PBS vs hTau PBS ($p<0.001$) and WT LPS vs hTau LPS ($p<0.05$). B: the graph shows the results obtained from RPPA using PHF1 as the antibody, to detect the phosphorylated tau in the samples. Statistical significance is shown by use of stars between WT PBS vs hTau PBS ($p<0.001$) and WT LPS vs hTau LPS ($p<0.001$). C: the western blot representation of the experiment, seen with GAPDH (red) and phosphorylated tau (green).

The overall trends seen in both graphs (figure 8A and 8B), showes similarity in all genotypes and treatment effects. A higher variability was seen in the western blotting results as compared to the RPPA, in particular for results seen in hTau+/-.

An overall statistical significance was found between genotypes in PHF1 for both western blot and RPPA. Pairwise comparisons for western blotting show that there were statistically significant differences between mTau LPS vs hTau LPS ($p=0.0001$) and mTau PBS vs hTau PBS ($p<0.001$).

Pairwise comparisons were carried out between the different genotypes and treatments administered in the reverse phase protein array experiments. Statistical significance was seen between mTau+/- LPS vs hTau+/- LPS ($p<0.001$) and mTau+/- PBS vs hTau+/- PBS ($p<0.001$).

6.c. Discussion

No statistical significance was seen between genotypes or treatment groups when measuring synaptophysin in either of the experiments. This means there were no differences in the pre-synapses for this set of samples. No significance was noted in the post-synaptic density either (PSD95).

Statistical significance was observed between genotypes in both western blotting and RPPA in the total amount of tau in these samples. This was expected, as the mice were those of tau pathology, and hence an increase in tau should have been seen for the hTau mice. This was also the case for phosphorylated tau for both western blotting and RPPA when looking at the genotype. However, the mice which were tau knockout (mTau-/-), should have shown no tau, however did show low levels of tau. This could be due to minor non-specific binding of the antibody.

Overall, there was lower variability in the RPPA experiments as compared to the western blots. This could be due to the presence of gel to gel differences which are often seen in western blot results. There was also variability in the type of gel used for the experiments, including self-made smaller gels, and larger pre-made gels. This is another factor which could introduce variability between experiments in to the experiment.

Where an increase in protein intensity was seen for RPPA not reciprocated in western blot (e.g. Tau46 for hTau sample experiment), this could have been due to the nature of the RPPA method. For RPPA, the intensity observed cannot be differentiated by molecular weight. Only the complete intensity of protein is seen as a single dot. If there was hence any cross-reactivity of the antibody, it would not be seen as a separate signal, rather as a combined whole signal.

There could have also been varying the post synaptic density in the two experiments, which again could have been due to unresolved background or non-specific binding.

7. Discussion

7.a. Aims and objectives:

The main aims and objectives of this project, were to be able to develop a method in which multiple Alzheimer's disease biomarkers could be identified and probed in a simultaneous manner, and with the ability to use numerous different sample types for this purpose.

Within the work of this project, we were able to develop this method with the use of micro-arrays. Within the time frame of the project, 4 antibodies were validated using western blotting techniques, and later used in reverse phase protein micro-arrays, to print on single microscope slides. Hence it can be said that the aims and objectives of the study were achieved. Using tau pathology mouse brain samples of the frontal cortex, it was seen that tau antibodies showed significant differences when comparing wild type and tau pathology brains. The optimisation steps included the use of a new technology in brain tissue, which was considered a fairly novel and new method.

7.b. Current work using RPPAs

Currently, reverse phase protein arrays are being used for a wide range of purposes in research. This is particularly relevant for research in cancer, in order to evaluate the molecular level genomic makeup of difference tumours in order to facilitate new therapeutic approaches (Zhao, Li and B. Mills, 2017). Significant progress has been made in the understanding of breast cancer (Ramirez-Ardila et al., 2017) activation pathways in different proteins, with the use of RPPA technology. Other than this, work has been done in understanding the characterization of lower grade gliomas (Lehrer et al., 2017), small-cell lung cancer (Sen et al., 2017), and pancreatic ductal carcinoma (J. Raphael, 2017), all with the help of RPPAs. However, to our knowledge, this is the first study to use the RPPA method to successfully detect Alzheimer's disease proteins using mice brain tissue.

RPPAs provide a quick and easy method for diagnosis of Alzheimer's disease in those patients who are suspected of having it. This could be done in a non-subjective manner. Currently diagnosis is only being made when a number of different factors show in the patients. These include the mental state of the patient, which is something that may be subjective to the clinician performing the exam. Others include tests such as lumbar puncture, which in particular for the age group associated with AD, could be considered invasive and uncomfortable.

In the future, RPPA technology could be extended for use with clinical practice in personalizing medicine or for use in diagnostics (Huang and Creighton, 2015). RPPA technology provides the ability to analyse specific proteins from disease pathways. This could enable further understanding of Alzheimer's disease pathways and hence in turn provide access to better diagnostic abilities as well as therapeutic potentials.

7.c. Limitations with western blotting:

Some discrepancies are seen between results from western blot and RPPA. This could be due to certain limitation factors which play a part when western blots are performed. Western blotting can only be done on a limited number of samples at the same time. This means that if there are more than 10-15 samples, multiple blots will have to be done. By doing this, an introduction of blot to blot or gel to gel differences comes in to play. This can provide variances between blots for the same antibody, increasing the standards of error. This is something which is not seen in RPPA, as all samples are spotted onto the same blot.

Another limitation linked with western blots is the fact that most of the technique is done by hand. For example, the loading of the samples onto the gel is done manually, and this could mean an inclusion of human error in amount of sample added. This is not the case with RPPA, as printing is done by the microarrayer, meaning all proteins are exactly the same size and hence identical protein concentration. It makes for more accurate results in RPPA, something which is also proven in the results for this project.

To avoid these drawbacks in the future, it could be possible to use larger gels in general, resulting in the decrease in variability for the gel to gel differences, as less gels would be used. It would also mean a consistency in all western blots.

7.d. RPPA as a technique; limitations and advantages:

The advantage of using western blots however is that any cross-reactivity to other proteins can be readily seen, and not added in any calculations. This is not possible for RPPA, as it is seen as a single entity. Therefore, the step of antibody validation is very important. Which makes the use of RPPA limited. However, once the antibody has been validated, it makes for the RPPA technique to be highly reliable and accurate technique.

For small protein targets such as Aβ, the RPPA method may be more beneficial as compared to western blot. This is because there would not be any possibility of the protein from running off the gel, or any limitations linked with its size. It would be possible to view the protein as an intensity as has been done for the others.

7.e. Amyloid beta; negative results in western blotting

Several optimisation western blots were done using amyloid beta-42 antibody. However, these were not met with success. This could be down to the fact that Aβ is an extremely small protein (Aβ- 5kD), and is passing through the running gel too quickly, resulting in loss of the protein from the gel. The 50kD bands seen in the western blots could be aggregated amyloid beta, which was not able to fully resolve

during the boiling process. Aβ is an extremely difficult protein to detect via western blotting, and further work is required in this area to optimise results.

7.f. Future direction:

In the future, this work could be expanded to include further biomarkers of Alzheimer's disease pathology, including those such as amyloid beta which could not be validated in this project. Other biomarkers include those which are linked to inflammation in AD.

8. Conclusions

Alzheimer's disease is an incredibly debilitating disease, which has clear biomarkers for disease presence and progression, namely amyloid beta plaques and phosphorylated tau tangles. In this project, we have been able to correctly validate four antibodies which are synaptophysin, PSD95, Tau46 and PHF1, and use these in reverse phase protein arrays in order to map out a panel of biomarkers on Alzheimer's disease pathology samples. The aim at the start of this project of developing such a method has been fulfilled.

The work done within this project lays down a foundation of work which has the potential for further expansion to take place within it. RPPA technology is fast growing, and has proven to be reliable, quicker and more efficient when compared with other conventional methods of protein detection. In terms of quantitative analysis, much time could be saved allowing for more rapid progress to be made in this field.

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