Modulatory effect of mild inflammation on tau phosphorylation in a human tau mouse model of Alzheimer's disease

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Abstract:

Alzheimer's disease (AD) is the most common form of dementia and involves the pathological hyperphosphorylation and aggregation of the microtubule associated protein tau. Aetiological evidence, and evidence from post-mortem studies, suggests neuroinflammation to be an early mechanistic driver of AD. In murine tau models, systemic inflammation induced by either chronic or acute lipopolysaccharide (LPS) administration has been reported to be a strong inducer of early tau pathology through augmenting tau hyperphosphorylation. However, the method by which LPS has been utilised to model inflammation in these studies has not always been representative of the underlying mild inflammation occurring in AD. For example, following chronic LPS administration, rapid tolerance occurs which is not representative of AD. While acute LPS administration results in a rapid pro-inflammatory response, the doses which have generally been utilised are more analogous with sepsis rather than the underlying inflammatory response observed in AD. This thesis is aimed at furthering clarifying the contribution of systemic inflammation to early tau pathology through systemic administration of low doses of LPS in the hTau model. The hTau model is thought to be the most relevant AD tau model for the expression of all 6, non-mutated, human tau isoforms on a murine tau (mTau) knockout (KO) background. However, hTau mice are associated with systemic pathologies and an increased ratio of 3R:4R tau isoforms which is not representative of AD. As the systemic pathologies are linked to the KO of mTau which consists solely of 4R tau isoforms, hTau mice were bred on a partial mTau background in the hope to avert the systemic pathologies, improve the isoform ratio and conserve the development of tau pathology. Heterozygous mTau expression in hTau/mTau$^{+/-}$ mice resulted in ablation of systemic pathologies, an increase in 4R tau isoforms and augmented tau hyperphosphorylation compared to hTau/mTau$^{-/-}$ mice, indicating the model ideal for understanding early pathological tau alterations. To determine the effect mild inflammation might have on early tau pathology in AD, tau phosphorylation, localisation and aggregation were assessed after 0, 100, 250 and 330 µg/kg (*i.v.*) LPS administration in 3 month old hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice during the height of the pro-inflammatory response at 4h following administration. LPS administration resulted in dose-dependent decreases in the pre-tangle associated phosphorylation epitope; pS202 in both genotypes while the post-tangle associated epitope; pS396/404 levels were selectively decreased in hTau/mTau$^{+/-}$ mice. On the other hand, LPS did not induce tau aggregation. To determine whether less immediate effects on tau pathology occurred, tau pathology was assessed 24h following 250 µg/kg (*i.v.*) LPS administration in hTau/mTau$^{+/-}$ mice. Tau

dephosphorylation persisting at both pS202 and pS396/404 epitopes at this time point. These results suggest that systemic inflammation might play a beneficial role on tau pathology in AD.

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Contents

Chapter 1 General introduction:

1.1 Alzheimer's disease overview:

Dementia, a collective term for a group of neurodegenerative disorders, affects up to 850,000 people in the UK, costing an estimated £24 billion (ARUK, 2014). Alzheimer's disease (AD) is the most common form of dementia and is characterised by the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs). Currently, there is no effective treatment at slowing disease progression. AD is a multi-faceted disease involving severe brain atrophy leading to diminished cognitive function, impaired learning and memory, delusions and a wide range of other behavioural abnormalities (Mega et al., 1996). There are two forms of AD: early onset familial AD and late onset sporadic AD, the latter of which is the more prevalent of the two. While specific genetic mutations which are involved in the generation of the Aβ peptide are associated with familial forms of AD, the pathogenesis of sporadic AD is much more complex (Prince et al., 2015). Importantly, AD is a progressive disorder giving a therapeutic window in which disease progression could be slowed. Existing treatments for the disease have poor efficacy, providing only limited symptomatic benefits. Current focus is to further the pathogenic understanding of the disease to aid in the discovery of so called "disease modifying therapies". Delaying the onset of the disease by just 5 years could reduce the number of sufferers by 36% and save the UK economy over £14 billion annually by 2030 (ARUK, 2014). Recently, systemic inflammation has been suggested to be integral to AD pathogenesis and modulating inflammatory processes in the disease might provide an effective disease modifying therapy within the disease.

1.1.1 Pathological features of Alzheimer's disease:

AD is associated with specific pathological signatures, with amyloid plaques and NFTs considered hallmarks of the disease. Amyloid plaques are generated through the pathological extracellular aggregation of the amyloid beta Aβ peptide. NFTs are produced through the pathological intracellular accumulation of the microtubule associated protein tau. A histological slide depicting amyloid plaques and NFTs is given in Figure 1.1. While both amyloid and tau deposition likely do contribute to disease progression, recently tau has received greater emphasis in the pathogenesis of AD.

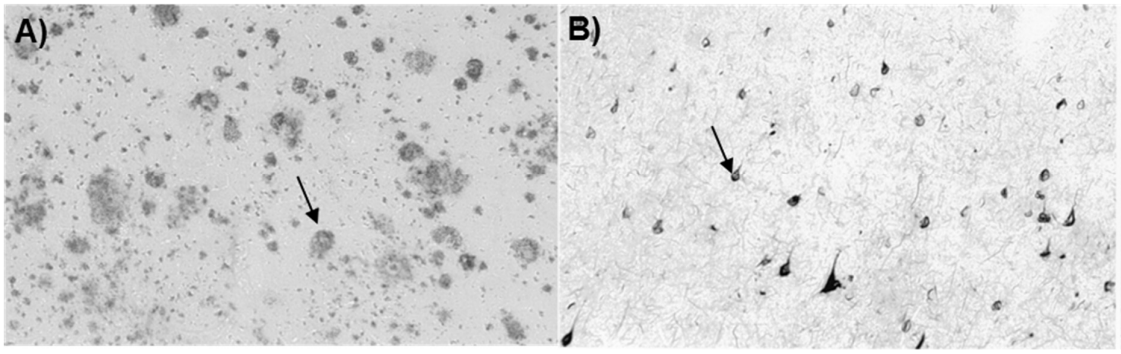

*Figure 1.1: Hallmarks of AD: **A)** Staining of amyloid plaques using anti-Aβ$_{42}$. Amyloid plaques are extracellular deposits of Aβ **B)** Staining of intracellular NFTs with the PHF antibody (Cummings et al., 2002).*

AD is a neurodegenerative disorder featuring overt brain atrophy. AD patients have up to 90% fewer neurons in the entorhinal cortex compared to healthy individuals (Gómez-Isla et al., 1996). A consequence of the neurodegeneration is a shrinking of cortical thickness and widening of the ventricles compared to non-demented individuals (Querbes et al., 2009) as observed in Figure 1.2. In addition to neuronal loss in AD, there are large decreases in the number of synapses which cannot be attributed to sheer neuronal loss alone (Davies et al., 1987). The pattern of pathology is localised to the limbic, neocortical and basal forebrain regions (Braak and Braak, 1991, Auld et al., 2002), with both cholinergic and glutamatergic projections being heavily affected (Francis, 2005).

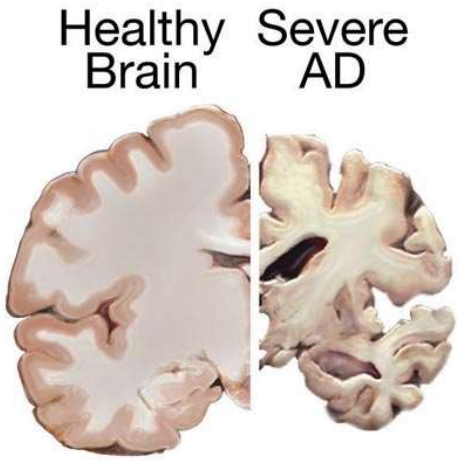

Figure 1.2: A comparison between a healthy brain and that from a severe AD patient. Picture sourced from the National Institute of Health (NIH).

1.1.2 Current treatment strategies in Alzheimer's disease

There are four licenced treatments available for AD: donepizil, rivastigmine, galantamine and memantine – all of which provide symptomatic benefit and fail to alter

progression of the disease (Doody et al., 2001, Raina et al., 2008). Donepezil, rivastigmine and galantamine are all classified as anti-cholinesterases. Acetylcholine (ACh) is a major excitatory neurotransmitter within the CNS, being heavily involved in learning and memory (Hasselmo, 2006). Upon being released into the synaptic cleft, ACh is rapidly metabolised by a group of enzymes called acetylcholinesterases (AChE) (Čolović et al., 2013). Profound loss of cholinergic neurons in AD means disruption of cholinergic signalling, affecting learning and memory processes (Francis et al., 1999). By inhibiting AChE, anti-cholinesterases increase the concentration of ACh at the muscarinic ACh receptor (mAChR), boosting signal transduction in an attempt to minimise the effect of cholinergic loss (Lane et al., 2006).

Memantine is the newest addition to the licenced treatments and is prescribed in moderate to severe AD cases. Memantine is a non-competitive N-methyl-D-aspartate receptor (NMDAr) antagonist (el Nasr et al., 1990). Unlike ACh, glutamate does not get metabolised in the synaptic cleft. Instead decreasing glutamate concentrations following signal transduction relies on uptake into neighbouring astrocytes where metabolism occurs (Anderson and Swanson, 2000). In AD, the propensity for astrocytes to uptake and store glutamate decreases, causing an excess of glutamate in the synaptic cleft (Lauderback et al., 2001, Kulijewicz-Nawrot et al., 2013). The increase in residual glutamate has two effects in AD. Firstly, increases in background signalling noise make glutamatergic transmission less efficient and impair signal transduction (Danysz and Parsons, 2012). Secondly, in a similar manner to stroke, excess glutamate induces excitotoxicity, aiding in the neurodegenerative process (Dodd et al., 1994). Through NMDAr antagonism, memantine is thought to block both detrimental effects of glutamate (Danysz et al., 2000). A depiction of the current treatments in AD is given in Figure 1.3.

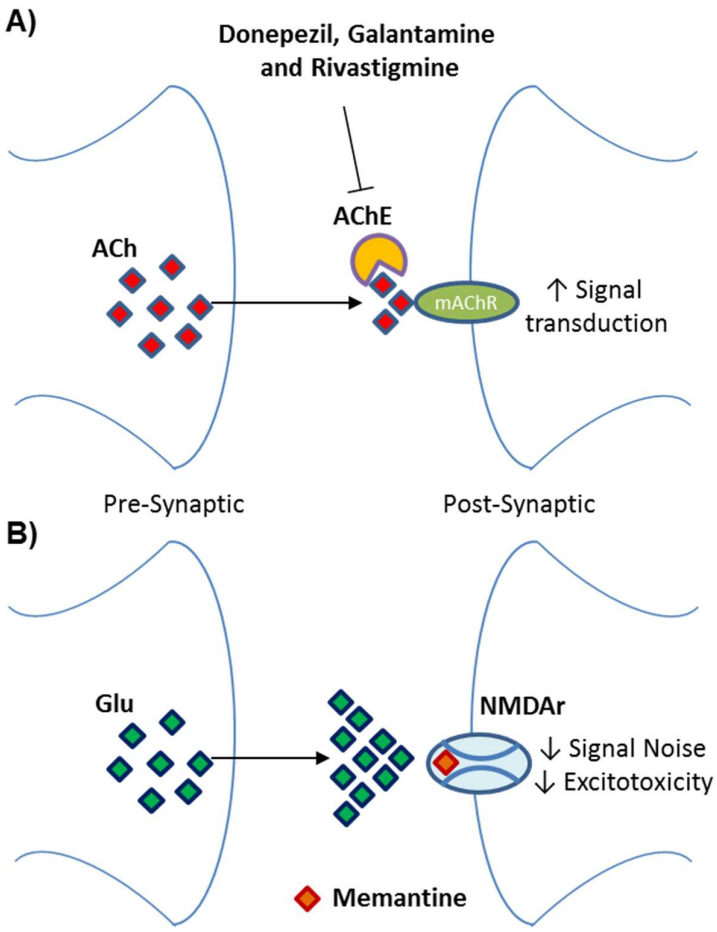

*Figure 1.3: Mechanism of action of current AD therapies. **A)** Donepezil, Galantamine and Rivastigmine are AChE inhibitors which increase the synaptic concentration of ACh and boost cholinergic signalling. **B)** Memantine is an NMDAr antagonist and decreases signal noise and excitotoxicity associated with increased levels of glutamate (Glu) in the synaptic cleft in AD. Figure drawn by author.*

1.1.3 Inflammation is associated with Alzheimer's disease

Neuroinflammation:

Chronic neuroinflammation – that is inflammation originating from within the central nervous system (CNS) is another overt feature of AD. Even in his early observations did Alois Alzheimer note that resident immune cells were in a state of reactive gliosis while conducting post-mortem examinations from AD patients (Strassnig and Ganguli, 2005). This observation was reinforced by immunohistological (IHC) studies in subsequent years that confirmed two resident immune cells within the CNS: microglia and astrocytes exist in a persistent state of activation (Pike et al., 1995, McGeer et al., 1987). Of even more importance, was the finding that activated microglia and astrocytes are found in close proximity to both amyloid plaques and NFT containing neurons (Itagaki et al., 1989, Serrano-Pozo et al., 2011, Cras et al., 1991), indicating an interaction between the hallmarks of the

disease and the neuroinflammatory environment. Accompanying glial activation, a wide range of pro-inflammatory mediators including IL-1β, IL-6, TNFα as well as prostaglandins have been found to have upregulated mRNA and protein levels in brains from AD patients (Morimoto et al., 2011, Yasojima et al., 1999b, Rao et al., 2011). However, due to the poor quality of post-mortem tissue, the association of pro-inflammatory mediators with brain tissue from AD patients is often conflicting (Lopez-Gonzalez et al., 2015, Chen et al., 2016) and hinders accurate interpretation. More recently, development of positron emission topography (PET) tracers to image microglial activation have provided valuable insight into the timeframe of the neuroinflammatory response during AD. For example, use of the tracer: [^{11}C](R)-PK11195 found the pattern of microglial activation, occurs early in the disease (Cagnin et al., 2001) and can even be detected in mild cognitive impaired (MCI) patients (Okello et al., 2009). Furthermore, use of the more specific [^{11}C]-PBR28 compound found microglial activation correlated with progression of the disease (Kreisl et al., 2016). Interestingly, early onset AD patients have higher uptake of [^{11}C]-PBR28 compared to late onset patients (Kreisl et al., 2013). Together, these studies suggest that neuroinflammation is an early event within AD and that it may play a role in driving the disease.

A key debate within the AD field is whether neuroinflammation is a cause or consequence of the disease – is it contributing to the disease or aimed at repair. Supporting the former, a variety of both environmental and genetic risk factors affecting the neuroinflammatory environment have been associated with AD. For example, lipopolysaccharide (LPS) is a component of gram –ve outer membrane and a potent activator of the innate immune system through Toll-like Receptor 4 (TLR-4) agonism (Chow et al., 1999). Under healthy conditions, LPS is endogenously located in the gut where there is an abundance of gram –ve bacteria (Marshall, 2005). However, a link between the microbiome, a “leaky gut” and AD has been suggested (Cristiano et al., 2016). While LPS is absent within the CNS of healthy individuals, it is found localised around plaques of AD patients, potentially highlighting LPS as an endogenous driver of the disease (Zhan et al., 2016). Furthermore, the herpes simplex virus (HSV) which affects the CNS has been associated with increased incidence of AD (Itzhaki et al., 1997), indicating long term neuroinflammation as a risk factor. Likewise, head trauma, which can be associated with chronic neuroinflammation (Breunig et al., 2013), has been found to increase the risk of AD (Mortimer et al., 1991, Li et al., 2017).

Categorically strengthening the notion of neuroinflammation being a causative factor within AD has been the use of recent genome wide association studies (GWAS) in AD. These studies have found polymorphisms in a number of genes involved in inflammation and microglial function to be associated with increased risk of developing AD. The most high profile of which is the gene encoding for triggering receptor expressed on myeloid cells 2 (TREM2) whereby the rare loss of-function missense mutation: rs75932628-T conferred to a 3 times greater risk of developing AD, a similar odds ratio to possessing a single allele of the so far strongest genetic risk factor in sporadic AD: the Apolipoprotein E (ApoE) ε4 allele (Jonsson et al., 2013, Ulrich et al., 2017). TREM2 is expressed on macrophages and microglial cells and its activation is thought to inhibit pro-inflammatory signals while stimulating phagocytic microglial responses (Colonna and Wang, 2016). Several additional inflammatory associated genes have been associated with AD including; CR1, CD33, CLU, EPHA1, HLA-DRB5/HLA-DRB1, the MS4A cluster and PLXNA4 genes albeit with lower risk (Lambert et al., 2013, Naj et al., 2011, Jun et al., 2014). CR1 encodes the complement receptor 1 which modulates the complement cascade through binding and phagocytosis of C3b immune complexes (Crehan et al., 2012). CD33 is a transmembrane sialic-acid-binding immunoglobulin-like lectins (SIGLECS) which produces anti-inflammatory action through inhibition of TLR signalling (Cao and Crocker, 2011). CLU encodes for clusterin, which has been shown to inhibit the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) resulting in decreased transcription of pro-inflammatory mediators (Santilli et al., 2003). EPHA1 encodes for ephrin type-A receptor 1 and is involved in leukocyte migration during infection (Ivanov and Romanovsky, 2006). The MS4A cluster encodes for the family of membrane-spanning 4A proteins which have roles in T-cell activation and trafficking across the blood brain barrier (BBB) (Ma et al., 2015). The PLXNA4 gene encodes for plexin-A4, a receptor for semaphorins, which aids in TLR signalling (Wen et al., 2010). Finally, GWAS studies have implicated genes involved in microglial phagocytosis including such as ABCA7, INPP5D and PICALM1 in AD aetiology (Lambert et al., 2013, Malik et al., 2015).

Systemic inflammation:

Despite initial assumptions that the CNS was immune privileged, there is crosstalk between the central and peripheral immune systems. This effect is mediated through a variety of routes including: neural such as vagal afferents, humoral through circumventricular organs, through effects on the BBB (Miller and Raison, 2016, Pardon, 2015). As such, it is not surprisingly that systemic inflammation which is associated with AD

has been implicated with disease progression. Unlike brain tissue from post-mortem studies, peripheral inflammatory mediators can be measured accurately in blood samples from AD patients. A meta-analysis of 40 different studies assessing peripheral cytokine concentrations in AD patients found increased concentrations of a number of cytokines in AD patients, including that for IL-1β, IL-6 and TNFα (Swardfager et al., 2010). Similar to neuroinflammation, upregulation of pro-inflammatory mediators in the periphery occurs during the MCI stage of the disease (King et al., 2017, Magaki et al., 2007) and progressively declines as AD severity increases (King et al., 2017, Motta et al., 2007). Potentially contributing to the underlying systemic inflammation in AD, elevated concentrations of circulating LPS have been observed in AD patients compared to healthy controls despite being infection free at the time of the study (Zhang et al., 2009c).

Corroborating a potential role for systemic inflammation in AD, conditions which induce systemic inflammation have also been associated with the disease. For example, individuals suffering from long-term peripheral atopic disorders such as asthma and eczema have an increased risk of developing AD (Chen et al., 2014). Conversely, rheumatoid arthritis (RA) has generally been associated with a decreased risk of AD – likely due to their long term use of anti-inflammatory agents (Policicchio et al., 2017). Supporting this, both long term use of NSAIDS and steroidal anti-inflammatory treatments in arthritis patients have been linked with a decreased incidence of the disease (McGeer et al., 1996). Furthermore, obesity, diabetes mellitus and smoking – conditions which are associated with increased systemic inflammation increase the risk of developing AD (Kivipelto et al., 2001, Kivipelto et al., 2005, Ott et al., 1998, Arvanitakis et al., 2004). In addition to systemic inflammatory conditions, systemic infections are associated with cognitive decline in AD patients. For example, AD patients who suffer from a systemic infection develop cognitive impairments lasting over 2 months following resolution of the infection (Holmes et al., 2003). A further study found that AD patients who had an acute systemic inflammatory event (infection or trauma) were found to have greater rate of cognitive decline over a 6 month period (Holmes et al., 2009). Interestingly, the authors report that patients which had a high baseline of circulating TNFα prior to the inflammatory event had an even greater rate of cognitive decline, indicating the level of systemic inflammation impacts on cognitive decline. Further, studies have found a link between periodontitis and cognitive decline in AD patients (Ide et al., 2016) while increased titres of common periodontal bacteria is associated with increased risk of AD (Noble et al., 2014). Together, these studies highlight an association between systemic

inflammation and AD aetiology, highlighting systemic inflammation as a potential therapeutic target.

Randomized controlled anti-inflammatory trials within AD

The association between both systemic and neuroinflammation with AD led to the notion that anti-inflammatory treatment in AD may provide a much needed disease modifying treatment. To date, several randomised trials with anti-inflammatory agents have been conducted in AD. Despite the use of multiple classes of anti-inflammatory treatments – whether non-steroidal anti-inflammatory drugs (NSAID) or steroidal; all have failed to demonstrate clear clinical efficacy (Jaturapatporn et al., 2012). However, a recent updated systematic review still argues in favour of their use for AD prevention (Wang et al., 2015a). As AD pathogenesis develops years prior to symptom manifestation, the authors suggest that anti-inflammatory agents are potentially beneficial when administered prodromal. However, the largest prophylaxis anti-inflammatory trial involving the use of NSAIDs on subjects at risk of developing AD failed to show benefits on AD incidence (Breitner J, 2013). The role of inflammation in AD is complex, providing both positive and negative effects such as clearance of debris and exacerbation of both amyloid and tau pathologies. The failures of anti-inflammatory treatments in AD could be down to non-selectively trying to dampen the immune system rather than modulate it (Heneka et al., 2016). Further research has the potential to further understand how the immune system impacts the disease and determine its therapeutic potential.

1.1.4 Summary

AD is a neurodegenerative disorder for which there are limited effective treatments. There are two hallmarks: amyloid plaques and NFTs. Extensive neuroinflammation is another apparent feature within the brain, with reactive immune cells in close proximity to NFT containing neurons and suggests an interaction between the neuroinflammatory environment and tau pathology. AD patients have increased levels of systemic inflammation which has the potential to contribute to neuroinflammation within the disease and drive the pathological progression of NFTs. This interaction will be reviewed in detail further on within this chapter. Several peripheral inflammatory conditions have been reported to either increase the risk of AD or increase the rate of cognitive decline suggesting that treating systemic inflammation might be of therapeutic potential within AD. Despite this, all randomized controlled trials have failed in AD. Inflammatory processes are complex and the non-selective nature of the agents used in these trails could possibly contribute to their

failings. Investigating the interaction between tau pathology and inflammation could provide useful insights into specific inflammatory processes which affect AD progression.

1.2 Tau pathology and its association with AD

1.2.1 Tau pathology closely correlates with Alzheimer's disease:

In recent years, tau has received a considerable amount of attention due to its correlation with AD progression. Histopathological examination of AD patients appears to highlight tau as the pre-requisite to developing AD (Schonheit et al., 2004). Furthermore, AD severity is graded through IHC BRAAK staging – a process which maps the spread of tau pathology (Braak et al., 2006). Recent PET studies have corroborated the role of tau as mechanistic driver of AD. For example, assessment with the amyloid and tau tracers: florbetapir and T807 respectively, found tau but not amyloid deposition increased with clinical diagnosis of AD (Brier et al., 2016). Furthermore, T807 retention actually appear to predict severity of clinical manifestations (Schwarz et al., 2016). T807 has a high selectivity for PHF tau over straight filament tau and other aggregated protein such as amyloid plaques (Marquie et al., 2015). However, the authors did report non-specific binding to neuromelanin- and melanin-containing cells resulting in binding in the eyes, midbrain and basal ganglia meaning T807 binding in these regions are unreliable. However, a subsequent report found non-specific binding of T807 to monoamine oxidase (Hostetler et al., 2016) although the initial report failed to find monoamine oxidase binding (Xia et al., 2013). As monoamine oxidase is ubiquitously expressed in the brain (Bogdanski et al., 1957), this could hinder the interpretation of data involving T807. Finally, while there are no mutations in the tau gene which have been associated with AD (Kwon et al., 2000), several tau mutations which promote its aggregation are associated with various forms of dementias where tau deposition is the only pathological feature (Hutton et al., 1998, Houlden et al., 1999). Altogether, these observations highlight tau aggregation as a potential driver of AD.

1.2.2 The structure of the tau protein:

In humans, full length tau is a 441aa protein which exists natively in a unfolded state and consists of 4 microtubule binding repeats and 2 n-terminal inserts (Gamblin, 2005). Depending on its alternative splicing, tau exists in 6 isoforms which differ in the number of n-terminal inserts and whether they possess 3 or 4 microtubule binding repeats so that: 0N3R, 0N4R, 1N3R, 1N4R, 2N3R and 2N4R tau isoforms exist (Goedert et al., 1989). Generally, these are classed into 3R and 4R tau isoforms depending on the number of microtubule

binding repeats they possess. Figure 1.4 represents the full length structure of the tau protein.

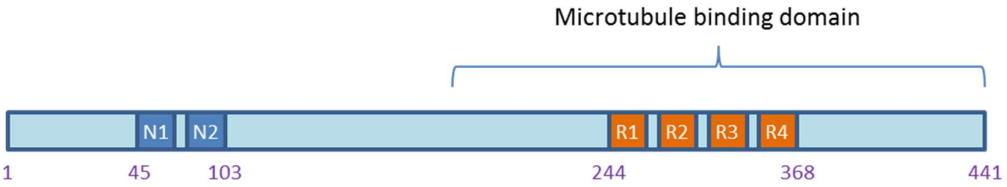

Figure 1.4: The longest isoform of Tau is a 441aa protein, consisting of two N-terminal repeats and 4 microtubule binding domains. Figure adapted from (Barré and Eliezer, 2013).

1.2.3. Generation of neurofibrillary tangles

Tau is a microtubule-associated protein predominately expressed in neurons and binds to microtubules and regulates their stability, cellular transport and cell morphology (Avila et al., 2004). In addition to stabilising microtubules, tau is also present at low concentrations in dendritic and nuclear compartments where it aids in synaptic plasticity and DNA maintenance respectively (Mondragón-Rodríguez et al., 2012, Trepanier et al., 2012, Sjöberg et al., 2006). Under pathological conditions such as AD, tau is abnormally hyperphosphorylated, decreasing its affinity for microtubules (Bancher et al., 1989), a process which is represented in Figure 1.5. Following detachment from the axons, hyperphosphorylated tau redistributes to the somatodentritic compartment where it undergoes pathological aggregation into soluble paired helical filaments (PHF), tau oligomers and then insoluble NFT's (Flament and Delacourte, 1989, Papasozomenos and Binder, 1987).

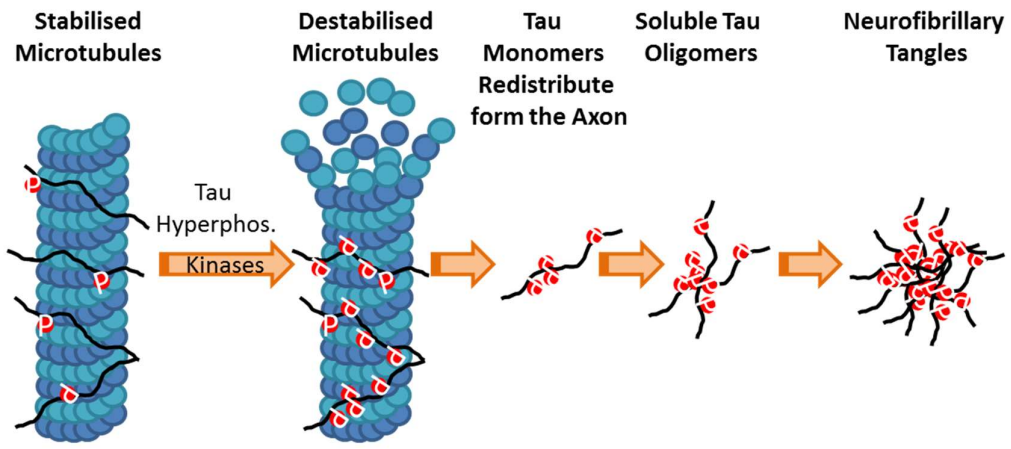

Figure 1.5: The tau aggregation process: Under physiological conditions tau regulates microtubule stabilisation. In tauopathies, tau hyperphosphorylation triggers a loss in microtubule affinity. Soluble tau aggregates into pathological soluble tau oligomers, ultimately forming pathological insoluble neurofibrillary tangles (NFT). Figure drawn by author and adapted from the National Institute of Ageing (NIA) and (Barron et al., 2017).

In a process known as seeding, tau pathology has the ability to self-propagate through the spread of tau oligomers, potentially explaining the sequential development of tau pathology in various brain structures in AD. The first evidence of tau seeding was achieved when aggregated tau from P301S mice was injected into the brain of mice overexpressing human tau but do not express tau pathology (Clavaguera et al., 2009). The authors not only reported NFT formation at the site of injection, which increased in severity with age, but an age-dependent spread in tau pathology to neighbouring brain regions. This landmark study had two important implications. Firstly, it demonstrated that extracellular tau oligomers are internalised into local neurons and initiate the pathological tau aggregation process. Secondly, it established that once overt tau pathology is expressed, it propagates into neighbouring neurons which explains the spread in tau pathology starting in the locus coeruleus and eventually spreading to neocortical regions in AD patients (Brettschneider et al., 2015, Braak et al., 2006). Although this study failed to identify which tau species is responsible for tau propagation, a subsequent study found that injection of tau oligomers but not PHF are required *in vivo* to induce tau seeding (Lasagna-Reeves et al., 2012b). Multiple mechanisms for tau secretion exist. Free/non-vesicular, exosome and extosome mechanisms have been documented *in vitro* while ectosome but not endosome mediated secretion has been demonstrated *in vivo* (Dujardin et al., 2014, Chai et al., 2012). These studies demonstrate that there are multiple mechanisms for tau seeding, but further studies are required to determine the exact mechanisms involved *in vivo* under different model conditions. An elegant study involving growing of primary neuronal cultures in

microfluidic chambers further found that tau oligomers but not monomers nor aggregates were endocytosed, further indicating them as the source of tau propagation (Wu et al., 2013). Studies have indicated the synapse as the likely location for tau propagation between neurons (Wang et al., 2017, Sokolow et al., 2015, Tai et al., 2012), although there is the possibility for tau to seed extra-synaptically (Medina and Avila, 2014).

1.2.4 Post translational modification of tau alters its propensity to aggregate in AD:

Phosphorylation is a key modulator of tau activity:

Tau is a protein which is extensively post-translationally modified in order to modulate its function. Phosphorylation is one of the key modifications which modulates tau affinity to microtubules (Bancher et al., 1989) with over 80 phosphorylation sites which predominantly flank its microtubule binding region being documented (Wang et al., 2013). In addition to regulating microtubule binding, tau phosphorylation can increase its potential to aggregate and sequester other microtubule stabilising proteins (Liu et al., 2007, Alonso et al., 1997). In the normal brain, tau contains roughly 2-3 moles of phosphate per mole of tau compared to about 8 moles in AD (Köpke et al., 1993). Tau can be phosphorylated by a multitude of kinases which can phosphorylate tau. To decipher their pathological relevance, it is important to understand both the phosphorylation sites which they act on and the phosphorylation sites present in AD patients. In doing so, a review article compiled by Martin *et al*., 2013 managed to determine the most important kinases which phosphorylate the tau protein in AD and is shown in Figure 1.6. The kinases which act on the greatest number of phosphorylation sites include casein kinases 1 and 2 (CK1 & 2), glycogen synthase kinase-3β (GSK-3β), protein kinase A (PKA), PKA, extracellular regulated kinase (ERK 1/2), cyclin dependent kinase (CDK 5) and c-Jun N-terminal kinases (JNK) (Martin et al., 2013). While phosphorylation is only half of the story, protein phosphatase 2A (PP2A) has been highlighted as the main tau phosphatase (Qian et al., 2010). Attempts have been made to attribute specific phosphorylation sites to certain aspects of tau pathology. For example, phosphorylation of epitopes in the proline rich (177aa-251aa) and microtubule binding regions have been reported to decrease microtubule affinity, while phosphorylation of epitopes in the c-terminal region (368-441) increase its affinity for aggregation (Liu et al., 2007, Sengupta et al., 1998, Abraha et al., 2000, Schneider et al., 1999). In a possible mechanism to hinder tau aggregation in healthy individuals, phosphorylation sites which decrease its affinity to bind to microtubules also appear to inhibit the ability of tau to aggregate, while phosphorylation of epitopes which promote aggregation also promote microtubule binding (Liu et al., 2007, Schneider et al., 1999). In relation to AD severity,

phosphorylation in the proline rich and microtubule binding domain are implicated in pre-NFT pathology, where phosphorylation across all regions in post-NFT pathology (Augustinack et al., 2002).

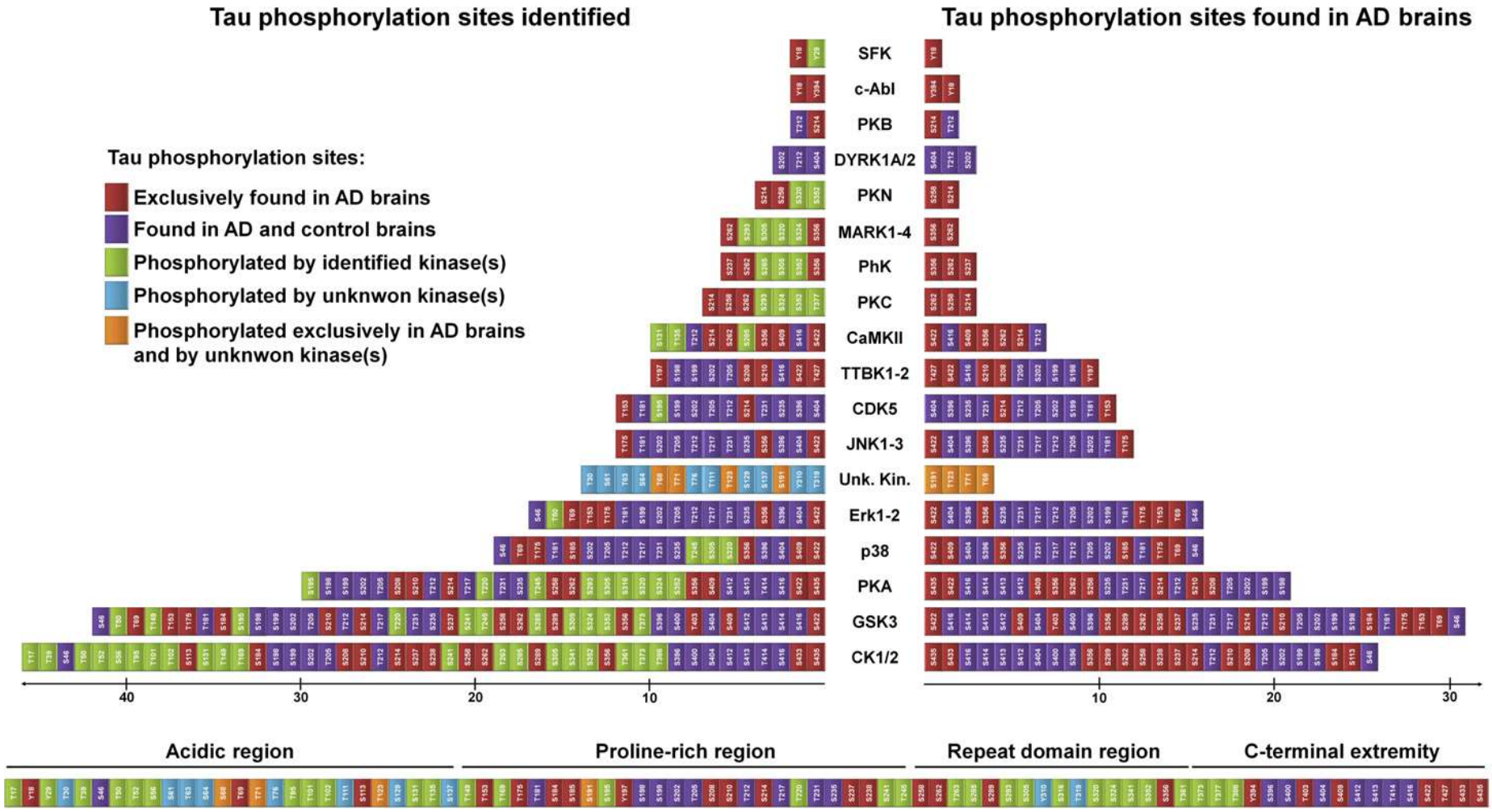

Figure 1.6 Kinases which have been implicated in the phosphorylation of the tau protein (Martin et al., 2013).

Tau truncation is associated with tau aggregation

In addition to tau phosphorylation, tau truncation has been suggested to contribute to the tau aggregation process. NFTs from AD patients contain truncated tau cleaved by both caspase 3 and caspase-6 (Horowitz et al., 2004, Basurto-Islas et al., 2008). Truncated tau is found incorporated in tau pathology from AD patients and is associated with increasing its propensity to aggregate *in vitro* (Rissman et al., 2004, Lee and Shea, 2012, Gamblin et al., 2003). As well as being more prone to aggregation, truncated tau can potentiate tau pathology through inducing aggregation of full length tau (Wang et al., 2007).

Nitration, glycosylation and methylation affect tau's ability to aggregate

Tau can undergo numerous additional post-translation modifications which increase its propensity to form tau pathology. Firstly, tau nitration both decreases its affinity for microtubule binding and increases its tendency to aggregate (Reynolds et al., 2006, Zhang et al., 2005). N-glycosylated tau is upregulated in AD brains and is more susceptible to phosphorylation than non-glycosylated tau (Liu et al., 2002). Conversely, O-glycosylated tau, which is downregulated in AD brains is less prone to phosphorylation (Liu et al., 2009) and protects against its aggregation (Yuzwa et al., 2012). Similar to O-glycosylation, tau methylation appears to confer to protection through decreasing its susceptibility for aggregation while not affecting its tubulin polymerisation function (Funk et al., 2014).

Tau isoform ratio influences its aggregation:

The ratio of 3R and 4R tau isoforms has in itself been suggested to alter the susceptibility to tau aggregation. An increase in 3R isoforms has been associated with certain tauopathies such as picks disease and Down's syndrome (Bronner et al., 2005, Shi et al., 2008). This compares to a ratio of about 1:1 in the healthy adult brain (Hong et al., 1998). 3R and 4R tau isoforms have altered microtubule binding dynamics. For example 3R tau isoforms have less affinity for microtubules making them more prone to aggregation (Panda et al., 2003). Paradoxically, an increased ratio of 4R tau isoforms has too been implicated in contributing to tau aggregation. Certain mutations which solely affect the alternate splicing of the tau gene and favour the generation of 4R over 3R tau isoforms are associated with the development of frontotemporal dementia with parkinsonism-17 (FTDP-17) (Goedert et al., 1999, Spillantini et al., 1998). When too much tau is bound to microtubules inhibition of microtubule transport of mitochondria, peroxisomes and neurofilaments occurs, making neurons susceptible to oxidative stress and neurodegeneration (Stamer et al., 2002, Mandelkow et al., 2003), possibly providing a mechanism for 4R tau isoforms to induce

neurodegeneration. Therefore, a fine balance between 3R and 4R tau isoforms is required for normal function of the tau protein and a shift in favouring either isoform can result in tau deposition. There is a typical ratio of about equal 3R and 4R isoforms in AD (Schmidt et al., 2001) indicating isoform ratio is not the main mechanism causing tau deposition within the disease.

1.2.5 Genetic tau models of AD:

Transgenic mouse models have the ability to model specific aspects of a disease and aid in understanding the underlying pathophysiological processes. For this reason, genetic tau models have been instrumental in elucidating the underlying pathological processes attributed to the tau aggregation process. To date, numerous tau models have been generated. These fall under three broad categories: mice which express aggregation prone mutant tau, mice which are knockout (KO) for tau and mice which overexpress Wt human tau. This section will aim at summarising some of the attempts to model tau *pathology* in respect to AD.

Mutant tau models:

The knowledge that specific mutations in the tau gene cause FTDP-17 (Hutton et al., 1998) led to the generation of mutant tau models expressing these. One of the most common mutated tau models is the JNPL3 model expressing 0N4R tau with the most common FTDP-17 mutation: P301L (Lewis et al., 2000). The authors reported tau deposition in brain and spinal cord regions by 4.5 months which increased in a progressive manner with age as well as astrocytosis and neuronal loss. Due to expression of tau pathology in the spinal cord, JNPL3 mice exhibit motor neuron degeneration and exhibit severe motor impairments by 10 months old, leading to paralysis and eventually death (Asai et al., 2014). Tau pathology and neuronal loss in the JNPL3 is spared in hippocampal and cortical regions – areas typically associated with cognitive function (Lewis et al., 2000) and as such, do not demonstrate cognitive impairments (Arendash et al., 2004, Morgan et al., 2008). The major advantage of the JNPL3 models is that robust tau pathology is expressed at an early age and that behavioural impairments, albeit in locomotor activity, are robust and easily measurable. This latter point means that the ongoing effectiveness of interventions can be tested through assessment of locomotor activity without needing to cull the animal. The major disadvantage of the model, and all mutated tau models in general, is their validity to AD. There are no known mutations in the tau gene in AD (Kwon et al., 2000) and expressing mutant tau which is prone to aggregation might not reflect the underlying pathological

mechanisms contributing to AD. This is reflected in the JNPL3 model in that the distribution of tau pathology is expressed in hindbrain and spinal cord regions which is regionally different from those areas affected in AD and consequently exhibits only locomotor impairments (Asai et al., 2014).

A few adaptions from the original JNPL3 model have been generated. Unlike the JNPL3 model, by expressing 2N4R tau with the P301L mutation, tau pathology is expressed in the forebrain as well as the spinal cord by 9 months (Terwel et al., 2005). Although the authors report locomotor impairments due to spinal cord expression of tau pathology, P301L mice develop cognitive impairments in spatial and recognition memory with the passive avoidance task being impaired from 5 months and novel object recognition (NOR) from 9 months (Maurin et al., 2014). Another adaption of the JNPL3 model is the rtg4510 model which is a suppressible version of the JNPL3 model through a tet-off system (SantaCruz et al., 2005). A key issue with all tau models is the early age of onset which fails to recapitulate any effects senescence might have on the pathology. As the P301L gene can be suppressed, the onset of tau pathology can be delayed to occur at older ages in the model, recapitulating any effects senescence might have on the tau aggregation process. This would be particularly appealing in understanding the effects of inflammation on tau pathology due to senescence that occurs in the immune system during the aging process (Streit, 2006), although to the best of the author's knowledge this has not yet been conducted. Finally, by substituting the leucine for a serine in the P301L model, a more severe phenotype is achieved compared to mutant models expressing the P301S mutation. P301S mice demonstrate tau deposition and microglial activation by 3 months of age, and neuronal loss by 9 months (Yoshiyama et al., 2007a). The authors report NFT formation in forebrain, brain stem and spinal cord regions as well as significant mortality at 9 months of age. Furthermore, P301S mice exhibit locomotor abnormalities and spontaneous alternation impairments at 3 months and impairments in the Morris Water Maze (MWM) by 6 months (Takeuchi et al., 2011), indicating impaired spatial working memory and spatial memory respectively (Vorhees and Williams, 2006, Hughes, 2004). While, these adapted P301 models have improved on the original JNPL3 model, the fundamental flaw that mutant, aggregation prone, tau does not reflect the aetiology in AD.

The final major mutated tau model is the 3xTg model, which again incorporates the P301L containing 0N4R tau mutation along with mutations in the presenilin 1 and APP genes (Oddo et al., 2003). The main advantage of the 3xTg model is the expression of both tau and

amyloid pathology. The authors report that mice develop amyloid deposition in forebrain regions from 6 months of age, tau aggregation from 12 months and synaptic impairments from 6 months (Oddo et al., 2003). In addition to both pathologies, IHC analyses reveals marked astrogliosis and microgliosis at 7 months in the model (Caruso et al., 2013). 3xTg mice are impaired in the Morris Water Maze (MWM) task at 4 months (Cañete et al., 2015) and the Barns Maze by 6 months (Stover et al., 2015). By 12 months, 3xTg mice exhibit impairments in passive avoidance, NOR and discriminant learning cognition tasks while being generally spared in motor impairments (Filali et al., 2012). In terms of mutated tau models, the 3xTg model has multiple advantages: both amyloid and tau pathologies are expressed, the spatial pattern of pathology is similar to AD and cognitive dysfunction with no overt motor impairments are observed. However, 3xTg mice do not exhibit substantial neuronal loss (Janelsins et al., 2008). Additionally, the fundamental issue of utilising models which are genetically pre-disposed to generate tau deposition does not reflect the aetiology of AD. Furthermore, by incorporating Aβ which is pre-disposed to aggregate, the model is even further disconnected from the conditions underlying sporadic AD.

Knockout models:

The second class of transgenic tau models focus on the ablation of tau. The main objective of these models was to understand the pathophysiological consequences of losing tau function in respect to microtubule stability. To this end, all the tau KO models have been largely disappointing due to a compensatory increase in microtubule associated protein 1A (MAP1A) among independent KO models (Ma et al., 2014, Dawson et al., 2001, Fujio et al., 2007, Harada et al., 1994). Despite a lack of overt microtubule effects, tau KO mice do exhibit behavioural abnormalities. Our group has found that tau KO mice to have altered locomotor activity as well as impairments in the spontaneous alternation and novel object recognition (NOR) tasks (Geiszler et al., 2016). Motor and cognitive impairments in tau KO mice have also been reported in the literature and are likely due to the loss of the interaction of tau with non-microtubule associated cellular proteins (Ke et al., 2012).

Overexpression models:

The final method of modelling tau pathology in mice involves the overexpression of non-mutated human tau. The most important of which is the human tau (hTau) model and is the model of most validity in terms of AD for the lack of mutations in the tau gene. The model involves the expression of all 6 non-mutated isoforms of human tau resulting from the expression of a single full length tau gene on a murine tau (mTau) KO background

(Andorfer et al., 2003). Abolition of the murine tau is required in the model as 8c mice, that is hTau mice on a Wt mTau background, do not show pathology or an overt behavioural phenotype (Duff et al., 2000). As the hTau gene is under the tau promotor, hTau mice develop tau pathology in an age dependent manner in areas which are similar to those affected in AD such as the neocortex and hippocampus (Andorfer et al., 2005, Andorfer et al., 2003). The authors report that hTau mice exhibit tau hyperphosphorylation and sarkosyl insoluble tau appearing at 2 months of age which increases with age. Furthermore the authors report that hTau mice have reduced neuronal counts with electron microscopy revealing morphological evidence of neuronal death by 10 months suggesting spontaneous neurodegeneration within the model. Additionally, antibody cytokine arrays of cortical lysates revealed a pro-inflammatory environment was observed from 3 months within the model (Garwood et al., 2010) and IHC analysis revealed significant microglial activation occurring by 12 months (Bhaskar et al., 2010). Subtle behavioural effects are observed at 4 months of age in tasks such as the food burrowing, a measure of daily living, and spontaneous alternation tasks (Geiszler et al., 2016) while impairments in the MWM are observed by 12 months (Polydoro et al., 2009). The latter of the studies also reported impairments in synaptic plasticity at this age and concurred with reports that hTau mice have altered dendritic spine morphology (Dickstein et al., 2010). However, the model does possess potential pitfalls. For example, hTau mice have a higher ratio of 3R:4R tau isoforms, meaning that despite the model not containing any genetic mutations, it is still genetically predisposed to developing tau pathology and is not fully representative of AD (Andorfer et al., 2003). Furthermore, in house observations have found hTau mice to have an increased incidence of systemic pathologies where 7 out of 10 mice had evidence of a systemic pathology which included tumours, cysts and spleen abnormalities. While this is likely an underreported feature, one study did report a significant increase in the incidence of tumours/lymphoma in hTau mice compared to control mice although the study is not comprehensive and it is unclear if control mice were of the same age (Levine et al., 2009). Finally the model involves overexpression of tau which in itself has been suggested to contribute to the tau aggregation process (Adams et al., 2009). However, overexpression in the hTau model isn't likely the sole source of tau aggregation as 8c mice do not develop NFTs (Duff et al., 2000). As the majority of tau pathology is pre-NFT, overexpression in the hTau model could theoretically be an issue irrespective of causing NFTs. Despite these issues, its recapitulation of tau pathology without expressing mutated tau, expression of all 6 tau isoforms, neuronal loss and behavioural manifestations, the hTau model is the seen as

the most AD relevant tau model. A summary of the transgenic tau models is given in Table 1.1.

Table 1.1: Advantages and disadvantages of various tau models

Model	Genetic modulation	Advantages	Disadvantages	Original reference
JNPL3	• P301L (ON4R) tau	• Develops tau pathology • Strong locomotor behavioural impairments • Gliosis • Neurodegeneration	• Mutated tau not related to AD • Pathology expressed in non-AD related areas • Locomotor impairments instead of cognitive • Paralysis at older ages	(Lewis et al., 2000)
P301L	• P301L (2N4R) tau	• Develops some tau pathology in AD related areas • Develops cognitive impairments	• Mutated tau not related to AD • Pathology expressed in non-AD related areas as well • Locomotor impairments present • Tau overexpression	(Terwel et al., 2005)
rtG4510	• P301L (ON4R) tau under tet-off system	• Ability to suppressible tau pathology • See JNPL3 advantages	• See JNPL3 disadvantages	(SantaCruz et al., 2005)
P301S	• P301S tau	• Aggressive tau pathology • Develops some tau pathology in AD related areas • Gliosis • Neurodegeneration • Cognitive impairments	• Mutated tau not related to AD • Pathology expressed in non-AD related areas as well • Tau overexpression • Locomotor impairments present • Paralysis and death at older ages	(Yoshiyama et al., 2007b)
3xTg	• P301L (ON4R) tau • APP KM670/ 671NL • PSEN1 M146V	• Amyloid and Tau pathology present • Areas affected similar to AD • Gliosis • Cognitive impairments	• Mutated tau and amyloid not related to AD • No overt neurodegeneration • Tau overexpression	(Oddo et al., 2003)
hTau	• mTau KO, • hTau (full length, generating all 6 isoforms)	• Non-mutated tau • All 6 isoforms expressed • Pathology expressed solely in regions associated with AD • Neurodegeneration • Gliosis	• Altered isoform ratio • Tau overexpression • Systemic pathologies present	(Andorfer et al., 2003)

1.2.6 Pathological effects of the tau aggregation process

Loss of function effects:

The tau aggregation process is associated with a multitude of neurotoxic and pathological effects: the first of which is thought to result from the loss of function of the tau protein. Tau functions as a microtubule stabiliser (Avila et al., 2004) and its hyperphosphorylation leads to detachment from microtubules and subsequent depolymerisation (Bancher et al., 1989, Drechsel et al., 1992). Neurons are long structures, and microtubules are important for intracellular transport such as trafficking of organelles, with disruption leading to increased susceptibility to oxidative and metabolic stress (Stamer et al., 2002, Mandelkow et al., 2003). In addition to stabilisation, tau regulates efficient microtubule transport (Dixit et al., 2008, Trinczek et al., 1999) which has the potential to exacerbate depolymerising effects. Aggravating the situation further, hyperphosphorylated tau sequesters non-pathogenic tau (Alonso et al., 1996) and other microtubule associated proteins (Iqbal et al., 2008).

In addition to disturbances in potential trafficking of intracellular organelles, microtubules are required for maintaining the Golgi apparatus structure. The Golgi apparatus is responsible for protein packaging, organisation and transport (Rios and Bornens, 2003). The Golgi apparatus has been found fragmented in post-mortem examinations from AD patients (Stieber et al., 1996). Indicating that tau can pathologically drive this process, overexpression of Wt human tau or the expression of several forms of mutant aggregation prone human tau induces Golgi fragmentation in primary neuronal cultures (Liazoghli et al., 2005). Interestingly, in AD Golgi fragmentation occurs prior to NFT formation, suggesting microtubule destabilisation rather than tau aggregation as a potential source of the disruption (Stieber et al., 1996).

Pathological gain of function effects of tau hyperphosphorylation and aggregation

While microtubule destabilisation contributes in part to the pathological effects of tau aggregation, toxic gain of functions resulting from the tau aggregation process are potentially even more pathogenic. NFTs have been traditionally thought as the pathogenic tau species due to their association with the disease. However, the fact that neurons containing NFTs potentially can survive for decades (Morsch et al., 1999) and that NFTs may not be required for neuronal loss (SantaCruz et al., 2005, Morsch et al., 1999, Wittmann et al., 2001) indicates that NFTs are not as pathogenic as originally hypothesised. Instead, soluble tau oligomers have been suggested as the species with greatest pathogenicity in AD,

with NFTs acting in a protective mechanism through sequestering of toxic tau oligomers (Patterson et al., 2011).

The au aggregation process has multiple potential neurotoxic effects. During the aggregation processes, tau relocates from the axon to the cell body and dendrites (Flament and Delacourte, 1989, Papasozomenos and Binder, 1987) and is able to disrupt synaptic function. Under physiological conditions, tau acts as scaffolding molecule for the Src family tyrosine kinase: Fyn – trafficking it to post-synaptic NMDAr and resulting in its stabilisation with post synaptic density-95 (PSD95) and aiding in glutamatergic signal transduction (Mondragón-Rodríguez et al., 2012, Trepanier et al., 2012). Enhanced levels of phosphorylated tau in the dendritic compartment increases the trafficking of Fyn to the synapse and augments NMDA signalling, resulting in altered synaptic plasticity (Mondragon-Rodriguez et al., 2012, Usardi et al., 2011). When injected *intracranially* into wild-type (Wt) mice, tau oligomers impair synaptic function and induces cognitive impairments (Lasagna-Reeves et al., 2011), substantiating a role for tau in disruption of synaptic signalling. Synaptic signalling is dependent on dendritic spines morphology, with a loss in the number of spines or synaptic densities impairing effective signalling transduction (Harris and Kater, 1994). In addition to altering synaptic plasticity, tau transgenic mouse models have been associated with decreased numbers of synaptic spines and post synaptic densities (PSD) (Maurin et al., 2014, Dickstein et al., 2010) indicating tau pathology affects synaptic morphology. Interestingly, in a transgenic amyloid model of AD, removal of tau oligomers ameliorates the abnormalities in dendritic spines (Castillo-Carranza et al., 2015). Furthermore, hyperphosphorylated tau in itself has been implicated in synaptic dysfunction due to its dendritic mislocalisation, indicating tau is not required to aggregate to affect synaptic function (Hoover et al., 2010). Tau induced impairments of synapses is not limited to post-synaptic densities, with abnormalities in pre-synaptic signalling caused by aggregated tau in mice expressing truncated, aggregation, prone tau (Decker et al., 2015). Furthermore, pre-synaptic injection of hyperphosphorylated tau into giant squid axons was shown to inhibit synaptic function through impairing vesicular release (Moreno et al., 2011). Altogether, these studies indicate that both hyperphosphorylated and oligomeric tau disrupt synaptic function, with its mislocalisation to the synapse the route cause.

In addition to altering synaptic plasticity, pathological tau species have the ability to exert neurotoxicity, potentially leading to the overt neurodegeneration observed in the disease. For example, overexpression of hyperphosphorylated tau induces neuronal

apoptosis *in vitro* (Fath et al., 2002, Chung et al., 2001). While this demonstrates that tau can induce neuronal apoptosis, *in vivo* tau models indicate both apoptotic and non-apoptotic mechanisms of tau-induced neurodegeneration (Zehr et al., 2004, Allen et al., 2002, Tanemura et al., 2002, Andorfer et al., 2005). While it is impossible to determine the exact mechanisms of tau-induced neurodegeneration in humans, pre-clinical tau models have helped to enlighten possible mechanisms by which tau induces its neurotoxicity.

AD is a disorder associated with substantial excitotoxicity (Dodd et al., 1994) – a feature which tau plays a part in. A consequence of the enhanced recruitment of Fyn to the synapse is an increased susceptibility to excitotoxicity through increased PSD95-NMDAr stabilisation (Mondragon-Rodriguez et al., 2012, Usardi et al., 2011). This is further exacerbated by increased levels of glutamate in the synaptic cleft in AD (Lauderback et al., 2001, Kulijewicz-Nawrot et al., 2013). Demonstrating a role for tau in excitotoxicity, selective blockage of the NMDAr but not the α-amino-3 hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr) ameliorates neuronal loss in a cellular tau model (Amadoro et al., 2006). Likewise, tau has been implicated in NMDAr induced excitotoxicity *in vivo*. Tau knockout mice are resistant to kainate-induced excitotoxicity (Roberson et al., 2007). This suggests that under physiological conditions, tau facilitates excitotoxicity to highly excitotoxic stimuli, with its role in the stabilisation of the NMDAr-PSD95 complex as a likely mechanism. Therefore, it is reasonable to believe that increased synaptic localisation of tau in AD could facilitate excitotoxicity to stimuli which are not normally excitotoxic. To date, this has been relatively poorly investigated. One study found that overexpression of Wt murine tau in primary cortical neurons increased susceptibility to NMDAr-induced excitotoxicity and that tau phosphorylation is required (Miyamoto et al., 2017). Supporting such a mechanism, expression of truncated tau that does not have a dendritic localisation, decreased the concentration of Fyn at the synapse, lowered levels of the NMDAr-PSD95 complex and conferred to protection against excitotoxicity (Ittner et al., 2010). Together these studies suggest enhanced synaptic location of tau could facilitate significant excitotoxicity in AD.

It is clear that NMDAr-mediated excitotoxicity is not the only mechanism of neurotoxicity in AD as memantine only provides brief symptomatic benefit (Doody et al., 2001, Raina et al., 2008). Another mechanism by which tau can induce neurotoxicity is through disruption of normal intracellular organelle activity. Mitochondria are the energy factories of the cell and disturbance in their regular function has wide reaching effects including increasing susceptibility to oxidative and metabolic stress (Grant et al., 1997) and

initiation of the apoptosis process (Desagher and Martinou, 2000). Mitochondrial abnormalities are a common feature in AD patients (Bosetti et al., 2002, Sekar et al., 2015). Preclinical evidence supports a role for pathogenic tau in driving mitochondrial neurotoxicity. Firstly, in immortalised cortical neurons, truncated tau induces mitochondria dysfunction (Quintanilla et al., 2009). Similar observations have been corroborated *in vivo* in the P301L model (David et al., 2005). Indicating tau oligomers as the mitochondrial toxic tau species *in vivo*, administration of tau oligomers but not monomers or fibrils in Wt mice induces both mitochondrial damage and neuronal loss (Lasagna-Reeves et al., 2011).

The final known mechanism by which tau pathology exerts neurotoxicity is through interference with normal cell cycle mechanisms. The cell cycle consists of four phases: G_1 – where the cell prepares for DNA synthesis, S – where DNA is replicated, G_2 – where the cell prepares for division and M – where mitosis occurs. Additionally, when the cell is not replicating it can transition from the G_1 to the resting G_0 phase (Vermeulen et al., 2003). Apart from in areas of neurogenesis, neurons are considered to be terminally differentiated – that is permanently in a state of G_0. In AD, neurons in areas associated with neurodegeneration aberrantly enter the cell cycle and progress up to the G_2 phase (Busser et al., 1998, Nagy et al., 1997, Bonda et al., 2009) but fail to progress to the M phase (Ogawa et al., 2003). If a cell progresses past the G_1 phases, it cannot return to G_0 and must undergo mitosis, with failure to do so resulting in apoptosis (Meikrantz and Schlegel, 1995). While the relationship between tau and cell cycle re-entry in AD has been poorly investigated, a few studies have indicated a connection. In a mutant tau drosophila model, neurodegeneration was associated with cell cycle re-entry (Khurana et al., 2006). Likewise, cell cycle re-entry has been associated with neurodegeneration in the hTau model (Andorfer et al., 2005). However, the authors report that cell cycle re-entry is distinct from cells which possess tau pathology, a similar observation is found in most-mortem examinations from AD patients (Vincent et al., 1998). Together these studies suggest a potential indirect role for tau pathology inducing cell-cycle re-entry. A summary of the pathogenic and neurotoxic tau species is given in Figure 1.7.

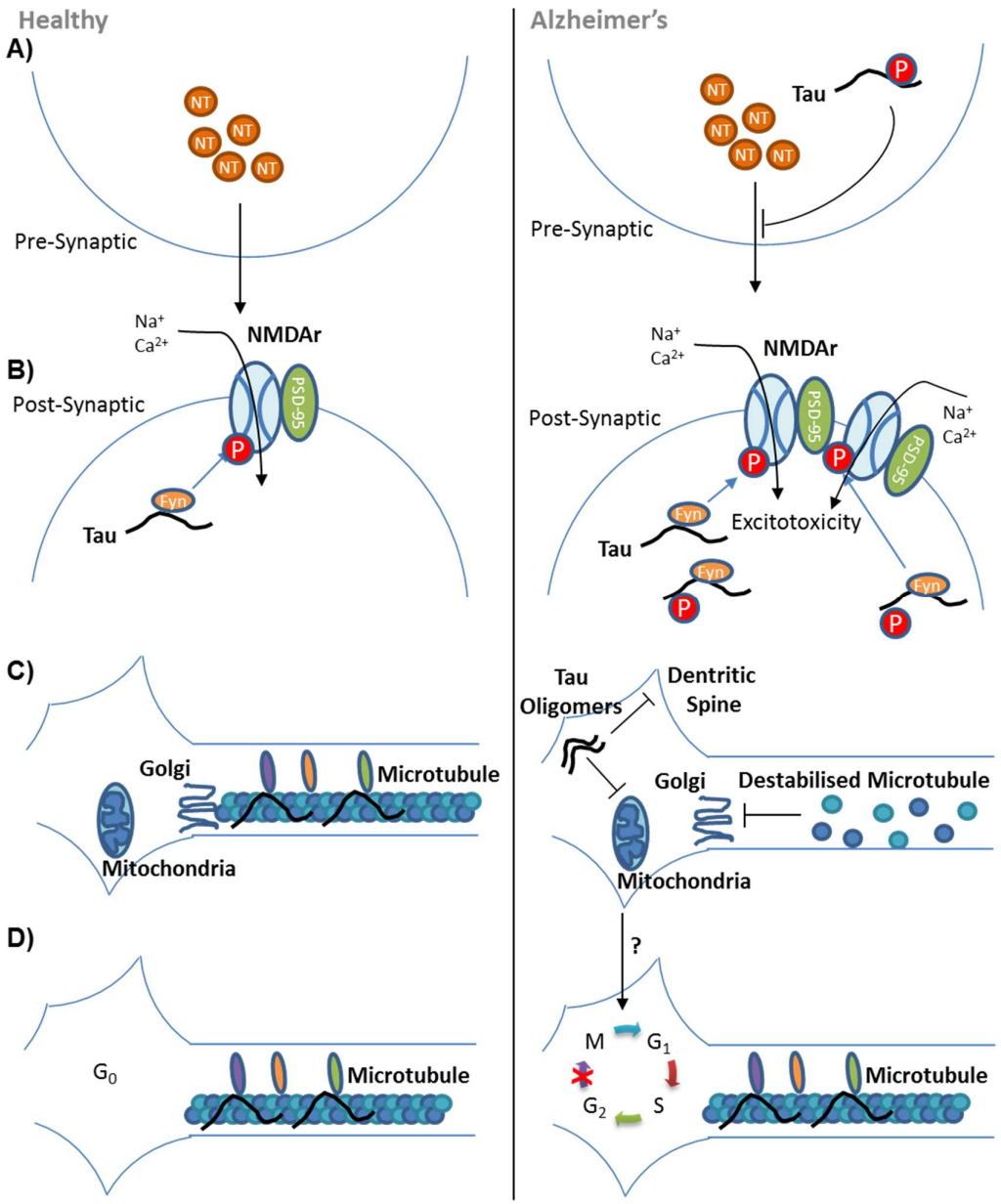

Figure 1.7: The pathogenic features of tau. ***A)*** *In AD, hyperphosphorylated tau interrupts neurotransmitter (NT) release at pre-synaptic positions.* ***B)*** *Under physiological conditions, dendritic tau acts as a scaffolding molecule for Fyn, causing it to locate to the synapse. Fyn causes the phosphorylation of the NMDAr leading to stabilisation with PSD95, facilitating glutamate signal transduction. In AD, an increased concentration of dendritic tau causes over stabilisation of the NMDAr-PSD95 complex resulting in excitotoxicity.* ***C)*** *Under physiological conditions tau stabilises microtubules, facilitating microtubule transport and stabilisation of the golgi apparatus. In AD, tau hyperphosphorylation leads to impaired microtubule transport and destabilisation causing an increase in susceptibility to stressors and golgi apparatus fragmentation. Tau aggregation leads to impaired mitochondrial function and altered dendritic spine morphology.* ***D)*** *Under normal conditions neurons are considered terminally differentiated and do not enter the cell cycle, instead residing in the G_0 phase. In AD, the pathological tau aggregation process leads to aberrant cell cycle re-entry of unaffected neurons by an unknown mechanism. Despite entering the cell cycle, AD neurons not progress to the mitosis phase and the uncompleted cell cycle can lead to apoptosis induction. Figure generated by author.*

1.2.7 Targeting tau in clinical trials

Due to its close association with the disease, tau has emerged as a popular clinical target in AD. While targeting tau kinases and phosphatases is problematic due to their wide reaching effects, numerous other mechanisms for halting the effects of tau pathology are in clinical trials. These include: microtubule stabilising agents, anti-tau aggregation inhibitors and passive/active immunisation therapies (Panza et al., 2016). To date there has only been one large scale Phase III clinical trial targeting tau pathology which has reached completion for AD. The trial involved the administration of Leuco-methylthioninium bis(hydromethanesulfonate) LMTM – a derivative of methylene blue which acts as a tau aggregation inhibitor (Schirmer et al., 2011). To great disappointment, LMTM failed to show efficacy in all primary endpoints (Gauthier et al., 2016). While the outcome of the trial is disappointing, it is not uncommon for first attempts on therapeutic targets to fail in clinical trials. With future trials the true potential for tau as a target in AD will be further elucidated.

1.2.8 Summary

Tau is a microtubule associated protein which highly undergoes post-translational modification. Tau phosphorylation is one of such modifications which is thought to be integral to the tau aggregation process which highly correlates with AD progression. Various tau species have a variety of neurotoxic properties and the tau aggregation process is able to self-propagate into healthy regions as the disease progresses. Numerous genetic tau models have been generated to aid in understanding the pathological effects of tau aggregation. The hTau model is the most relevant to AD for lack of mutations, expression of all 6 human isoforms and tau aggregation occurring solely in areas similar to those affected in AD. Despite some fundamental issues in the hTau model, for example their tau isoform imbalance and expression of systemic pathologies, it is currently the most attractive for modelling tau pathology in respect to AD.

1.3 Neuroinflammatory features of AD

1.3.1 Neuroinflammation overview

Inflammation is the response to injury and infection involving a diffuse set of biological responses. The aim of the inflammatory response depends on the type of immune stressor involved. In response to injury, the aim is to clear potentially toxic material while facilitating repair. In response to infection, inflammation is aimed at eradicating the

infection often at the cost of cellular toxicity. Neuroinflammation is inflammation which, does not necessarily originate, but occurs within the CNS. Immunological responses are classed into two sub-categories: those which are part of the innate immune system, and those which are part of the adaptive immune system with the former primarily responsible for neuroinflammatory responses. The innate immune system involves inflammatory responses to nonspecific triggers known as pathogen- or damage- associated molecular pattern molecules (PAMPs or DAMPs) depending on whether the immunological stressor originates from outside or within the host respectively. Recognition of PAMPs and DAMPs by pattern recognition receptors (PRRs) on numerous immunological cells results in a diffuse and variable response. There are multiple immune cells within the CNS which are responsible for propagation of the neuroinflammatory response including: astrocytes, microglia and non-parenchymal macrophages. While the neuroinflammatory response is critical for clearance of infection and facilitating repair to injury, if left unchecked it can lead to substantial neurotoxicity. This section will summarise some of the chronic inflammatory features AD is associated with, how they dynamically change with the disease and the evidence of how specific mediators affects tau pathology.

1.3.2 Immunological cells involved in AD

Microglia

Microglial cells are a key Immune cell within the CNS with a dedicated immune function. Microglia express a whole host of PRR's receptors including TLR's, nod-like receptros (NLR's) and scavenger receptors, enabling them to monitor their environment (Wilkinson and El Khoury, 2012). Under resting conditions microglia are nomadic surveyors of the CNS, searching for potential infectious or toxic triggers, including Aβ and tau aggregates (Block and Hong, 2005, Halle et al., 2008, Morales et al., 2013). Upon activation they can elicit two functions. The first is releasing a host of inflammatory mediators including cytokines, complement factors and prostaglandins (Zhang et al., 2009a, Smith et al., 2012, Walker et al., 1995, Kreutzberg, 1996). The second involves the phagocytosis of potentially infectious or neurotoxic materials (Cherry et al., 2014). Depending on the immunogen, altered combinations of the two occur. To hinder the spread of infection, microglia activation by PAMPs results in phagocytic and pro-inflammatory responses. Conversely, to prevent inflammatory driven neurotoxicity, microglial activation by DAMPs results in a phagocytic response accompanied by secretion of anti-inflammatory mediators (Neumann et al., 2009). In the healthy brain, the latter process is important to aid in the clearance of extracellular toxic proteins and cellular debris without causing self-damage.

While microglia have been well documented to phagocytose aggregated amyloid species in an important clearance mechanism (Koenigsknecht and Landreth, 2004), recent evidence suggests that they can also aid in the clearance of extracellular tau species. For instance, primary and immortalised microglial cells were found to phagocytose synthetic extracellular tau oligomers following treatment with the TLR4 agonist: lipopolysaccharide (LPS) (Majerova et al., 2014). Supporting this role, C57BL/6 mice injected with both soluble and aggregated human tau were shown to have microglial internalisation of both species (Bolos et al., 2015) which could potentially aid in slowing tau seeding.

There is a growing body of evidence that the phenotype of microglia alters during the ageing process. In rodents, ageing is associated with increased microglial activation (Ogura et al., 1994), as well as increased microglial activation to exogenous stressors such as LPS (Huang et al., 2008, Godbout et al., 2005). This heightened immune response is due to a process known as priming in which aged microglia develop a hypersensitive, pro-inflammatory phenotype characterised by high levels of major histocompatibility complex-II (MHC-II) (Godbout et al., 2005, Frank et al., 2006, Perry and Holmes, 2014). The phenomenon of microglia priming is not restricted to the ageing process and has been documented in a number of neurodegenerative disorders including AD. Recent advances in single cell RNA sequencing (RNAseq) in AD models have truly furthered our understanding of how microglia dynamically change in AD. Using the technique, both the amyloid APPswe/PS1dE9 and the tau rtg4510 models were found to possess microglial that had signatures of microglial priming but not of those associated with acute LPS activation (Holtman et al., 2015). Importantly, this suggests that while amyloid and tau pathologies are adequate to induce microglial priming, they are insufficient in driving the neuroinflammation observed in the disease. Instead, additionally immunological stressor such as systemic inflammation might be required to elicit overt neuroinflammatory responses. Supporting this notion, primed microglia in the ME7 prion model showed significant activation following peripheral immune stimulation with LPS, whereas control animals administered with LPS or ME7 mice administered with saline did not (Murray et al., 2012). There is evidence for microglial priming occurring in AD patients too. Histological examination from AD patients reveals senescent rather than activated microglia present in brain tissue (Streit et al., 2009). Interestingly, the authors report that senescent microglia only occur in proximity to NFT containing neurons whereas microglia surrounding plaques were spared suggesting tau as the driver of microglia dysfunction.

Neuroinflammation in AD is a dynamic process. The pattern of microglial activation, occurs early in the disease (Cagnin et al., 2001) and can even be detected in mild cognitive impaired (MCI) patients (Okello et al., 2009). Recent attempts have been made to untangle the dynamic alterations in microglial function in AD through temporally performing single cell RNAseq in microglia from AD models. In a landmark study, Keren-Shaul *et al*., 2017 found that compared to Wt mice, amyloid 5xFAD mice had two additional microglia populations; "disease associated microglia" (DAM) and an intermediary phenotype. The authors report that not only were DAMs localised around plaques, but were found to have upregulated genes for lipid metabolism and phagocytosis. On the other hand, the intermediary microglial phenotype was found to have a similar profile to that of DAMs apart from many of the phagocytic genes were absent (Keren-Shaul et al., 2017). Furthermore, the authors report that DAMs have a higher ability to phagocytose Aβ. This indicates that at least in response to amyloid pathology, microglial cells develop a protective phenotype; a revelation which has previously been suggested (Fiala et al., 2007, D'Andrea et al., 2004, Herber et al., 2004) and is line with senescent microglia not being observed around plaques in AD brains (Streit et al., 2009). When conducted over several time-points within the 5xFAD model, a shift towards transient microglia was observed at 3 months of age when plaque deposition occurs and DAMs appearing at 8 months; extremely late within the model (Keren-Shaul et al., 2017). Similar RNAseq has been conducted in another model of neurodegeneration; the CK-p25 model which overexpresses p25 and involves early amyloid and later tau pathology. The model was also shown to have two distinct populations of microglia; one early intermediate and one late stage at 2 and 6 weeks respectively (Mathys et al., 2017). Interestingly, the majority of the genes altered in DAMs, were found to be affected in the late stage microglia population from CK-p25 mice; indicating homeostatic changes associated with AD are sufficient in driving changes in microglial phenotype. A limitation of both studies is the failure to assess wider reaching neuroinflammatory changes, although the Mathys *et al*., 2017 study did observe changes in some inflammatory transcripts including that the inflammatory cytokine macrophage migration inhibitory factor (MIF) was found elevated in the intermediate microglia population which persisted into the late stage population. While more information is required in terms of the wider inflammatory properties of these distinct microglia populations, this could suggest that intermediate microglia populations are detrimental for lack of phagocytic properties that late stage microglial have. These studies are adding to a growing body of evidence that if anti-inflammatory treatment is to succeed in AD, early intervention is required and that a

targeted approach is required to spare beneficial microglial responses (Krause et al., 2010, Heneka et al., 2016).

While both the Keren-Shaul *et al*., 2017 and Mathys *et al*., 2017 studies provide evidence of how amyloid pathology might drive a beneficial microglial phenotype, they do not address what impact tau might have. The mice utilised in the Mathys *et al*., 2017 study were 2w and 6w old, far younger than the 27 weeks required to develop NFTs in the model (Cruz et al., 2003). This compares to $A\beta_{42}$ accumulating prior to 2w within the model (Cruz et al., 2006). As dystrophic microglia accumulate selectively around NFT containing neurons in AD patents (Streit et al., 2009), it stands to reason the impact of tau on microglial populations might differ to that from amyloid. A recent meta-analysis (which added a few original data-sets) examined RNAseq data for microglial and brain myeloid cells from 16 different models including 3 amyloid (PS2APP, 5xFAD and APPswe/PS1dE9), 2 tau (P301L and P301S), mice administered with LPS as well as comparing to AD tissue (Friedman et al., 2018). The study compared each model under a specific set of microglial phenotype "modules" including one for neurodegeneration, LPS-related, interferon related and microglia-related. Unfortunately, the study didn't try to elucidate specific individual microglial populations, making it difficult to discern specific microglial phenotypes and function. (Friedman et al., 2018). However, the study reported a strong increase in the LPS-related module among post-mortem tissue from AD patients which weren't recapitulated in any of the amyloid and tau models. The failure of AD models to recapitulate the microglial phenotype from AD patients casts doubt on validity from single-cell RNAseq data from AD models and instead provides a more hypothetical view how a specific pathology might affect microglial phenotype. Additionally, it could indicate again that amyloid or tau pathology is not enough to recapitulate microglial alterations which are observed in AD and that an additional stimulus is required such as systemic inflammation. Nevertheless, it is imperative to use a similarly exhaustive approach as the Keren-Shaul *et al*., 2017 and Mathys *et al*., 2017 in tau models to elucidate the temporally dynamic effect tau pathology might have on microglial function and how this altered function might impact on tau pathology.

Astrocytes

Under physiological conditions, astrocytes have a wide range of functions including roles in metabolic homeostasis, signal transduction and are a key connection between neurons and the vasculature (Bélanger and Magistretti, 2009). Astrocytes host numerous PRRs including NLRs, TLRs and RIG-like receptors (RLRs) (Kigerl et al., 2014) and are able to

induce a neuroinflammatory response (Krasowska-Zoladek et al., 2007). In addition to PRR's, astrocytes can propagate the neuroinflammatory response after being activated by inflammatory mediators. Activation of astrocytes by PAMPs, DAMPs or neuroinflammatory mediators can release numerous inflammatory mediators including cytokines, complement factors and prostaglandins (Heneka et al., 2010). In AD, astrocytes are in a state termed reactive astrogliosis – whereby they are increased in number and in a state of activation. Alois Alzheimer first noted reactive gliosis in his original observations and a tendency for astrocytes to locate around amyloid plaques (Strassnig and Ganguli, 2005). Recent evidence suggests astrocytes not only localise around amyloid plaques, but are in close proximity to NFT containing neurons (Serrano-Pozo et al., 2011). Additionally the authors report that astrogliosis is a relatively early event in AD, which lineally increases with the duration of the disease, a feature which has been identified previously (Webster et al., 2006). While the interaction of tau and astrocytes has been poorly investigated, various Aβ species induce astrocyte activation *in vitro* and *in vivo* (Garcia-Matas et al., 2010, White et al., 2005). Activation of astrocytes in AD, whether through amyloid or pro-inflammatory mediators, leads to secretion of pro-inflammatory mediators, contributing to the chronic neuroinflammation in AD. Furthermore, reactive astrocytes have a lower propensity to uptake and store glutamate in AD, causing an excess of glutamate in the synaptic cleft and contributing to excitotoxicity (Lauderback et al., 2001, Kulijewicz-Nawrot et al., 2013). Finally, astrocytes have a role in Aβ uptake and clearance, a function which is potentially impaired in AD (Iram et al., 2016).

Role of the blood brain barrier:

Separating the peripheral and central compartments is the blood brain barrier (BBB). Forming an integral property of the BBB, tight junctions exist between endothelial cells from capillaries in the BBB, forming a diffusion barrier. Pericytes further make up the BBB and have a variety of functions including initiating the formation of BBB. Encasing the pericytes and endothelial cells is the basal lamina which stabilises the unit. Finally, astrocyte-end-feet surround the basal lamina and are important for maintenance of the BBB in addition to functioning as an important member of the neuro-vascular unit (NVU) (Ballabh et al., 2004, ElAli et al., 2014). Together, these cells form a barrier which not only prevents peripheral cells from entering the CNS, but also most molecules. The NVU is depicted in Figure 1.8. Despite forming a barrier between the periphery, systemic inflammatory stimuli are able to induce a central neuroinflammatory response (Pardon, 2015).

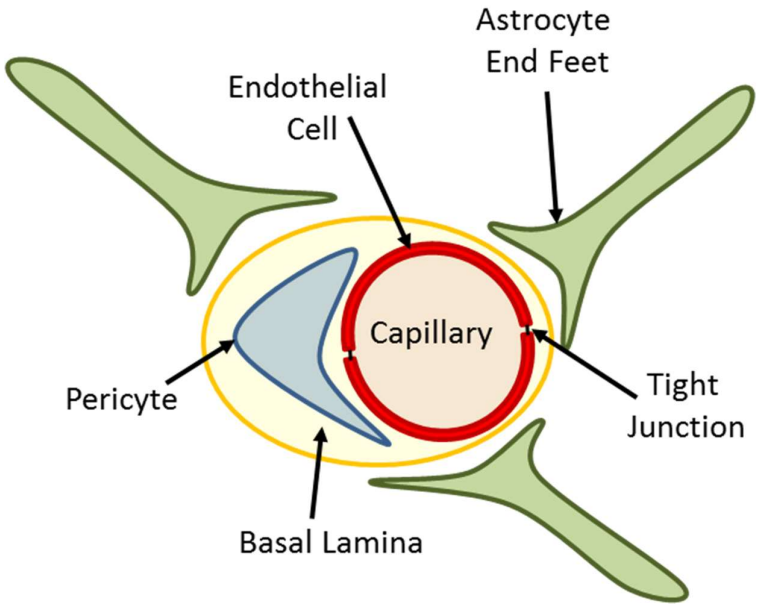

Figure 1.8 The cellular composition of the blood brain barrier. Figure drawn by author.

The BBB is vital in the transmission of systemic inflammation into the CNS through a variety of its functions. The BBB is important for the transport of molecules from the periphery to central compartments by a multitude of transport mechanisms (Zlokovic, 2011). A variety of cytokines including: IL-1β, IL-6 and TNFα all have saturable transport mechanisms across the BBB whereas IL-2 and TGF-β do not (Banks et al., 1995, Kastin et al., 2003). There is evidence that systemic inflammation in itself can increase the saturable transport of certain cytokines across the BBB. For example, TNFα is transported across the BBB through receptor mediated endocytosis in brain endothelial cells (Pan and Kastin, 2002). Systemic LPS administration in rats has been shown to increase the transport of labelled TNFα across the BBB through increased expression of both TNF receptors (Osburg et al., 2002). Therefore, the BBB is not only responsible for transmission of certain peripheral inflammatory mediators into the central compartment, but systemic inflammation can increase their transport mechanisms. Potentially contributing to the transport of peripheral inflammatory mediators into the brain, there is evidence of a dysfunctional BBB in AD (Sweeney et al., 2018). In post-mortem examinations increased levels of blood derived proteins such as prothrombin and fibrinogen have been observed in brains of AD patients compared to non-demented individuals (Zipser et al., 2007, Ryu and McLarnon, 2009). However, using modern dynamic contrast-enhanced magnetic resonance imaging (DC-MRI) techniques to assess BBB permeability has provided conflicting data. Increased BBB

permeability has been reported in MCI and AD measured by DC-MRI (Montagne et al., 2015, Montagne et al., 2016, van de Haar et al., 2016). This compares to studies which have failed to report any changes in BBB permeability when measured by DC-MRI (Starr et al., 2009, Caserta et al., 1998), possibly due to the extremely low power in the studies. Nevertheless, any increase in BBB permeability has the potential to contribute to the infiltration of peripheral immune mediators including LPS in AD.

Endothelial cells possess a whole host of receptors which respond to peripheral inflammatory mediators *in vitro* including endogenous mediators such as IL-1β, IL-4, IL-6 and INFγ (Fasler-Kan et al., 2010) as well as exogenous mediators such as LPS which do not cross the BBB (Pugin et al., 1993, Banks and Robinson, 2010). However, the question remains is whether luminal (blood compartment) recognition by endothelial cells promotes the abluminal (brain compartment) secretion of inflammatory mediators. To answer this question, an *in vitro* assay compromising luminal and abluminal compartments separated by a mouse brain endothelial monolayer has been developed. A landmark study found that LPS administered to the abluminal compartment induced luminal secretion of IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNFα (Verma et al., 2006). Interestingly, the authors report that the transluminal secretion appeared selective to these cytokines as IL-1β, IL-2, IL-4, IL-12, and IFNγ were not induced. Indicating that systemic inflammation can induce transluminal secretion of cytokines, LPS when added to the luminal compartment in the same experimental model, induced selective abluminal secretion of; IL-6, IL-12(p40), G-CSF, MCP-1, KC, MIP-1α and RANTES (William et al., 2017). There are two important implications from both studies. Firstly, transluminal stimulation of brain endothelial cells could occur and that it is a viable route for transmission of systemic inflammation into the brain. The second is that inflammatory mediator induction was selective; although the effect of different inflammatory mediators needs to be assessed. However, brain endothelial cells are not isolated but instead act as in unison with other cells from the BBB. Both astrocytes and pericytes have been reported to be capable of initiating inflammatory responses (Pieper et al., 2014, Choi et al., 2014). To address this issue, William *et* al., 2017 utilised the endothelial monolayer setup but with tricultures containing mouse astrocytes, pericytes and endothelial monolayers. An increase in abluminal cytokine secretion was observed compared to solely monolayers as well as induction of cytokines which were not previously induced including IL-1β, TNFα and MIP-1β. By eloquently culturing mouse endothelial monolayers with human astrocytes and pericytes, the authors were able to determine the contribution of each to transluminal cytokine secretion. They

report that abluminal IL-6, GM-CSF, IL-8, TNFα were resulting from selective astrocyte/pericyte secretion, abluminal MCP-1 was primarily resulting from astrocyte/pericyte secretion and abluminal G-CSF was resulting from endothelial cell secretion (William et al., 2017). In a similar manner, perivascular macrophages are thought to contribute in the systemic to central immune transmission. Depletion of perivascular macrophages has been reported to reduce COX-2 mRNA induction in the perivascular space as well as reduce CSF PGE_2 levels in response to systemically administered TNFα in rats (Yu et al., 2010). Similarly, perivascular macrophage depletion in rats is associated with a decrease in the number of COX-2 positive cells as well as decreased PGE_2 levels in the vascular area following systemic IL-1 administration (Serrats et al., 2010). Interestingly, the authors report an increase in COX-2 positive cells following systemic LPS administration in the vascular areas due to increased induction in brain endothelial cells. This was accompanied by an increase in PGE_2 concentrations suggesting that perivascular macrophages are not only important in propagating the inflammatory response, but also in regulating endothelial cell function depending on the immune stressor. Together, these studies demonstrate that the BBB plays an integral role transferring systemic inflammation into the central compartment.

Infiltration of peripheral immune cells:

An important function of the BBB is to prevent circulating immune cells from infiltrating the central compartment unless certain stimulators are present. The general absence of peripheral immune cells within the CNS is an important feature to minimise potential unnecessary neuroinflammatory responses. Peripheral immune cell infiltration has been suggested to be important in the pathogenesis of AD (Zenaro et al., 2016). However the role of infiltrated peripheral immune cells in AD is controversial. For example, while slightly elevated numbers of T-cells have been reported within brains from AD patients, the T-cells present are not effector cells and are not likely to contribute to the neuroinflammatory environment (Togo et al., 2002). Peripheral monocyte infiltration has received much attention in AD. In murine models of amyloid deposition, peripheral monocytes have been shown to infiltrate across the BBB and phagocytose Aβ (Lebson et al., 2010, Stalder et al., 2005). However, evidence is lacking that, apart from perivascular, choroid plexus and meningeal macrophages, that peripheral monocyte infiltration occurs in AD (Hohsfield and Humpel, 2015). Even this special subset of CNS orientated macrophages appears to be more closely related to microglia than peripheral macrophages (Goldmann et al., 2016). Furthermore, the authors report by using advanced fate mapping approaches and

parabiosis that these non-parenchymal macrophages do not originate from peripheral infiltration, but originate from the yolk sack in a similar manner to microglia. In fact, peripheral monocyte infiltration in AD appears only in cases where stroke was a comorbidity (Wisniewski et al., 1991), likely due substantial breakdown of the BBB following ischaemic injury (Sandoval and Witt, 2008). Aβ is known to induce monocyte trafficking *in vitro* (Humpel, 2008, Giri et al., 2000), and as such peripheral monocyte infiltration in murine amyloid models could simply be resultant of overexpression of Aβ which is not recapitulated in AD patients. Similar to peripheral monocytes, neutrophils have been shown to infiltrate the CNS in murine amyloid models (Zenaro et al., 2015), but fail to do so in AD patients (Hohsfield and Humpel, 2015). Therefore, unlike more aggressive neurological disorders such as stroke and multiple sclerosis which involve the breakdown of the BBB, the role of peripheral leukocyte infiltration in AD appears to be minimal (Prinz and Priller, 2017).

1.3.4 Inflammatory mediators in AD

A consequence of sustained gliosis in AD, is altered expression of many inflammatory mediators in brains from AD patients. This section will summarise some of the most important inflammatory mediators in respect to AD. However, substantial issues exist when trying to assess these in post-mortem examinations from AD patients (Gomez-Nicola and Boche, 2015). For example, the tissue is of poor quality which can have substantial effects on both mRNA and protein levels. Furthermore, the group sizes which are used in many of the studies assessing inflammatory mediators are simply not big enough to draw definitive conclusions for a multi-faceted disease such as AD which involves multiple co-morbidities, stages or even possibly aetiologies. For these reasons, many of the inflammatory mediators in AD have been found both up and down regulated depending on the study. However as a whole, there appears a bias towards upregulation of pro-inflammatory mediators, corroborating observations of a discreet inflammatory environment in AD.

Cytokines

Cytokines are small secreted inflammatory signalling proteins and are integral to the inflammatory signalling cascade. Cytokines can be classified generally into those that possess greater pro- or greater anti-inflammatory properties, although most can exert both pro- and anti-inflammatory functions based on the situation.

IL-1β

IL-1β is the prototypical pro-inflammatory cytokine and is important for inducing and propagating the inflammatory cascade (Weber et al., 2010). Furthermore, IL-1β has been implicated in impairing cognition *in vivo* (Terrando et al., 2010) through depressing LTP (Gonzalez et al., 2013). Despite IL-1β being thought as integral to neuroinflammatory processes in AD (Shaftel et al., 2008) whether it is upregulated in AD is controversial. Table 1.2 provides a summary of studies assessing IL-1β levels in AD. Despite lack of a clear upregulation of IL-1β with AD, increased IL-1 positive microglia have been found surrounding both amyloid plaques and NFT containing neurons (Sheng et al., 1997, Griffin et al., 1989), suggesting both hallmarks induce IL-1β in the disease. Similarly, increased levels of circulating IL-1β has been reported in AD patients (Swardfager et al., 2010).

Table 1.2: Studies assessing IL-1β concentrations in AD patients

Brain region	Subjects (mean age)	Stage of AD			Notes	Reference
		Mild	Moderate	Severe		
Frontal cortex	15 control (63), 11 mild (74), 9 mod. (79) and 20 severe (81).	↑mRNA, ↑Protein	↑mRNA, ↑Protein	↑mRNA, =Protein	Non-aged matched controls	(Lopez-Gonzalez et al., 2015)
Orbito-frontal cortex	11 control (53), 10 mild (75), 15 mod. (80) and 6 severe (77).	=mRNA	↑mRNA	=mRNA		
Entorhinal cortex	7 control (53), 11 mild (77), 13 mod. (79) and 15 severe (81).	=mRNA	=mRNA	↑mRNA		
Frontal white matter	20 control (79) and 16 severe (84)			↓Protein		(Chen et al., 2016)
Entorhinal cortex	5 control (82) and 11 mod./severe		↓mRNA, ↓Protein			(Wood et al., 2015)
Frontal cortex	37 control (84), 23 mild (87) and 16 severe (86)	↑mRNA		↑mRNA		(Sudduth et al., 2013)
Frontal cortex	10 control (70) and 20 severe (70)			↑mRNA, ↑Protein		(Rao et al., 2011)

IL-6

IL-6 is a pleiotropic cytokine which is highly upregulated in infection and inflammatory states (McGeough et al., 2012). IL-6 is thought to function as both anti-inflammatory through signalling through its membrane bound receptor and pro-inflammatory through its soluble receptor (Scheller et al., 2011). Similar to IL-1β, whether IL-6 is upregulated in AD brains is controversial with Table 1.3 summarising IL-6 concentrations in AD patients. AD patients exhibit increased concentrations of circulating IL-6 (Swardfager et al., 2010).

Table 1.3: Studies assessing IL-6 concentrations in AD patients

Brain region	Subjects (mean age)	Stage of AD			Notes	Reference
		Mild	Moderate	Severe		
Frontal cortex	15 control (63), 11 mild (74), 9 mod. (79) and 20 severe (81).	↑mRNA, ↑Protein	↑mRNA, ↓Protein	=mRNA, =Protein	Non-aged matched controls	(Lopez-Gonzalez et al., 2015)
Orbito-frontal cortex	11 control (53), 10 mild (75), 15 mod. (80) and 6 severe (77).	↑mRNA	=mRNA	↑mRNA		
Entorhinal cortex	7 control (53), 11 mild (77), 13 mod. (79) and 15 severe (81).	=mRNA	↑mRNA	↑mRNA		
Frontal white matter	20 control (79) and 16 severe (84)			↓Protein		(Chen et al., 2016)
Entorhinal cortex	5 control (82) and 11 mod./severe		=mRNA, ↑Protein			(Wood et al., 2015)
Frontal cortex	37 control (84), 23 mild (87) and 16 severe (86)	↑mRNA		↑mRNA		(Sudduth et al., 2013)

INFγ

INFγ is a type 2 interferon and is important for the transition from the innate to adaptive immune response (Schroder et al., 2004a). The primary response to INFγ is production of antiviral mediators and to a lesser extent antibacterial mediators and has been implicated in the development of autoimmune disorders in the CNS (Schroder et al., 2004b). Furthermore, INFγ in the CNS appears to be an inducer of neurogenesis (Baron et al., 2008) and stimulate microglia activation (Tsuda et al., 2009). While the concentration of IFNγ has been less widely assessed in the brains from AD patients, IFNγ mRNA was found upregulated in mild and severe AD while protein concentrations were found to increase in severe stages of the disease (Wood et al., 2015).

TNFα

TNFα is a pro-inflammatory cytokine and is highly involved in regulation of immune cells as well as acting as an endogenous pyrogen. TNFα signalling is associated with induction of pro-inflammatory mediators in addition to being able to induce apoptosis (Guadagno et al., 2013). The concentration of TNFα in the brains from AD patients is controversial with studies assessing TNFα concentration summarised in Table 1.4. In contrast, increased concentrations of circulating TNFα has been observed in AD patients.

Table 1.4: Studies assessing TNFα concentrations in AD patients:

Brain region	Subjects (mean age)	Stage of AD			Notes	Reference
		Mild	Moderate	Severe		
Frontal cortex	15 control (63), 11 mild (74), 9 mod. (79) and 20 severe (81).	=mRNA	=mRNA	=mRNA	Non-aged matched controls	(Lopez-Gonzalez et al., 2015)
Orbito-frontal cortex	11 control (53), 10 mild (75), 15 mod. (80) and 6 severe (77).	=mRNA	=mRNA	=mRNA		
Entorhinal cortex	7 control (53), 11 mild (77), 13 mod. (79) and 15 severe (81).	=mRNA	=mRNA	=mRNA		
Entorhinal cortex	5 control (82) and 11 mod./severe		↓mRNA, ↑Protein			(Wood et al., 2015)
Frontal cortex	37 control (84), 23 mild (87) and 16 severe (86)	↑mRNA		↑mRNA		(Sudduth et al., 2013)
Frontal cortex	10 control (70) and 20 severe (70)			↑mRNA, ↑Protein		(Rao et al., 2011)

IL-10

IL-10 is the prototypical anti-inflammatory cytokine and is important in suppressing un-necessary inflammatory reactions and terminating the inflammatory cascade once initiated (Couper et al., 2008). IL-10 has consistently been found upregulated in the CNS from AD subjects, with Table 1.5 summarising the key findings in post-mortem studies. Paradoxically, the elevation of IL-10 in AD supports an underlying inflammatory environment as the cytokine is upregulated in response to inflammatory insults (Erickson and Banks, 2011).

Table 1.5: Studies assessing IL-10 concentrations in AD patients:

Brain region	Subjects (mean age)	Stage of AD			Notes	Reference
		Mild	Moderate	Severe		
Frontal cortex	15 control (63), 11 mild (74), 9 mod. (79) and 20 severe (81).	=mRNA,	=mRNA,	↑mRNA	Non-aged matched controls	(Lopez-Gonzalez et al., 2015)
Orbito-frontal cortex	11 control (53), 10 mild (75), 15 mod. (80) and 6 severe (77).	↑mRNA	=mRNA	↑mRNA		
Entorhinal cortex	7 control (53), 11 mild (77), 13 mod. (79) and 15 severe (81).	↑mRNA,	↑mRNA	↑mRNA,		
Entorhinal cortex	5 control (82) and 11 mod./severe		=mRNA, ↑Protein			(Wood et al., 2015)
Frontal cortex	37 control (84), 23 mild (87) and 16 severe (86)	=mRNA		↑mRNA		(Sudduth et al., 2013)

Chemokines

Like cytokines, chemokines are small inflammatory signalling molecules secreted by inflammatory cells. Where cytokines generally function as effectors of the inflammatory responses, chemokines are the chemoattractants. The function of chemokines is to induce chemotaxis of immune cells, recruiting them to the site of injury. In addition to functioning as chemoattractants, certain chemokines have been shown to modulate the inflammatory response. Numerous chemokines are increased in brains from AD patients, including MCP-1, MIP-1 and RANTES. (Liao et al., 2011, Kester et al., 2011, Wood et al., 2015). On the other hand, IL-8 has been found both up (Wood et al., 2015) and down (Chen et al., 2016) regulated in AD patients, while IP-10 levels have been found to decrease (Chen et al., 2016).

Complement factors

The complement cascade involves a complex set of serine-protease cleavages of complement fragments and can activate the classical or alternatively pathways; with the former requiring an antigen-antibody complex. The end product of the complement cascade is the membrane attack complex (MAC) which disrupts the plasma membrane of cells; leading to cell death (Müller-Eberhard, 1988). Importantly, MAC disruption has been reported to affect healthy, neighbouring, "bystander" cells (Park et al., 1997). In addition to generation of the MAC, the complement cascade is associated with induction of pro-inflammatory cytokines and activation of innate immune cells (Merle et al., 2015). Both aggregated amyloid and tau have been reported to induce the complement system, resulting in the formation of the MAC (Shen et al., 2001). Not surprising then have multiple components of the complement system been associated with amyloid plaques (Afagh et al., 1996, Ishii and Haga, 1984) and NFT containing neurons (McGeer et al., 1989). Supporting a strong role for complement activation in AD, virtually all complement factors involved in the cascade are upregulated in AD (Shen et al., 1997) and increased levels of the MAC been observed in AD patients (Webster et al., 1997).

Prostaglandins

Prostaglandins are lipid signalling molecules generated from the biosynthesis of arachidonic acid and involve catalysis by COX enzymes which are the target of non-steroidal anti-inflammatory drugs (NSAIDs). Prostaglandins have diverse functions and have a wide range of biological and homeostatic functions when driven by COX-1 catalysis which is constitutively active. Conversely, catalysis by COX-2 which is induced by inflammation increases the concentration of prostaglandins and resulting in inflammation (Ricciotti and FitzGerald, 2011). COX-2 is well documented to be upregulated in areas affected in AD (Yasojima et al., 1999a, Pasinetti and Aisen, 1998) and occurs at early stages within the disease (Hoozemans et al., 2005, Hoozemans et al., 2004).

ROS and RNS

One of the end products any inflammatory response is the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) inducing oxidative stress. Oxidative stress is known to damage lipids, proteins, DNA and mitochondrial DNA (mtDNA) resulting in necrosis or apoptosis if left unchecked (Cho et al., 1999, Aprioku, 2013). There is substantial oxidative stress in AD, with AD post mortem examinations showing oxidative damage to lipids, proteins, mtDNA and DNA (Lyras et al., 1997, Ansari and Scheff, 2010, Mecocci et al.,

1994). Furthermore, oxidative stress appears to be an early event within the disease (Nunomura et al., 2001).

Specialised pro-resolving mediators

While pro-inflammatory mediators are responsible for inducing acute inflammation, resolution pathways involving specialised pro-resolving mediators (SPM's) are responsible ensuring inflammation does not become chronic. SPM's are lipid mediators which are produced from the metabolism of arachidonic acid, eicosapentaenoic acid or docosahexaenoic acid generating five classes of SPMs; lipoxins, E-resolvins, D-resolvins, protectins and maresins (Serhan et al., 2015). SPM's possess a variety of pro-resolving functions including the removal of debris, resolution of inflammation and regeneration of tissue (Basil and Levy, 2016). The induction of SPM's is delayed compared to that of pro-inflammatory mediators which enables a dynamic balance between the pro-inflammatory response and its resolution (Sansbury and Spite, 2016). Take for instance lipoxins which are metabolised from arachidonic acid which is also required for the production of prostaglandins and leukotrienes (Serhan et al., 2015). Prostaglandin and leukotriene synthesis occurs early in the response to TNFα treatment in a mouse air pouch model while lipoxin synthesis occurs at a distinct later time-point (Levy et al., 2001). Furthermore, the authors report that PGE_2 is responsible for the inhibition of leukotriene production and promotion of lipoxin synthesis in a process known as class switching. The process of class switching is important in ensuring inflammatory processes do not become chronic (Serhan and Savill, 2005), making SPMs of interest in chronic inflammatory conditions such as AD. While receiving less attention than pro-inflammatory pathways, there is limited evidence that pro-resolution pathways are impaired in AD. Compared to control subjects, lower levels of lipoxin A_4 and higher levels of PGE_2 are present in the hippocampus of AD patients indicating there might be a failure to initiate class switching despite the higher concentrations of PGE_2 (Wang et al., 2015b). Interestingly, the authors report increased hippocampal expression of the lipoxin A_4 receptor and the resolving E1 receptor; ChemR23. The increase in SPM receptors within the AD brain could be resulting from a compensatory mechanism linked to increased levels of inflammation. Furthermore, neuroprotectin D1 and its precursor, docosahexaenoic acid, were reported to be reduced in the hippocampus from AD patients (Lukiw et al., 2005). Accompanying decreases in protection D1, the authors reported reduced levels of 15-lipoxygenase – a key enzyme in the generation of neuroprotection D1 synthesis. Further suggesting an impairment of the docosahexaenoic acid pathway in AD, liquid chromatography-tandem mass spectrometry of entorhinal cortex

samples from AD patients revealed decreased levels of resolving D1, maresin 1 and protectin D1 (Zhu et al., 2016). Together, these results suggest there might be impaired biosynthesis of SPM's in AD, although further research is required to understand the biological relevance in light of compensatory increases in their receptor expression.

1.3.3 Crosstalk between inflammation mediators and tau phosphorylation:

Mapping inflammatory signalling pathways and their common tau kinases

By understanding the signalling pathways which are affected by inflammatory stimuli, we can determine the potential for neuroinflammation to modulate tau pathology. Figure 1.9 represents common inflammatory pathways modulating kinases and phosphatases which have been linked with the tau phosphorylation. Inflammatory signals that signal through G_s receptors such as the prostaglandin EP2/4 receptors induce PKA which inhibits both GSK-3β and CK1δ through their phosphorylation (Giamas et al., 2007, Yokoyama et al., 2013, Fang et al., 2000). Additionally, PKA can indirectly induce the activation of GSK-3β and CK1ε through increasing the activity of PP2A (Vinyoles et al., 2016, Qian et al., 2010). Similar to G_s, receptors which signal through the G_q mechanism such as purinergic P2X7 and prostaglandin EP1 receptors inhibit both GSK-3β and CK1δ through their phosphorylation by PKC (Meng et al., 2016, Rundhaug et al., 2011). Three of the common pathways associated with inflammatory signalling are MAPK, PI3K and NF-κB. MAPKs are induced by a variety of inflammatory receptors including TLR4, IL-1RI and TNFR1/2 and results in enhanced activity of ERK1/2, JNK and p38 – all of which have been implicated in tau phosphorylation in AD (Martin et al., 2013, Guo and Friedman, 2010, Risbud and Shapiro, 2013, Aggarwal, 2003). A result of ERK1/2 activation is increased CDK5 activity through p35 transcription and CDK5 transcription while JNK and p38 all decrease CDK5 activity through inhibiting p35 expression (Shah and Lahiri, 2014). Additionally, p38 enhances the activity of CK2 which can further activate NF-κ2 trough actions similar to IKK (Yu et al., 2006, Sayed et al., 2000). PI3K signal transduction is initiated by a multitude of receptors including TLR4 and TNFR2 which results in the inhibition of GSK-3β through its phosphorylation by Akt (Guo and Friedman, 2010, Urschel and Cicha, 2015, Manning and Toker, 2017). Additionally, PI3K pathways have the potential to affect tau phosphorylation through modulation of the MAPK pathway (Quan et al., 2015). In a similar manner to JNK and p38, NF-κB inhibits the transcription of p35, reducing CDK5 activity (Shah and Lahiri, 2014). Although LRRK2 is a kinase predominately associated with Parkinson's disease (Zimprich et al., 2004), INFγR and TLR4 signalling through ERK5 induces LRRK2 activation (Kuss et al., 2014, Schapansky et al., 2014, Dzamko et al., 2012) - a potential interaction which could occur in AD. Finally, ROS

production, a common consequence of inflammation, can affect tau phosphorylation through induction of PP2A (Sheth et al., 2009). The common kinases affected by inflammation and those implicated in tau phosphorylation suggest that inflammation can effect most tau phosphorylation sites which have been found in AD brains (Martin et al., 2013).

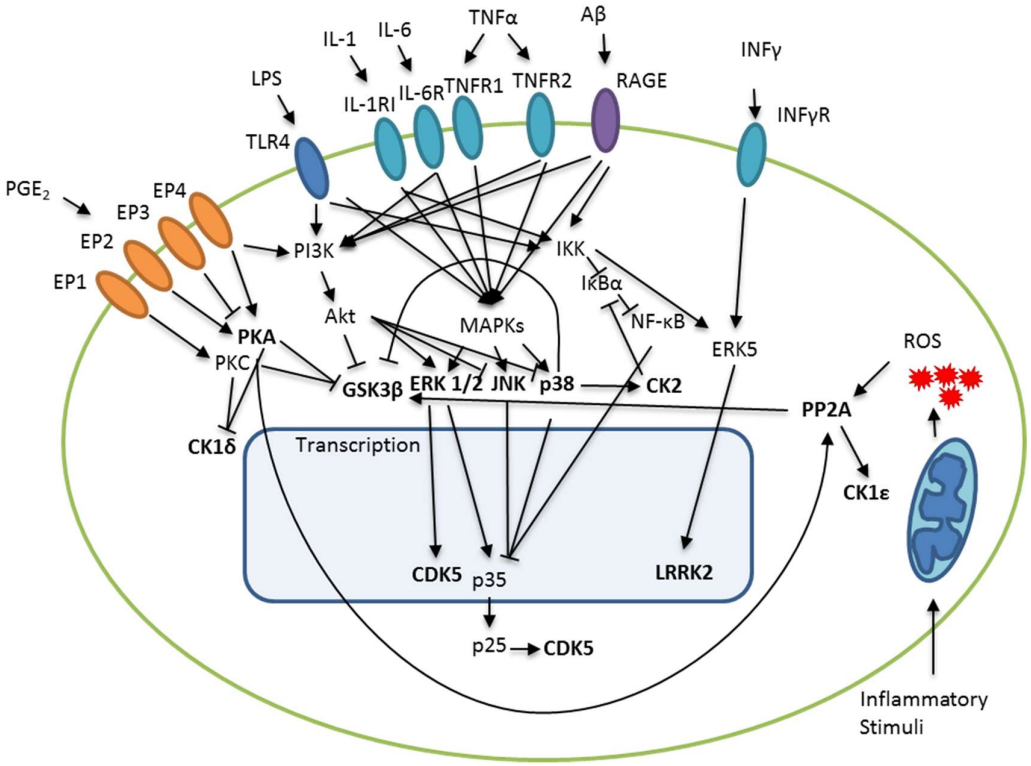

Figure 1.9: Inflammatory signalling pathways which affect kinases which are important in tau phosphorylation. Bold represents kinases which phosphorylated tau and the blue box indicates transcription induced by inflammatory signalling. Figure drawn by author.

Experimental evidence for overlap between inflammatory signalling and tau phosphorylation

Several attempts have been made to understand the potential effect of specific inflammatory mediators on tau phosphorylation. Treatment of primary cortical neurons with IL-1β was reported to increase tau phosphorylation in a p38 dependant manner (Li et al., 2003). The inducible expression of IL-1β selectively in the brains of 3xTg mice by crossing with IL-1β^{XAT} mice was shown to increase hippocampal pT202/205, pT231 and pS396/404 tau (Ghosh et al., 2013). Together, these studies indicate that IL-1β, or one of its downstream effectors, is an inducer of tau phosphorylation. IL-6 has been reported to induce tau phosphorylation in primary hippocampal neurons at the pS202/205 epitope in a

CDK5 dependant manner while decreasing levels of dephosphorylated tau (Quintanilla et al., 2004). The effect of INFγ on tau pathology is controversial. Adenoviral over expression in the rtg4510 model found an increase in soluble pS396/404 tau levels and a decrease in total tau levels (Li et al., 2015). The authors additionally reported an increase in pS396/404 tau in JNPL3 mice expressing the same P301L mutation. This is in contrast to adenoviral overexpression of IFNγ in the 3xTg model which resulted in tau dephosphorylation at multiple pre-tangle associated phosphorylation sites (Mastrangelo et al., 2009). The contrast between the two studies could be resulting from the presence of amyloid in the latter model, with further investigation required to elucidate the effect INFγ has within AD. Chronic adenoviral overexpression of TNFα additionally caused an increase in the pre-tangle-associated pT231 epitope (Janelsins et al., 2008). However, ablation of both TNF receptors in the 3xTg model paradoxically resulted in increased tau phosphorylation (Montgomery et al., 2011), suggesting too little and too much inflammation can result in tau phosphorylation. Finally, the effect of IL-10 on tau phosphorylation has been relatively understudied. One study found that chronic overexpression of IL-10 failed to impact tau phosphorylation in an amyloid model (Chakrabarty et al., 2015) but further studies are required to understand its effect in tau models. Together these studies indicate that inflammatory signalling cascades can indeed induce the phosphorylation of tau.

1.3.4 Summary of neuroinflammation in AD

Neuroinflammation in AD is a dynamic process. Two key immunological cells within the CNS: astrocytes and microglia are upregulated and activated at an early stage in AD and localise around both hallmarks. Microglia and astrocytes are important cellular mediators of the neuroinflammatory response. Recent advances in technology are furthering our understanding of how microglia dynamically change during the course of the disease. In models of amyloid deposition, powerful single cell RNAseq techniques demonstrate that microglia undergo a phenotypic change to a protective state. Further investigation is required to understand how DAM's affect the progression of tau pathology. While similar strategies have not been conducted in tau models, evidence from post-mortem examinations suggests that tau may differentially affect microglia and induce their senescence. The BBB is integral in transferring the systemic inflammatory response into the brain; with recognition of blood born factors able to stimulate neuroinflammatory changes. Through analysis of common inflammatory signalling cascades it is clear that there is a strong overlap with kinases induced by inflammation and those responsible for tau phosphorylation. While assessing inflammatory mediators in the brain has its pitfalls, many

inflammatory mediators which share common overlap between inflammatory signalling pathways and kinases which phosphorylated tau have been shown to induce tau phosphorylation in *in vitro* or *in vivo* models. This suggest that systemic inflammation could be a strong inducer of tau pathology through modulating the neuroinflammatory environment.

1.4 The interaction between systemic inflammation and tau pathology

1.4.1 Methods for modelling systemic inflammation in animal models

To truly understand the contribution of inflammation on tau pathology, systemic inflammation needs to be modelled in animal models in a relevant manner to AD. Systemic inflammation in AD is a chronic mild inflammatory event with concentrations of circulating cytokines in the low pg/ml range (Wu et al., 2015, Choi et al., 2008, Holmes et al., 2009, Dursun et al., Chao et al., 1994, Yasutake et al., 2006, Singh and Guthikonda, 1997). There are a plethora of models to induce systemic inflammation in animal models. These can be classed into three broad categories; those which use challenges to model systemic inflammation, those which model specific inflammatory conditions and those which model systemic infection. A fourth method exists through overexpression of inflammatory mediators, although unless specifically under a promoter in the peripheral compartment, are expressed in centrally as well. As this is often the case, deciphering whether effects are due to systemic or neuroinflammation is difficult. Due to the diverse nature of any person's inflammatory history, solely using one model of systemic inflammation is not enough to decipher its role in AD. Instead only by modelling systemic inflammation under different conditions will we be able to understand the contribution of various inflammatory stimuli in the progression of AD.

Models of specific peripheral inflammatory conditions

There are several peripheral inflammatory conditions that exist in humans which can be modelled in rodents. Without being drawn into the debate of how well these models resemble the human condition, they do provide an interesting opportunity to induce chronic systemic inflammation in AD models. A further added bonus is that many of these peripheral inflammatory conditions are in fact risk factors for AD and can provide insights into the contribution of these comorbidities to the disease. For example, rheumatoid arthritis (RA) is thought to both be a risk factor in the development of AD (McGeer et al., 1996) and involves the upregulation of multiple circulating pro-inflammatory cytokines including; TNFα, IL-4 and IL-12 (Azizieh et al., 2017). There are multiple models of RA in rodents including but not

limited to; collagen induced arthritis (CIA), antigen induced arthritis and streptococcal cell wall induced arthritis (Kannan et al., 2005). A few studies have attempted to assess the systemic inflammatory phenotype of RA models. While the streptococcal cell wall model does indeed induce systemic IL-1, IL-6 and TNFα, this response appears only in the acute phase due to the streptococcal cell wall injection rather than the RA (Fuseler et al., 1997). Furthermore, a longitudinal study of serum cytokines in an antigen induced arthritis model found a complete absence in the induction of serum IL-1β, IL-2, IL-6, IL-9, IL-13, IL-17, IL-18 and INFγ following methylated bovine serum albumin (BSA) injection into synovial joints (Paquet et al., 2012). Conversely, increased serum IL-6 and TNFα were observed in 19 days after the last collagen injection in the CIA model (Amdekar et al., 2011). Supporting long term systemic inflammation during CIA, a further study found elevated serum IL-1β, IL-6 and TNFα 21 days after the collagen injection (Tsubaki et al., 2015). Importantly, the largest change in serum expression was a change of around 200 pg/ml in the former study indicating mild inflammation was present. Furthermore, CIA has been reported to induce significant microglial activation in mice (Park et al., 2011). A disadvantage of the CIA model is that it induces depression in locomotor activity which might affect behavioural studies (Hartog et al., 2009). Taken together, these studies suggest CIA as an interesting model to induce mild, chronic, systemic inflammation.

Further potentially suitable models for peripheral inflammatory conditions are those for atopic disorders such as asthma. Similar to RA, asthma is both a risk factor for AD (Eriksson et al., 2008) and asthmatics have elevated levels of circulating pro-inflammatory mediators such as IL-4, IL-5 and INFγ (ten Hacken et al., 1998). The most common asthma model in rodents is the ovalbumin sensitisation model. Following chronic ovalbumin administration in mice, there is a chronic upregulation in serum pro-inflammatory cytokines IL-4, IL-5 and Il-13 and the anti-inflammatory cytokine IL-10 (Srivastava et al., 2010). The authors report the changes in each cytokine to be in the region of 100-400 pg/ml, again suggesting the model to involve chronic, mild, systemic inflammation. Ovalbumin sensitisation additionally results in neuroinflammation characterised by increased microglial and astrocyte activation (Klein et al., 2016, Spaziano et al., 2015). However, due to its skew towards a Th2 phenotype (Barnes, 2001), asthma models are not optimal for understanding the effect of general systemic inflammation on tau pathology. This is because the Th2 phenotype is characterised by high levels of IL-4 (Barnes, 2001), which is responsible for shifting microglia to a, protective, anti-inflammatory phenotype (Liu et al., 2016b). Therefore, the model only provides utility in aiding with the understanding of how atopic

disease may contribute to AD. Furthermore, models of atopic disease are characterised by increased levels of IgG's and IgE's (Sarlus et al., 2012). The heavy chain of these have a molecular weight of 54-55 kDa and 68 kDA respectively (Bazin et al., 1974). As both are of similar molecular weight to tau depending on its post-translational modifications, technological considerations need to be made when assessing tau phosphorylation by western blotting in atopic models such as using TrueBlot antibodies or using heat stable fractions (Petry et al., 2014). A further limitation is that for sensitisation to occur, a mouse strain susceptible to allergies is required such as the BALB/c strain and might require changing the background strain of common tau models (Zhu and Gilmour, 2009, Sarlus et al., 2012).

Finally models which model lifestyle choices such as obesity that are associated with systemic inflammation provide an additional method for inducing mild, chronic, systemic inflammation. Obesity is a risk factor for Alzheimer's disease (Vagelatos and Eslick, 2013) and is associated with chronic systemic inflammation such as elevated circulating levels of IL-6 and TNFα (Chen et al., 2013, Pickup et al., 2000). Rodents fed with a high fat diet are a common model for type 2 diabetes which involves the development of insulin resistance (Winzell and Ahrén, 2004). Likewise, chronic systemic inflammation is associated with the high fat model of diabetes is associated with chronically elevated circulating levels of IL-1β, IL-6 and TNFα (Kim et al., 2012). Importantly, the authors report the systemic inflammation expressed in the model is mild, with the highest increase in concentration for TNFα at 150 pg/ml. Despite the mild inflammation expressed, the high fat diet is associated with significant microglial activation adding relevance to AD (Knight et al., 2014). However, a major drawback of the model, and models of diabetes in general, is that they are associated with insulin resistance (Vagelatos and Eslick, 2013) which in itself has been found to affect tau pathology (El Khoury et al., 2014). Therefore, confusion can occur as to whether the systemic inflammation or insulin resistance is inducing pathological alterations. As such, Type-2 diabetes models are not suitable for understanding the effects of systemic inflammation on tau pathology.

Models of systemic infection

A major mechanism for systemic inflammation in humans is through infection. This can come through various forms, whether an extreme acute infection such as a blood infection or a sustained mild infection such as a periodontal infection. As with peripheral inflammatory conditions, many systemic infections can be modelled in rodents. One model

of systemic infection in rodents is through the systemic administration of attenuated strains of bacteria. For example, a single *intraperitoneal* (*i.p*) injection of the SL3261 *Salmonella typhimurium* strain in mice results in increased circulating: INFγ peaking 7 days after infection, IL-12 which remained elevated 3 weeks post-infection but failed to affect serum IL-1β levels (Püntener et al., 2012). Accompanying increases in serum cytokines, the authors report microglia activation and long term increases in IL-1β and IL-12 expression within the brain. A further study found increased serum INFγ, TNFα, IL-2, IL-4, IL-5, IL-6, IL-10 and IL-12 four weeks following infection with SL3261 *Salmonella typhimurium* administered *intranasally* in mice which involved a booster 14 days after initial infection (Moustafa et al., 2015). Similarly, mice *intravenously* (*i.v.*) infected with attenuated *Salmonella typhimurium* strain; BRD509 showed increases in serum INFγ, TNFα and IL-6, peaking 3 weeks following infection (Kupz et al., 2014). These studies demonstrate that attenuated bacteria strains are a viable model to induce chronic systemic inflammation. One issue arising when utilising infectious models is, depending on the pathogen, that different inflammatory profiles are expressed. For example, *i.p.* infection with *Campylobacter jejuni* in mice results in increased serum INFγ but no effect on serum IL-6 concentrations while the reverse is true for infection with *Listeria monocytogenes* (Abram et al., 2000). A further issue with models of systemic infection is that the level of inflammation is too strong to be comparable with the underlying pathogenesis occurring within AD. A comparison between two models of systemic infection: peritoneal contamination and cecil ligation/puncture, found the inflammatory response and severity were generally similar to that from 5 mg/kg LPS administration (Seemann et al., 2017) – a dose which is severe enough to induce chronic neurodegeneration (Qin et al., 2007). This could highlight the need to use attenuated bacterial strains, although even these have a higher inflammatory profile than those expressed in AD (Püntener et al., 2012, Swardfager et al., 2010). As such, they offer an excellent opportunity to understand exacerbating effects of systemic infection on AD progression.

Models involving systemic immune challenges

There are various models which induce systemic inflammation through the administration of a molecule which stimulates a specific aspect of the immune system. These can be split into two categories; those which utilise endogenous biologics and those which involve the administration of exogenous immune stimulators. While the former can involve a large variety of biologics, two of the most common models have been the systemic administration of IL-1β and TNFα. Administration of both pro-inflammatory cytokines results in a pyrogenic response, sickness behaviours and induce a range of systemic inflammatory

mediators including – IL-1β, IL-6 and TNFα (Skelly et al., 2013, Biesmans et al., 2015). Furthermore, systemic administration of both molecules induces hippocampal expression of IL-1β, IL-6 and TNFα mRNA in addition to inducing microglial activation (Skelly et al., 2013, Biesmans et al., 2015, Burrows et al., 2016). There are a few advantages of modelling systemic inflammation in this manner. It is a method of inducing a wide range of peripheral and central inflammatory mediators through a mechanism which occurs naturally. Furthermore, the biologic chosen can be one that is upregulated in the periphery of MCI and AD patients, increasing the validity. Finally, they can be informative into the specific contribution of endogenous inflammatory mediators in the AD process and highlight possible avenues for therapeutic intervention. An additional advantage, if expressed genetically, then the biologic can be expressed chronically. However, disadvantages exist when utilising this class of model. The concentrations utilised are often much higher than that is seen under physiological conditions. Together with the fact that many inflammatory mediators penetrate across the BBB, means that the models in themselves are not truly representative of systemic inflammation (Banks et al., 1995). Furthermore, by stimulating one specific aspect of the immune system, there are vast differences in the inflammatory profiles that are expressed depending on the inflammatory mediator administered (Skelly et al., 2013). This again highlights the value of these models relies in deciphering the contribution of a specific inflammatory mediator on a given pathology although might not necessarily be fully representative of systemic inflammatory conditions in AD.

Instead a more balanced approach is required when trying to understand the contribution of systemic inflammation in AD, which comes in the form of systemic administration of exogenous immune stimulators. Two of the most common are the systemic administration of polyinosinic:polycytidylic acid (Poly I:C) and LPS. The former is a mimetic of double stranded RNA (dsRNA) which is present in some viruses and acts as a TLR-3 agonist (Li et al., 2005). The latter is a component of –ve bacteria cell wall and is a TLR-4 agonist (Chow et al., 1999). Despite both molecules stimulating inflammation associated with either bacterial or viral infection, both challenge models are not considered effective models of infection due to their simplicity. Instead, a more appropriate terminology is models of systemic inflammation where the choice between the two relies on the specific inflammatory profile required. Through activation of TLR-3 signalling: Poly I:C is predominately associated with both systemic and central upregulation of IL-1β, IL-6, TNFα as well as viral inflammatory mediators such as type 1 interferons (Kumar et al., 2006, Murray et al., 2015). Similar to LPS, systemically administered Poly I:C has been reported to induce

microglia activation (Gibney et al., 2013) although may require prior microglial priming (Field et al., 2010). Additionally, Poly I:C appears to be relatively resistant to tolerance, making it more suitable for chronic models (Cunningham et al., 2007, Soszynski et al., 1991). Although Poly I:C is synthetic and not found naturally, the dsRNA dependent kinase; PKR is upregulated in brain tissue from AD patients positive for HSV-1 (Bullido et al.) However, TLR3 activation has been documented to break down the BBB, enabling both viruses and their dsRNA to enter the brain (Wang et al., 2004). In a similar manner, Poly I:C has been found to induce breakdown of the BBB and induce microglia activation in rodents (Ifuku et al., 2014). The ability for Poly I:C to disrupt the BBB means that as an inflammatory model, Poly I:C is not strictly a systemic model due to its ability to elicit a direct central inflammatory response.

Systemically administered LPS provides an interesting model of inflammation with specific relevance to AD. A phenomenon known as “leaky gut” has been suggested to occur in AD (Cristiano et al., 2016) which results in higher levels of circulating LPS in AD patients compared to control subjects (Zhang et al., 2009c). Furthermore, obesity which is a risk factor for developing AD (Alford et al., 2018), is associated with metabolic endotoxemia which is characterised by chronic elevated levels of circulating LPS and high levels of systemic inflammation (Boutagy et al., 2016). Together these studies not only suggest that circulating LPS is present at higher concentrations in AD, but higher levels of circulating LPS is a risk factor for developing the disease. Systemic LPS administration in mice is associated with a marked upregulation of various peripheral and central inflammatory mediators including IL-1β, IL-6 and TNFα as well as microglial activation (Erickson and Banks, 2011, Bhaskar et al., 2010). Importantly, LPS does not cross the BBB (Banks and Robinson, 2010). As the inflammatory response is dependent on endogenous production/release of inflammatory mediators, the inflammatory response mediated is more natural in nature compared to administration of a single endogenous mediator. Furthermore, increased levels of circulating and central LPS has been reported in AD patients: adding further relevance of the model to AD (Zhan et al., 2016, Zhang et al., 2009c). Furthermore, RNAseq analysis of post-mortem AD tissue reveals many genes upregulated in microglia are shared with those associated with systemic LPS administration in rodents; suggesting a similar neuroinflammatory response between the two (Friedman et al., 2018). The association of endogenous LPS with AD, as well as the similar microglial phenotypes seen in AD and those evoked by LPS administration in rodents makes systemically administered LPS one of the most relevant models of systemic inflammation in AD. For that reason, this thesis will focus

on LPS as a model to understand the contribution of systemic inflammation to tau pathology in AD. One caveat of the model is that if injected in too high concentrations, LPS is a model of sepsis (Thomas et al., 2014) rather than the mild chronic inflammation associated with AD. A summary of the different models of inflammation is given in Table 1.6.

Table 1.6: Summary of systemic inflammatory models

Class	Model	Advantages	Disadvantages
Peripheral inflammatory conditions	Ovalbumin (asthma)	• Atopic diseases a risk factor in AD • Induces systemic and neuroinflammation • Mild inflammation • Suitable for chronic studies	• Characterised by a Th_2 response – protective in brain • Increased levels of IgG's mean special considerations are required when assessing tau
	CIA (rheumatoid arthritis)	• Rheumatoid arthritis risk factor in AD • Induces systemic and neuroinflammation • Mild inflammation • Suitable for chronic studies	• Impairs locomotor activity
	High fat diet (Type-2 diabetes)	• Type-2 diabetes a risk factor in AD • Induces systemic and neuroinflammation • Mild inflammation • Suitable for chronic studies	• Associated with additional pathological features such as insulin resistance
Systemic infection	Attenuated infections (e.g. SL3261 *Salmonella typhimurium*)	• Systemic infections associated with exacerbation of AD symptoms • Induces systemic and neuroinflammation • Suitable for chronic studies	• Nature of response is pathogen dependent • Can induce strong inflammatory alterations
Challenge	Biologics (IL-1β, TNFα, etc…)	• Induces systemic and neuroinflammation • Useful for understanding contribution of specific inflammatory pathways	• Unbalanced inflammatory response (relevance to AD?) • Some cross the BBB – not models of systemic inflammation • Often administered at concentrations much higher than physiologically possible • Tolerance (not suitable for chronic studies) • Causes sickness syndrome
	LPS	• Found in brains of AD patients • Induces systemic and neuroinflammation • Increased levels of circulating LPS in AD patients • Does not cross the BBB • Induces similar microglial phenotypic alterations which are found in AD	• Model of sepsis if administered at too high concentrations • Tolerance (not suitable for chronic studies) • Causes sickness syndrome
	Poly I:C	• Evidence for dsRNA in brains from AD patients • Induces systemic and neuroinflammation	• Causes breakdown of BBB – not a model of systemic inflammation • Tolerance (not suitable for chronic studies) • Causes sickness syndrome

1.4.2 Lipopolysaccharide as a model for systemic inflammation in vivo

LPS signalling pathway

Because of its high validity for modelling inflammation in AD, the most utilised method for modelling the effect of systemic inflammation on tau pathology is through systemic administration of LPS. LPS elicits its inflammatory effects through acting as a TLR4 agonist (Chow et al., 1999). Figure 1.10 summarises the TLR4 signalling pathway in response to LPS. Upon host infection, LPS-binding protein (LBP) extracts LPS from gram –ve bacterial and binds to CD14. This induces binding with the TLR4-MD2 complex, inducing myD88 dependent and independent cascades. The myD88 dependent results primarily in production of pro-inflammatory cytokines, while the independent pathway in type 1 interferons (Lu et al., 2008).

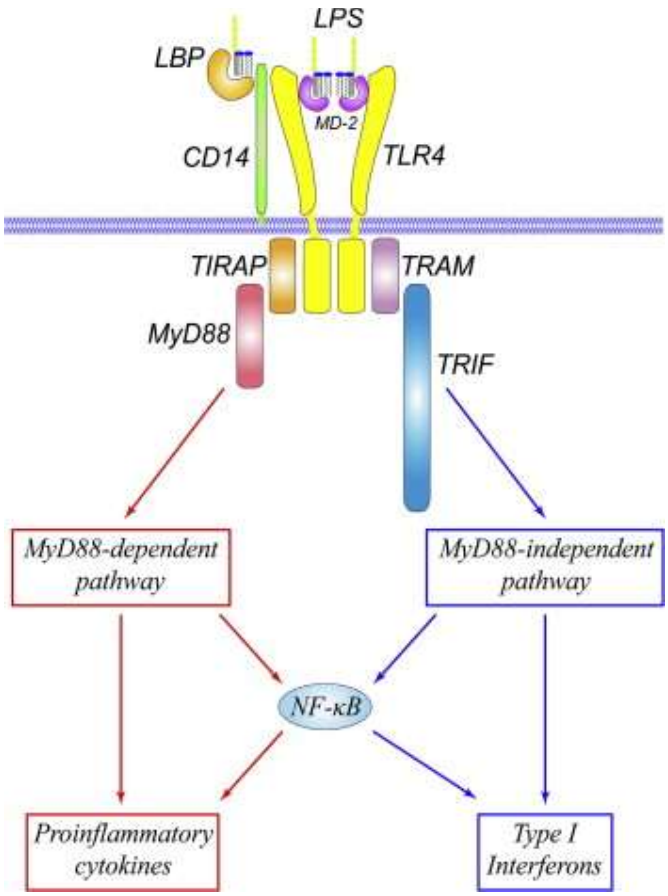

Figure 1.10: LPS induces pro-inflammatory signalling through TLR4 induction. LPS bound to LBP associates with CD14 and TLR4 causing induction of the pro-inflammatory cascade. Intracellular signalling through a MyD88-dependent pathway induces pro-inflammatory cytokines while signalling through the MyD88-independent pathway stimulates the transcription of type 1 interferons (Lu et al., 2008).

Systemic administered lipopolysaccharide induces neuroinflammatory changes in mice

Systemically administered LPS does not cross the blood brain barrier (Banks and Robinson, 2010) but is able to elicit central inflammatory responses nonetheless (Erickson and Banks, 2011) due to crosstalk between the peripheral and central immune system. This is mediated through a variety of routes including: neural such as vagal afferents, humoral through circumventricular organs, infiltration of peripheral monocytes and through effects on the BBB (Miller and Raison, 2016, Pardon, 2015). Thus, systemic inflammation in itself induces a central neuroinflammatory response. For example, systemically administered LPS induces microglial and astrocyte activation in addition to upregulation of a multitude of pro-inflammatory mediators such as IL-1β, IL-6, IL-10, IP-10, CXCL1, MCP-1, MIP-1α, COX-2, RANTES and TNFα within the central compartment (Erickson and Banks, 2011, Griffin et al., 2013, Kitazawa et al., 2005, Norden et al., 2016). One question that arises is whether the profile of neuroinflammatory mediators induced by LPS is similar to those altered within AD. As of yet, this is unknown due to simply not knowing enough about the underlying inflammatory conditions within the brains from AD patients. However, one recent study that utilised powerful single-cell RNAseq technology found that there was a similar pattern of genes affected in microglia from rodents systemically administered with LPS and from those taken from bulk tissue from AD patients (Friedman et al., 2018). Although more work is required to understand the inflammatory environment of the AD brain, this does suggest that systemically administered LPS is an interesting model for understanding how systemic inflammation might drive neuroinflammation and affect tau pathology.

Chronic or acute? Which has greater relevance to AD

One issue which arises when utilising LPS as a model to induce systemic inflammation is whether to conduct an acute or chronic treatment regime. When conducting chronic LPS studies, a phenomenon called tolerance occurs whereby subsequent inflammatory responses are suppressed or even inverted. This prevents detrimental over stimulation of the immune system following an infection when potentially immunogenic debris is leftover. While the basis of LPS tolerance was initially thought to involve receptor desensitisation (Medvedev et al., 2002, Medvedev et al., 2000), a further investigation in tolerant primary murine macrophages treated with LPS found TLR4-induced genes which are involved in the inflammatory cascade such as pro-inflammatory cytokines were silenced (Foster et al., 2007). Interestingly the authors reported TLR4-induced genes which are involved in the antimicrobial response were even more upregulated compared to naïve

macrophages treated with LPS. These studies indicate that a bias in signalling rather than receptor desensitisation is the source of LPS tolerance in mice.

Exploring the underlying inflammatory response during LPS tolerance provides profound implications for studies utilising chronic LPS. For instance, BALB/c mice administered with a single 2 μg LPS injection show greatly diminished IL-1β, IL-6, INFγ and TNFα responses when administered with a subsequent 100 μg LPS injection 72 hours following (Fensterheim et al., 2017). This demonstrates that increasing the dose does not necessarily negate LPS tolerance. Furthermore, compared to vehicle pre-treatment, 1, 5 or 20 μg/kg LPS administration followed by a 100 μg/kg injection in BALB/c mice again reported a decrease in the pro-inflammatory cytokines: IL-12 and TNFα in serum while increasing the prototypical anti-inflammatory cytokine IL-10 (Wysocka et al., 2001). Similarly, LPS-induced TNFα induction is ablated in immortalised macrophages tolerant to LPS, whereas IL-10 induction is increased by a second LPS incubation (Frankenberger et al., 1995). What's more, the greater the number of LPS injections, the more severe the tolerance becomes (Erroi et al., 1993). A useful review on the downregulated and upregulated responses following repeated LPS administration is found here (Ziegler-Heitbrock, 1995). Repeated LPS administration therefore shifts the inflammatory response towards anti-inflammatory and can have profound implications for studies.

However, in a disease such as AD which involves chronic exposure to endogenous LPS, the question is whether LPS tolerance is more reflective of the pathological conditions. The chronic, underlying systemic inflammation observed in AD suggests this might not to be the case (Swardfager et al., 2010). Supporting a role for an absence of LPS tolerance in AD, treatment of peripheral blood mononuclear cells (PBMCs) from AD patients with LPS either results in an increased inflammatory response (Vida et al., 2017, Ciaramella et al., 2010) or a similar inflammatory response compared to control subjects (Magaki et al., 2007, Rosenberg et al., 2009). However, one study found LPS treated PBMCs from AD patients to have a slightly augmented inflammatory response compare to control subjects but only in moderate-severe cases (De Luigi et al., 2001). Together, these studies indicate at least during the early stages of the disease when the systemic inflammatory response is at its greatest that LPS tolerance does not occur. Together with the fact that tolerance occurs much more readily in mice compared to humans (Warren et al., 2010) this thesis focussed on acute LPS administration in order to understand the effects of systemic inflammation on early tau pathology.

However, there is a limitation to using LPS in an acute manner to model systemic inflammation in AD. That is the method fails to recapitulate any chronic effects of the inflammation within the disease. The systemic inflammatory response following acute LPS administration is a dynamic process, resulting only in a temporary pro-inflammatory phase followed by a resolution phase (Srinivasan et al., 2010). Following acute systemic LPS administration in mice, pro-inflammatory mediators peak between 2-4 hours following administration which return to baseline levels within 24 hours (Qin et al., 2007, Biesmans et al., 2013, Erickson and Banks, 2011, Srinivasan et al., 2010). In comparison, while anti-inflammatory mediators do peak in a similar time-frame, they remain elevated even past 24 hours (Srinivasan et al., 2010, Erickson and Banks, 2011). It is this balance between the pro- and anti-inflammatory responses which determines the overriding nature of inflammatory response and determines its resolution. Despite only inducing a temporary systemic response, acute LPS administration is useful in determining the effect of a pro-inflammatory response on a specific pathology.

LPS confounds behavioural models through induction of a sickness syndrome

Systemic inflammatory events are well known to depress cognitive function (Noble et al., 2009a, Wichmann et al., 2014). Similarly, LPS has been suggested to impair hippocampal dependent cognition tasks in mice (Murray et al., 2012, Sparkman et al., 2005b, Sparkman et al., 2005a, Terrando et al., 2010). One issue when administering LPS in animal models is the LPS-induced sickness syndrome. Sickness syndrome describes all the physical and behavioural aspects of the feeling unwell feeling associated with infection. Typical aspects of sickness syndrome involve effects on motivation, pyrogenicity, anhedonia, anorexia, mood, locomotion and hyperalgesia (Lacosta et al., 1999, Kaur et al., 2005, Teeling et al., 2007, Nava and Carta, 2000). As many of these behaviours are utilised to measure performance in cognition models, LPS significantly confounds these tasks (Cunningham and Sanderson, 2008). Even sub-pyrogenic doses as low as 1 μg/kg are able to influencing some of these behaviours, demonstrating how potent LPS is at inducing sickness behaviours (Teeling et al., 2007). Therefore, careful consideration must be taken when interpreting cognition studies following LPS administration. Nevertheless, in studies which have accounted for the LPS-induced sickness syndrome, LPS does indeed appear to impair hippocampal dependent cognition (Terrando et al., 2010, Murray et al., 2012).

1.4.3 Systemic inflammation and its effect on tau pathology in animal models

To date no study has assessed the effect of systemic inflammation on tau pathology in humans. Traditionally there has been a strong implication for neuroinflammatory modulation of tau pathology in pre-clinical models. Despite this, recent studies have provided conflicting evidence, confusing as to the exact role of systemic inflammation on tau pathology. Attempts at modelling the effects of systemic inflammation on tau pathology in animal models are summarised in Table 1.7, with LPS being the most common model to induce inflammation. Significant variation in batch to batch activity in LPS and universal absence in reporting of batch numbers/activity units in the studies means caution is required when comparing between the studies.

Table 1.7: Studies reporting the effect of inflammation on tau pathology

Model	Challenge	Time to cull	Effect on tau	Kinases implicated	Reference
Primary neuronal and microglia cultures	LPS (30ng/ml)	n/a	Tau phosphorylation (epitope not specified)	↑P38 MAPK	(Li et al., 2003)
3x Tg-AD (Amyloid + Tau)	LPS (*Escherichia coli 055:B5*, 6 weeks, twice per week, 0.5mg/kg, *i.p.*)	24h	↑pT231/pS235, **↑pS202/pT205, =pS396/404**	↑CDK5, =GSK-3β, =JNK, =p38 MAPK	(Kitazawa et al., 2005)
3xTg-AD (Amyloid + Tau)	LPS (6 weeks, twice per week, 0.5mg/kg, *i.p.*)	48h	↓Total tau, **↑pS202/pT205, ↑pS214, ↑pT212/pS214, = pT212** ↑Insoluble tau	↑GSK-3β =CDK5	(Sy et al., 2011)
Tau P301S	LPS (12 weeks, twice per weel, 150µg/kg, *i.p.*)	24h	↓Insoluble tau **↓pS202/pT205**	↑Autophagic degradation =GSK-3β =p38	(Qin et al., 2016)
hTau (Tau)	LPS (10mg/kg, *i.p.*)	24h	**↑pS202/pT205,** ↑pT231	-	(Bhaskar et al., 2010)
C57BL/6 (Wt)	LPS (10mg/kg, *i.p.*)	24h	**↑pS202/pT205,** ↑pT231, **↑pS396/404**	-	(Bhaskar et al., 2010)
C57BL/6 (Wt)	LPS (100µg/kg, *i.p.*)	0-4h	Transient : **↑pS396/404, ↑pS202/pT205**	↑GSK-3β, ↑CDK5, =ERK2, =JNK	(Roe et al., 2011)
C57BL/6 (Wt)	LPS (250µg/kg, *i.p.*)		**↓ pT205:total tau** =pT235:tsotal tau	-	(Pearson, 2014)
C57BL/6 (Wt)	Poly I:C (daily, 7, 14, or 21 days, 12mg/kg *i.p.*)	72h	7 days: = pS235 14 days ↑pS235 21 days ↓pS235	-	(White et al., 2016)
BALB**/c** (Wt)	Ovalbumin (5 week treatment regime *i.p, i.n.*)	24h	**↑pS202/pT205** ↑pT231	=ERK, =CDK5, =GSK-3β, =JNK, =p38	(Sarlus et al., 2012)
P301S (Tau)	CIA model	15d	↓Tau aggregation	↑Microglial clearance	(Lang et al., 2017)

Bold indicates tau phosphorylation epitopes associated with post-tangle pathology as described by Augestinack et al., 2002 and adapted from Barron et al., 2016.

Systemically administered LPS and its effect on tau pathology

The first direct evidence for a role of inflammation in exacerbating tau pathology stemmed from *in vitro* studies with primary microglial cells stimulated with Aβ or LPS prior to being co-cultured with primary neocortical neurons (Li et al., 2003). This landmark study showed that secretion of the pro-inflammatory cytokine IL-1β by microglial cells induced an increase in tau phosphorylation through activation of p38-MAPK. It was therefore postulated that inflammation could exacerbate tau pathology through increasing tau phosphorylation and driving the aggregation process. This has been confirmed *in vivo* predominantly using the 3xTg model with numerous kinases implicated. A chronic treatment regimen with LPS (0.5 mg/kg twice a week for 6 weeks) triggered tau hyperphosphorylation at multiple phosphorylation sites associated with both pre- and post-tangle tau pathology at both early and advanced pathological stages in the 3xTg model (Kitazawa et al., 2005, Sy et al., 2011). Furthermore, the latter study reported an increase in aggregated tau, indicating modulation of the aggregation process as a whole. Again, microglial activation and resulting secretion of IL-1β were implicated, *via* activation of either cyclin dependent kinase-5 (CDK-5) (Kitazawa et al., 2005) or glycogen synthase kinase-3β (GSK-3β) (Sy et al., 2011). The discrepancy in the kinases involved could be due to differences in age and pathological stages between the two studies.

While the 3xTg model is useful for studying interactions between amyloid and tau pathologies, tau specific models are valuable to decipher whether inflammation directly affects tau pathology. Both pre- and post-tangle phosphorylation sites were shown to be increased following a single 1 mg/kg dose of LPS in the hTau model, demonstrating that the disease-exacerbating effect of inflammation on tau pathology is not due to tau mutations (Bhaskar et al., 2010). To address the mechanisms involved, the authors utilised hTau mice deficient for the fractalkine receptors which are exclusively expressed on microglial cells in the CNS and known to stimulate anti-inflammatory and pro-phagocytic responses (Medina and Ravichandran, 2016). This intervention exacerbated the impact of LPS on tau phosphorylation (Bhaskar et al., 2010), again supporting a role for microglia function in pathological tau phosphorylation. Conversely, a protective role for systemic inflammation was found when a chronic 150 µg/kg LPS treatment regime was administered in P301S mice (Qin et al., 2016). The authors reported that there was a reduction in soluble and insoluble pS202/205 tau, with the decrease likely due to autophagic degradation rather than alteration in phosphorylation. The discrepancies between this and the earlier chronic LPS studies could be resulting from lack of amyloid pathology in the P301S model or the lower

dose utilised. Nevertheless, these studies demonstrate that systemic inflammation can induce tau pathology through its phosphorylation or induce its degradation through modulating autophagic flux.

Examining the effect of systemically administered LPS in Wt mice has proved useful to examine LPS effects on non-pathogenic tau. A low 100 µg/kg dose of LPS was found to induce CDK5-dependent tau phosphorylation at post-tangle associated epitopes as early as 20 minutes post-injection and which subsided within 4 hours (Roe et al., 2011) indicating that low levels of inflammation are sufficient to trigger tau phosphorylation transiently. Conversely, Bhaskar *et al*., 2010 demonstrated that a 10 mg/kg dose of LPS was not associated with changes in tau phosphorylation 24h following administration in WT mice. The failure to observe tau hyperphosphorylation could be attributed to the use of a later time-point than Roe *et al*. (2011). However, when the mice were deficient of the fractalkine receptor, the same 10 mg/kg dose increased microglial activation and tau phosphorylation 24 hours later at both pre- and post-tangle sites (Bhaskar et al., 2010). Furthermore, the authors report that IL-1 receptor signalling was again found to be the underlying mechanism of tau phosphorylation. On the other hand, a 250 µg/kg LPS injection was found to reduce high molecular weight pT205 while not affecting pT235 levels in Wt mice (Pearson, 2014). Again, this indicates that the effect of LPS on tau phosphorylation may be dose dependent. Altogether, these studies suggest that systemic inflammation induced by LPS appears to be a strong inducer of tau phosphorylation, a high dose may be required.

Is LPS being utilised in a relevant manner to Alzheimer's disease?

Despite being shown to modulate tau pathology, the manner which LPS has been utilised in the aforementioned models often has limitations which could contribute to some of the conflicting findings. The issue of tolerance following LPS administration in mice means acute LPS studies provide a better understanding of pro-inflammatory responses on tau pathology. However, the doses which have been utilised are often too high to represent the underlying inflammatory response associated with AD. Most doses which have been utilised are above 1 mg/kg. LPS acts in a dose-dependent manner with doses higher than 1 mg/kg considered to simulate sepsis rather than infection (Thomas et al., 2014, Woodske et al., 2009). While some AD patients do experience sepsis, it is much more common to suffer mild chronic inflammation. Only two studies utilised an acute dose of LPS which does not correspond to sepsis. The study from Roe et al., 2011 which utilised a lower dose of 100 µg/kg, thought to reflect mild systemic infection (Murray et al., 2012), only saw transient

increases in tau phosphorylation which appears to be an effect of injection rather than inflammation. The second study found a 250 µg/kg LPS injection had a very limited effect on reducing levels of high molecular weight pS205 (Pearson, 2014). This could demonstrate the need for severe inflammatory responses, which are not commonly experienced by AD patients, to affect tau pathology. This thesis is aimed at understanding the consequence of LPS administration on tau pathology at physiologically relevant doses in the hTau model.

The effect of systemic inflammation on tau pathology; looking past LPS

While the majority of attempts at understanding the effect of systemic inflammation on tau pathology utilise LPS, a few attempts at exploring the effect of other models of inflammation have been made. Although not strictly a model of systemic inflammation due to its ability to break down the BBB (Ifuku et al., 2014), the effect of systemically administered Poly I:C is controversial. Chronic LPS administration for 7 days, 14 days and 21 days was respectively found not to affect pS235 tau, increase pS235 tau or decrease levels of pS235 tau in Wt mice (White et al., 2016). Although the study failed to assess mechanisms as to the differential responses, similar total tau levels suggest a change in phosphorylation rather than production/degradation. In an attempt to elucidate the effect of atopic systemic inflammation on tau pathology, Sarlus *et al*., 2012 assessed tau phosphorylation in the ovalbumin asthma model in Wt mice. Despite no alterations in central cytokine expression or glial activation, the authors reported an increase in both pS202/pT205 tau in the hippocampus and parietal cortex while an increase in T231 tau was observed in the parietal cortex. However, the changes in phosphorylation in the study were measured by western blotting with antibodies raised in mouse. Due to the failure of employing a technique which would negate non-specific binding of the secondary antibody to heavy chains of IgG's and IgE's, the apparent changes in tau phosphorylation could simply be resulting from elevated levels of IgG and IgE in the study (Sarlus et al., 2012). In order to truly understand the effect of atopic inflammation on tau pathology, it is imperative to employ techniques which avoid potentially non-specific binding to endogenous Ig's which have a similar molecular weight to the tau protein.

Numerous attempts have been made to understand the effect of systemic inflammation induced by a high fat diet on tau pathology – largely to disappointing outcomes. The majority of studies failed to find an effect on tau phosphorylation in Wt animals (Moroz et al., 2008, Becker et al., 2012, McNeilly et al., 2012) or in the 3xTg model (Knight et al., 2014). In another study, Wt mice or mice expressing the ApoE4 allele and fed

with a high fat diet saw a reduction in tau phosphorylation at multiple sites (To et al., 2011). Only one study involving THY-Tau22, which expresses 4R tau containing G272V and P301S mutations, reported an increase in pS214, pS402 and pS422 tau following a high fat diet (Leboucher et al., 2013). However, the authors report the effect was only in THY-Tau22 mice which had a selective increase in insulin signalling which was not observed in Wt mice. As such, the increase in insulin signalling could potentially be the source of tau phosphorylation within the model. Together, this suggests systemic inflammation induced by high fat diet is insufficient to induce tau phosphorylation, although perturbations in insulin signalling make it difficult to draw definitive conclusions.

With CIA an interesting model to induce low grade, chronic inflammation, a recent attempt to determine its effect on tau pathology in the P301S model was made by Lang *et al*., 2017. The authors report that chronic systemic inflammation resulted in decreased axonal and extracellular pS202/205 staining as well as a decrease in the number of neurons containing insoluble tau as indicated by silver staining. Explaining the decrease in extracellular pS202/205 tau, the authors reported a significant increase in microglial phagocytosis of tau. Finally, P301S mice that underwent CIA but were immunized against collagen did not develop arthritis and had remarkably similar tau burden to non-CIA treated P301S mice, demonstrating peripheral inflammation rather than the collagen challenge as the source of the resolution in tau pathology (Lang et al., 2017). While further mechanism assessment is required in the model such as assessing kinases and autophagic flux, the study provides compelling evidence that mild chronic inflammation can have a beneficial effect on tau pathology. Therefore, recent evidence looking past LPS suggests that extremely mild inflammation might even play a beneficial role on tau pathology.

The effect of neuroinflammation on tau pathology

A couple attempts to model the effect of neuroinflammation on tau pathology have been conducted. Intracranial administration of an altered murine hepatitis virus (MHV) strain triggered an increase in tau phosphorylation (Sy et al., 2011). Likewise, a 10 µg intra-hippocampal injection of LPS in rtg4510 mice resulted in an increase in tau phosphorylation which persisted for at least seven days post injection (Lee et al., 2010). However, the authors report no effect on tau aggregation, possibly demonstrating the need for chronic inflammatory modulation to affect tau pathology. Together, these results indicate that modulating the neuroinflammatory environment alters the phosphorylation state of tau. The potential effect of neuroinflammation on tau pathology is depicted in Figure 1.11.

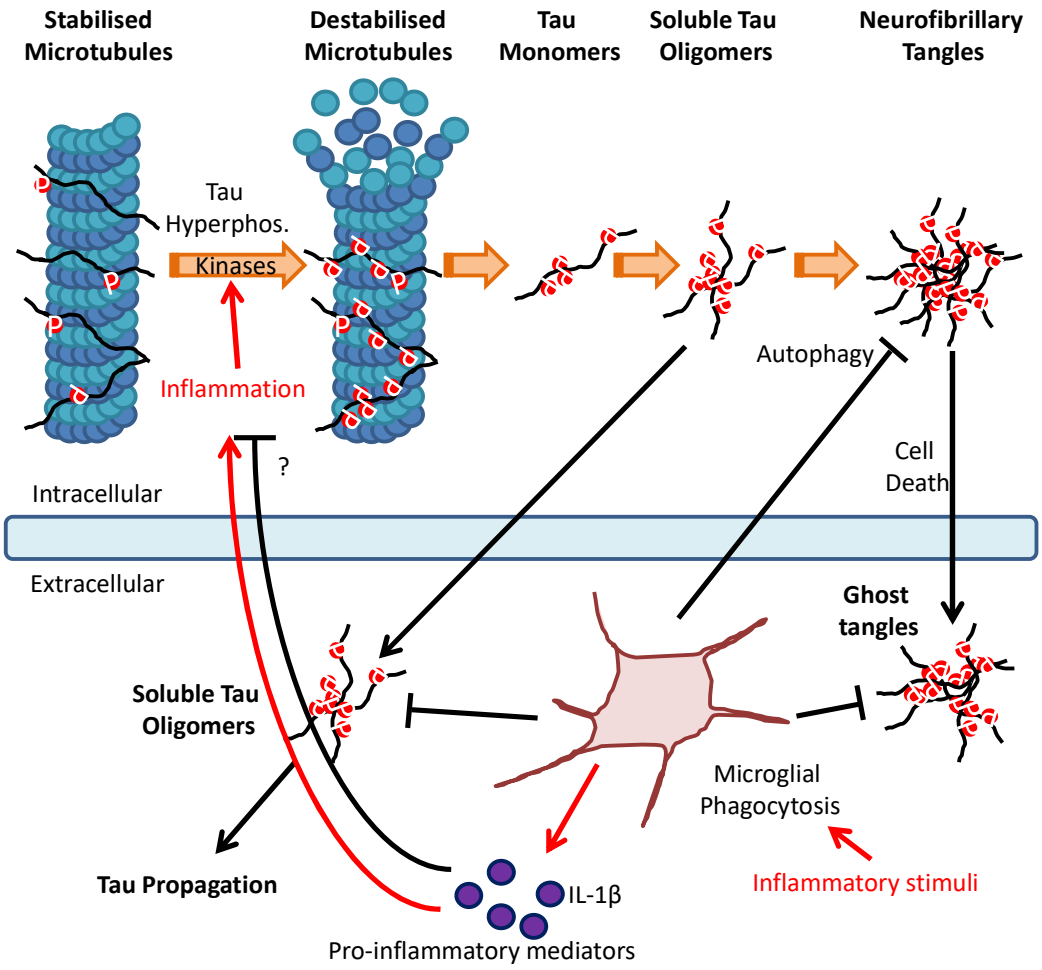

Figure 1.11: Current understanding of the relationship between neuroinflammation and tau pathology: Inflammatory stimuli, such as Aβ or pro-inflammatory cytokines, stimulate microglial production of pro-inflammatory mediators such as IL-1β leading to the up-regulation of kinases involved in tau phosphorylation and exacerbation of the pathology. However, recent evidence suggests mild inflammation which is more akin to AD might induce tau dephosphorylation. Furthermore, inflammation can have beneficial effects on tau pathology by inducing autophagy of NFT's or microglial phagocytosis of extracellular tau species. Image extended from Figure 6 and originally displayed in Barron et al., 2017.

The effect of anti-inflammatory treatments in tau models:

A few studies have aimed at assessing the effect of anti-inflammatory treatment in tau models. Chronic treatment with the NSAIDS: ibuprofen or r-flurbiprofen both failed to affect tau phosphorylation in the 3xTg model (Carreras et al., 2013, McKee et al., 2008). In the most beneficial study, treatment with the tetracycline derivative: minocycline, reduced both tau phosphorylation and aggregation in the hTau model (Noble et al., 2009b). Conversely, minocycline treatment in the 3xTg model resulted a mixed effect on tau phosphorylation with some epitopes increasing, decreasing or remaining unchanged (Parachikova et al., 2010). Therefore, whether anti-inflammatory treatment can have

positive effects in tau models is thus far inconclusive with further studies required to elucidate its potential.

1.4.4 Summary

Traditionally systemic inflammation has been viewed as a strong inducer of tau pathology through its phosphorylation. Often these studies have relied on either chronic LPS administration or high doses of LPS which do not necessarily reflect the underlying pathogenesis of AD. Nevertheless, it does appear systemic inflammation can induce tau phosphorylation, although a few reports have found beneficial effects of systemic inflammation on tau phosphorylation. Of the few studies which have assessed tau aggregation following LPS administration, there have been mixed findings. One found an increase in tau aggregation, potentially through its increase in tau phosphorylation, while two others found clearance of aggregated tau through either autophagic or microglial mechanisms. Taken together, these studies suggest that systemic inflammation might induce tau phosphorylation and stimulate its clearance, although the conflicting findings make any definitive conclusions difficult. Furthermore, there is a general failure to assess the effect of systemic inflammation on other post-translational modifications such as glycosylation or methylation, again making it difficult to decipher the true effect of systemic inflammation on tau pathology.

1.5 General Summary

Tau aggregation highly correlates with AD progression and a variety of tau species have been found to exert neurotoxic functions. Furthermore, systemic and neuro-inflammation have been linked with the aetiology of AD and the onset of inflammation occurs premature to clinical diagnosis. There is an overlap between the kinases affected by inflammatory cascades and those which phosphorylate tau. To understand this interaction, systemic inflammation has been modelled in mice prone to tau aggregation. This includes the hTau model which has the greatest relevance to AD for lack of mutations within the model. LPS is a valid method for assessing the effect of systemic inflammation in AD because it has recently emerged as a potential endogenous mediator of the disease, presence in the periphery and brain of AD patients, it does not cross the BBB and induces similar microglial phenotypic changes as to those in AD. In animal models, systemic inflammation has been reported to exacerbate tau phosphorylation, although recent studies have suggested it to be beneficial in the clearance of insoluble tau. While LPS is a relevant method for inducing systemic inflammation in respect to AD, the high doses in systemic administration studies

does not necessarily reflect the mild inflammatory conditions which occur in AD. As such, further study is required to understand whether systemic inflammation is a positive or negative force within AD.

1.6 Aims and objectives

The aim of this thesis is to understand the symptomatic and pathological effect of physiological relevant acute systemic inflammation on early stage tau pathology in AD:

To reach this aim, I will address the following objectives

1. Develop a cognition task which is not confounded by the LPS-induced sickness syndrome in the form of a contextual fear condition paradigm
2. Develop an *ex-vivo* magnetic resonance spectroscopy (MR spectroscopy) technique to aid in accurately quantifying neuroinflammatory alterations
3. Develop a tau model which represents AD through validating a hTau model that partially expresses mTau (hTau/mTau$^{+/-}$) which addresses the isoform imbalance and systemic pathologies within the model
4. Measure the effect of systemic inflammation induced by acute LPS administration on contextual fear conditioning in the hTau/mTau$^{+/-}$ model
5. Identify pathological alterations induced by systemic inflammation through acutely administered LPS in the hTau/mTau$^{+/-}$ model at a low dose

1.7 Hypotheses

By expressing hTau mice on a heterozygous mTau background, the author predicts that both the isoform imbalance and systemic pathologies will be improved while conserving tau pathology. Inflammation has been reported to affect similar cognitive domains as to those which are affected in both AD and in the hTau model. As such the author hypothesises that systemic inflammation induced by acute LPS administration will further exacerbate behavioural impairments which are observed in the model. This thesis is aimed at understanding the effect of systemic inflammation on early tau pathology which is predominately characterised by tau hyperphosphorylation and relocation to the dendritic compartment. While recent studies have suggested a beneficial effect of systemic inflammation on late stage tau pathology, the author hypothesises that, even with low doses of LPS, tau phosphorylation will occur during the pro-inflammatory phase. Furthermore, the author hypothesises that following the resolution of the LPS-induced inflammatory response

that tau phosphorylation levels will return to normal due to the constant balance between kinase and phosphatase activity.

Chapter 2 Methods Chapter:

2.1. Animals, husbandry and experimental groups

2.1.1 Animals and husbandry:

All experimental animals were bred in the University of Nottingham's Biomedical Support Unit (BSU) transgenic animal facility from breeding stock of APPswe/PS1dE9 and hTau mice were purchased from the Jackson Laboratories (Bar harbour, USA, stock number: 34829 JAX and 005491 JAX). Unless otherwise mentioned, all control animals in this thesis were wild-type (Wt) littermates. Animals were grouped housed in individually ventilated cages (IVC), usually 4 per cage. Food and water were provided *ad libitum* and access to a play tube and nesting material was given. Temperature, humidity and air exchange were automatically controlled and a 12h light/dark cycle was implemented. All animal procedures were carried out in accordance with the UK animals Scientific Procedures Act and approved by the University of Nottingham animal welfare committee.

2.1.2 Experimental cohorts

Six experimental cohorts were utilised to achieve the thesis objectives and are summarised in Table 2.1. Due to initial issues with breeding the hTau mice, APPswe/PS1dE9 mice, which are positive for amyloid pathology, were used to address Objective 1 and 2. The thesis objectives and the experimental cohorts used to achieve them go as follows:

1. Develop a cognition task which is not confounded by the LPS-induced sickness syndrome in the form of a contextual fear condition paradigm
    a. Experimental Cohort 1: 16 experimentally naïve 6-month old female APPswe/PS1dE9 mice and Wt littermates assessed in the comprehensive laboratory animal monitoring system (CLAMS^{TM}) apparatus following a 100µg/kg *intravenous* (n=8/genotype, *i.v.*) LPS injection. The experimental cohort was utilised to assess the temporal dynamics of the LPS-induced sickness syndrome to facilitate in designing a contextual fear conditioning task which would not be affected by confounding LPS affects.
    b. Experimental Cohort 2: 56 experimentally naïve 6-month old female APPswe/PS1dE9 mice and Wt littermates were utilised to validate a contextual fear conditioning involving a pre-exposure to the context

following either phosphate buffered saline (PBS) or 100µg/kg LPS administration (*i.v.*, n=12-16/group).

2. Develop an *ex-vivo* magnetic resonance spectroscopy (MR spectroscopy) technique to aid in accurately quantifying neuroinflammatory alterations
    a. Cortical samples from Experimental Cohort 2 were utilised for optimisation of an *ex-vivo* magic angle spinning (MAS)-nuclear magnetic resonance (NMR) spectroscopy technique. Optimisation of the technique was conducted in the APPswe/PS1dE9 model because it would enable comparison with previously published *in vivo* MR spectroscopy conducted within the model (Pardon et al., 2016).
3. Validate a hTau model that partially mTau (hTau/mTau$^{+/-}$) which addresses the isoform imbalance and systemic pathologies within the model
    a. Experimental Cohort 3: 23 cortical tissue samples from 6-month old male Wt, mTau$^{+/-}$ and hTau/mTau$^{+/-}$ which were on a mixed 129SvJ x C57Bl/6 background and 6-month old male Wt (aged match controls), mTau$^{-/-}$ and hTau/mTau$^{-/-}$ which were on a C57BL/6 background (n=3-4/genotype). The experimental cohort had undergone a previous battery of behavioural testing and was utilised for a pre-validation of tau pathology in the hTau/mTau$^{+/-}$ model.
    b. Experimental Cohort 4: 19 experimentally naïve 9-month old male Wt, mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ (n=3-4/genotype) were utilised to determine tau aggregation in the hTau/mTau$^{+/-}$ model.
4. Measure the effect of systemic inflammation induced by acute LPS administration on contextual fear conditioning in the hTau/mTau$^{+/-}$ model
    a. Development of strong allergies to the mice meant animal exposure had to be limited and this objective was not addressed.
5. Identify pathological alterations induced by systemic inflammation induced by acutely administered LPS in the hTau/mTau$^{+/-}$ model at a physiologically relevant dose
    a. Experimental Cohort 5: 176 experimentally naïve 3-month old Wt, mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/}$-and hTau/mTau$^{-/-}$ which had been administered with PBS or a 100, 250 or 330µg/kg dose of LPS (n=8-

9/group, *i.v.*) and were culled 4h after administration. The experimental cohort had two purposes:

i. Comparison between PBS treated animals to further validate the tau phenotype of the hTau/mTau$^{+/-}$ model (data in two chapters).

ii. Comparison among the experimental cohort as a whole to determine the effects of the initial pro-inflammatory phase of LPS administration on early tau pathology.

b. Experimental Cohort 6: 59 experimentally naïve 3-month old Wt, mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/-}$-and hTau/mTau$^{-/-}$ which had been administered with either PBS or a 250µg/kg LPS injection (n=9-10/group, *i.v.*) and were culled 24h following administration. The experimental cohort was utilised to determine the persistence of pathological alterations following the resolution of the initial pro-inflammatory response.

Table 2.1 Experimental groups utilised and their experimental chapters

Experimental Cohort and name	Genotypes	Treatment (*i.v*)	Behaviour	Aim of study	Chapter
1 – CLAMS™	APPswe/Ps1dE9 (n=8), Wt (n=8)	100µg/kg LPS	CLAMS™	Sickness syndrome dynamics	3
2 – Contextual fear	APPswe/Ps1dE9 (n=24), Wt (n=25)	PBS or 100µg/kg LPS	Contextual fear conditioning	Optimisation of behavioural task and *ex vivo* MRS	3
3 – Pre-validation	Wt, mTau$^{+/-}$ and hTau/mTau$^{+/-}$ (mixed background n=4/genotype) Wt (aged match controls), mTau$^{-/-}$ and hTau/mTau$^{-/-}$ (C57 background n=4/genotype)	No treatment	Previous battery of behavioural experiment	Pre-validation of hTau/mTau$^{+/-}$ model	4
4 – 9 month group	Wt, mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ (n=4/genotype)	No treatment	-	Assessment of late stage tau pathology in hTau/mTau$^{+/-}$ model	4
5 – LPS 4h	Wt, mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ (n=34-36/genotype)	PBS, 100, 250 and 330µg/kg LPS (n=8-9/group)	Food burrowing and spontaneous alternation	Analysis of PBS treated animals for validation of hTau/mTau$^{+/-}$ model	4
				Analysis of whole experimental group for effect of LPS on tau pathology during pro-inflammatory phase	5
6 - LPS 24h	Wt, mTau$^{+/-}$ and hTau/mTau$^{+/-}$ (n=19/20/genotype)	PBS and 250 µg/kg LPS (n=9-10/group)	Food burrowing and spontaneous alternation	Effect of LPS on tau pathology following resolution of pro-inflammatory phase	6

2.2 LPS preparation and treatment:

LPS (Escherichia coli 0111:B4, batch no: 114M4009V, Sigma) was dissolved in sterile PBS, aliquoted and stored frozen in silanized vials at -20˚C. Prior to administration, aliquots were defrosted and brought to room temperature. Treatment was given *i.v.* in the dorsal vein with a 300µl 29G x ½" insulin syringe (BS30M2913, Terumo) at a volume of 1µl/g of body weight. LPS administration was conducted *intravenously* as to mimic the circulating effects LPS might have in AD and to avoid local inflammation in the peritoneal cavity associated with *intraperitoneal* administration of LPS (Miyazaki et al., 2004). Animal welfare was monitored two, four and six hours following LPS administration by assessing whether the animal was mild, moderately or severely affected depending on subduedness, piloerection and other elements of the grimace scale. Animal welfare was additionally monitored at 24, 48 and 72 hours following LPS administration in a similar manner as well as recording bodyweight.

2.3 Behavioural procedures:

2.3.1 CLAMS™:

Animals underwent CLAMS™ assessment to determine the dynamics of the LPS-induced sickness syndrome. The CLAMS™ apparatus has the advantage over other tracking methods such as telemetry due to the procedure being non-invasive. Food intake, locomotor activity, respiratory exchange ratio (RER) and calorie expenditure were measured by simultaneous live recording with the CLAMS™ apparatus (Linton Instrumentation, Linton, UK/Columbus Instruments, Columbus, OH). The CLAMS™ apparatus involved 8 identical chambers with a centrally placed food hopper filled with mashed mouse chow in which each mouse's food intake was simultaneously monitored. Water was provided from bottles above and intake was not recorded. The chamber air was measured sequentially across each chamber for 1 minute every 9 minutes to assess oxygen (O2), carbon dioxide (CO2) and air temperature. Bodyweights were measured daily at 1pm. Changes in bodyweight were analysed by a one-way repeated measures ANOVA with genotype as the between subject factor and light cycle as the within subject factor. Where appropriate a Tukey's post hoc test was employed.

A water bottle leak during baseline recording meant food intake was only recorded for 7 Wt mice while all other parameters were recorded normally. RER signifies the ratio

between the amount of oxygen consumed and the amount of carbon dioxide exhaled, representing fuel metabolism and is influenced by food consumption (Paoli et al., 2011). Additionally, while calorie expenditure is representative of underlying metabolic activity, it is additionally heavily affected by physical activity (O'Neal et al., 2017). As well as the CLAMS^{TM} parameters, the recovery in bodyweight was recorded. Differences in baseline bodyweights were assessed by an unpaired *t*-test. Baseline recordings were split into light/dark cycle, with differences assessed by a one-way repeated measures ANOVA with genotype as the between subject factor and light cycle as the within subject factor. Where appropriate a Tukey's post hoc test was employed. The time course of each parameter was plotted in 1.5h bins. To track the recovery, the difference from baseline was calculated and split into 6h bins and a one-sample *t*-test was conducted to determine whether the response had returned to baseline. To assess whether the recovery was similar between genotypes, a repeated measures ANOVA was conducted with genotype as the between subject factor and bin as the within subject factor. Where appropriate a Tukey's post hoc test was employed.

2.3.2 Contextual fear conditioning:

Contextual fear conditioning (CFC) is a form of aversive learning which involves the pairing of a foot shock with the context of the conditioning chamber. CFC has special relevance to AD. Contextual fear conditioning is reported to be heavily dependent on hippocampal processes (Phillips and LeDoux, 1992, Rudy and O'Reilly, 1999), a brain region heavily affected in AD (Braak and Braak, 1991, Braak et al., 2006). Furthermore, associative learning tests have found impairments in AD patients (Quenon et al., 2015, Bastin et al., 2014). Similarly, inflammation is thought to impair cognitive function through disruption of common regional areas which are required for CFC. Namely, inflammation is thought to affect hippocampal dependent cognitive function. For example, IL-1β, TNFα or LPS application to organotypic rat hippocampal slices results in inhibition of LTP (Cunningham et al., 1996). Furthermore, acute and chronic systemic LPS administration have been reported to impair LTP *in vivo* (Hennigan et al., 2007, Maggio et al., 2013) as well as impair contextual fear in rodents (Pugh et al., 1998, Terrando et al., 2010). Therefore, AD and inflammation affect common pathways which are required for CFC, making the task ideal for the assessment of the synergism between the two.

All CFC behavioural testing occurred in the same conditioning chamber with the dimensions of: 25x25x38cm, comprising of three grey stainless walls. The fourth wall was transparent Perspex and the floor was a stainless steel grid spaced 1cm apart. There was no

roof to the box, and trials were video recorded from above. The grid flooring was connected to a shock generator (Campden Instruments, Loughborough, UK), allowing shocks (1s, 0.4mA) to be administered from the floor. The room was illuminated using uplighters to cause less aversion to the animals.

The CFC protocol utilised had two important elements. The first involved a pre-exposure session where the animal was required to learn the context of the conditioning chamber. The second involved an immediate shock deficit in the conditioning trial. An immediate shock deficit would mean that the mice do not have a chance to learn the context during the conditioning trial and must rely on the contextual memory formed in the pre-exposure session (Brown et al., 2011). In doing so, learning of the context could be impaired through administration of LPS in the pre-exposure session and ensure impairments are not due to any confounding effects of LPS.

The pre-exposure trial involved exposing the mice to the conditioning chamber for 5 minutes before being returning to their home cages. Following a 40s period in the home cage, the mice were returned to the conditioning chamber for 40s and subsequently returned to their home cage once more. The process of 40s in/out of the conditioning chamber was repeated until 5 repeats were conducted in total. Figure 2.1 depicts the pre-exposure protocol. The repeated exposure protocol was chosen as it has been reported to facilitate learning of the context (Brown et al., 2011). Immediately after the last exposure to the context, either PBS or LPS was administered. A period of 5-10s was given for transport to and from the conditioning chamber. Immobility in the first 5 minutes as well as the total time was assessed as a measure of locomotor activity. Differences in immobility levels between genotypes were assessed using an unpaired *t*-test.

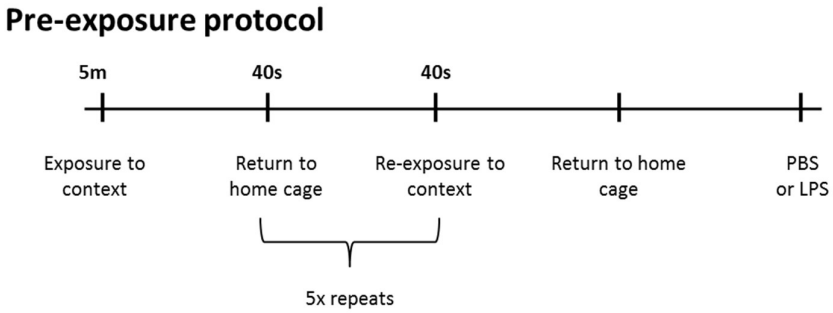

Figure 2.1 The pre-exposure session protocol. An initial 5 minute exposure to the context was given followed by the animal being returned to its home cage. After 40s the animal was returned to the conditioning chamber for another 40s exposure to the context. A set of 5x 40s repeats in and out of the conditioning chamber were given in total. Immediately after the last repeat, the animal was injected with PBS or LPS.

The conditioning trial commenced 48 hours following the pre-exposure to the context. The trial lasted 9 minutes with an immediate shock administered upon placement to the conditioning chamber (1s, 0.4mA). 9 subsequent shocks (01s, 0.4mA) were administered, one minute apart so that 10 shocks over the 9 minutes were administered. The immediate shock was chosen to avoid learning of the context during the conditioning trial (Wiltgen et al., 2001). Following the conditioning trial the mice were returned to their home cage. Recordings were divided into one minute time bins between each shock. Increasing immobility over each time bin was taken as an indication of the acquisition of contextual fear. The acquisition of contextual fear was compared using a two-way repeated measures ANCOVA where genotype and treatment were between-subject factors, shocks as the within subject factor and total immobility levels during the pre-exposure session taken as the covariate. Where appropriate, Tukey's post hoc analysis was conducted.

A retention trial was commenced 24 hours subsequent to the conditioning trial to assess retention of contextual fear. The retention trial length was 3 minutes and involved exposure to the context without shocking. Following the retention trial the animals were returned to their home cage. Immobility was analysed as an index of the retention of contextual fear. Expression of contextual fear was compared using a two-way ANCOVA where genotype and treatment were between-subject factors and total immobility levels during the pre-exposure session taken as the covariate. Where appropriate, Tukey's post hoc test was conducted.

To assess contextual fear memory extinction, an extinction trial was conducted 24 hours following the retention trial. Again, the trial consisted of a 3 minute exposure to the context without administration of a shock. Immobility during the extinction trial was used as an indication of contextual fear expression during the extinction trial. Extinction of contextual fear was compared using a two-way ANCOVA followed by Tukey's post hoc where genotype and treatment were the between-subject factors and total immobility levels during the pre-exposure session taken as the covariate. Where appropriate, Tukey's post hoc test was conducted. A simple extinction index: the immobility during extinction – immobility during retention was calculated in order to assess the extent of contextual fear extinction. To determine whether contextual fear was extinguished for each group, one sample *t*-tests were used to compare the extinction index to 0 (retention level).

2.3.3 Food burrowing task:

The food burrowing (FB) task was adapted from that originally described by (Deacon, 2009). It is thought to model activities of "daily living" in humans which are impaired early in AD (Sacco et al., 2012, Perneczky et al., 2006). FB is susceptible to hippocampal and fronto-cortical manipulations (Deacon, 2012) and is the most sensitive task to early tau pathology in hTau mice (Geiszler et al., 2016). For the practise food burrowing session, a jar containing 50g of equal sized food pellets were placed in the group cages between 5-6pm. The morning following the group practice, the jars were removed from the cage between 9-10am. On the evening subsequent, the animals were single housed between 5-6pm and a 50g food jar was added to the cage overnight. The amount of food burrowed was recorded between 9-10am the following day. A jar was utilised instead of the tube which had originally been described by Deacon 2012 as our group have found mice burrow nonetheless (Geiszler et al., 2016) and it would minimise accidental displacement of food pellets. The difference between this and the original 50g placed in the cage was taken as the amount of food burrowed. Data was represented as percentage of food displaced. For statistical analysis, the amount of food burrowed was rank transformed as food burrowing data is not normally distributed (Deacon, 2009). Differences in baseline burrowing behaviour among genotypes was assessed via a one-way ANOVA followed by Tukey's post hoc test where appropriate. In Chapter 6, food burrowing was assessed by a two-way repeated measures ANOVA with session as the within subject measure and genotype/treatment as the between subject measures. If appropriate, planned comparisons were conducted.

2.3.4 Spontaneous alternation task:

The primary rational for conducting the spontaneous alternation (SA) task was to measure locomotor activity and determine the extent of the sickness response. Additionally, the task was chosen to assess spatial working memory which again relies on hippocampal processing (Hughes, 2004). Assessing spatial working memory has similar advantages as CFC. Spatial working memory impairments have been reported in AD patients (Guariglia, 2007, Baddeley et al., 1991). However, while impairments have been reported in the task following systemic LPS administration in rodents, these could be due to confounding sickness effects (Custódio et al., 2013, Hritcu et al., 2011, Sayed and El Sayed, 2016). Nevertheless, the SA task is an interesting model for understanding the synergism between inflammation and tau pathology if conducted after the resolution of the sickness syndrome.

The Y-maze comprised of 3 clear Plexiglas arms, each 15 cm tall and 6 cm wide. The task involved placing the mouse in the centre of the maze which was then allowed to freely explore the maze for 5 minutes. A correct alternation entailed a mouse entering three different arms in a row and the alternation rate was calculated and taken as an indication of spatial working memory. Each session was recorded by a camera from above the maze and the distance moved was automatically tracked (Ethovision version 10, Noldus, NL). Both spatial working memory and locomotor activity were normally distributed and assessed by parametric analysis. Baseline spatial working memory and locomotor activity were compared with a one-way ANOVA followed by a Tukey's post-hoc where appropriate. To assess the effect of a LPS on spatial working memory and locomotor activity, a two-way ANOVA was conducted followed by planned comparisons where appropriate. In Chapter 6, spatial working memory and locomotor activity were assessed by a repeated measures two-way ANOVA with session as the within subject measure and genotype/treatment as the between subject measures. If appropriate, planned comparisons were conducted. Additionally, to assess whether the alternation rate occurred significantly above chance level (50%), a one-sample *t*-test was conducted.

2.3.5 Tissue Collection:

All mice were euthanized via cervical dislocation immediately after the last behavioural task and the brains were divided into two hemispheres. Depending on the experimental cohort, hemispheres were handled in 3 manners. The first involved the dissection of a hemisphere prior to being snap frozen in dry ice and stored at -80˚C. Cortical samples were used from the contextual fear conditioning study for NMR validation and hippocampal tissue was utilised for immunoblotting in subsequent studies. For sarkozyl extraction, a hemisphere was snap frozen in dry ice and stored at -80˚C. For IHC analysis, a hemi-brain was fixed for 6 hours using 4% paraformaldehyde (PFA) prior to being stored in 70% ethanol EtOH for 18h and subsequently embedded in paraffin wax. Trunk blood was collected and centrifuged in serum separating tubes containing lithium at 3000G for 10 min at 4˚C. There was an issue collecting trunk blood in the fifth experimental group and therefore only an n=6-8/group was achievable. The serum was collected and frozen at -80˚C. A breakdown of the tissue used is given in Table 2.2.

Table 2.2 Tissue procedures conducted on each experimental group

Experimental group number and name	Tissue	Number /group	Use for tissue
1 – CLAMS™	Tissue not used in thesis		
2 – Contextual fear	Cortex	6-9	NMR validation
3 – Pre-validation	Cortex	3-4	Western blotting of tau species
4 – 9 month group	Frozen hemi-brain	3-4	Sarkosy extraction
	Paraffin embedded hemi-brain	3-4	IHC analysis (microglia + tau)
5 – LPS 4h	Hippocampus	8-9	Western blotting assessment of tau species and PP2A activation
	Frozen hemi-brain	6	Sarkosy extraction and tau aggregation assay
	Paraffin embedded hemi-brain	3	IHC analysis (microglia + tau)
	Serum	6-8	Multiplex of cytokines
6 - LPS 24h	Hippocampus	9-10	Western blotting assessment of tau species and PP2A activation
	Frozen hemi-brain	6	Sarkosy extraction
	Paraffin embedded hemi-brain	3-4	IHC analysis (microglia + tau)
	Serum	9-10	Multiplex of cytokines

2.4 Tissue procedures:

2.4.1 *Ex-vivo* magic angle spinning ^{1}H-NMR

Loading of zirconia rotor:

In order to optimise the *ex vivo* MAS-NMR spectroscopic analysis of brain metabolite levels, snap-frozen cortical samples from the APPswe/PS1dE9 fear conditioning study were utilised. The decision to initially optimise on APPswe/PS1dE9 mice instead of hTau mice was taken because the spectra could be compared to *in vivo* MR spectroscopic analysis which had previously been reported in the model, albeit at different ages (Pardon et al., 2016). Subsequently, the technique was intended to be utilised in the hTau studies, although this ultimately was not conducted for reasons discussed later in Chapter 3. Tissue utilised for MAS ^{1}H-NMR was snap frozen and stored at -80˚C. Loss of samples meant that only an n=6-9 per group was achievable. While thawing, a ~40mg piece of cortical tissue was dissected on ice and loaded into a 40mm zirconia rota. Cortical samples were used for method

optimisation instead of hippocampal tissue because the latter had been utilised for western blotting of synaptic markers which is not included in this thesis. To facilitate loading of the rotor, the tissue was allowed to thaw slightly on ice so it could be cut into ~3mm strips but was still frozen enough to hold its shape and enable easy loading of the rotor. This step was important because if the tissue was kept frozen (by being prepped on dry ice), the tissue wouldn't slice but instead crack into pieces. If the tissue was allowed to thaw too much, it would lose its shape and considerably increase loading times which would have a negative impact on the metabolites being assessed. Between loadings of tissue, the rotor was shortly centrifuged to ensure the tissue would locate to the bottom. Following loading the tissue, a Teflon cap was fitted and the rotor placed in the NMR spectrometer.

^{1}H MAS-NMR protocol

^{1}H MAS-NMR was conducted on a Varian 400 MHz (VNMRS) spectrometer using a 4 mm T3 MAS NMR probe – a system optimised for high resolution biological solid-state NMR. Samples were rotated at 6kHz and 4˚C. The initial temperature of the probe was 10˚C which was brought down to 4˚C while the rotor was spinning. This was done so that the Teflon cap would not slip out while bringing the rotor up to speed. The pulse sequence consisted of a 1s water pre-saturation followed by a 5.7 µS direct excitation and acquire sequence at a flip angle of 90˚ and is depicted in Figure 2.2A. Without water suppression, the spectrum was dominated by the water resonance due to its abundance in biological tissue (Figure 2.2B), overwhelming the resonances of interest (Figure 2.2C). The application of a long, low powered, RF pulse resulted in saturation of signal for a narrow frequency around the water resonance. Increasing the power of the pre-saturation signal increases its effectiveness for water suppression but increases the range of frequencies suppressed. The current frequency was chosen as it sufficiently suppressed the water resonance without significantly affecting neighbouring resonances (Figure 2.2D). 512 repeats were conducted in total with an inter-pulse delay of 3s, lasting 25mins in total. A total of 3205 complex points were acquired at a spectral width of 6410Hz. Spectra were externally referenced to tetramethylsilane (TMS) in deuterated chloroform (Sigma, #87921).

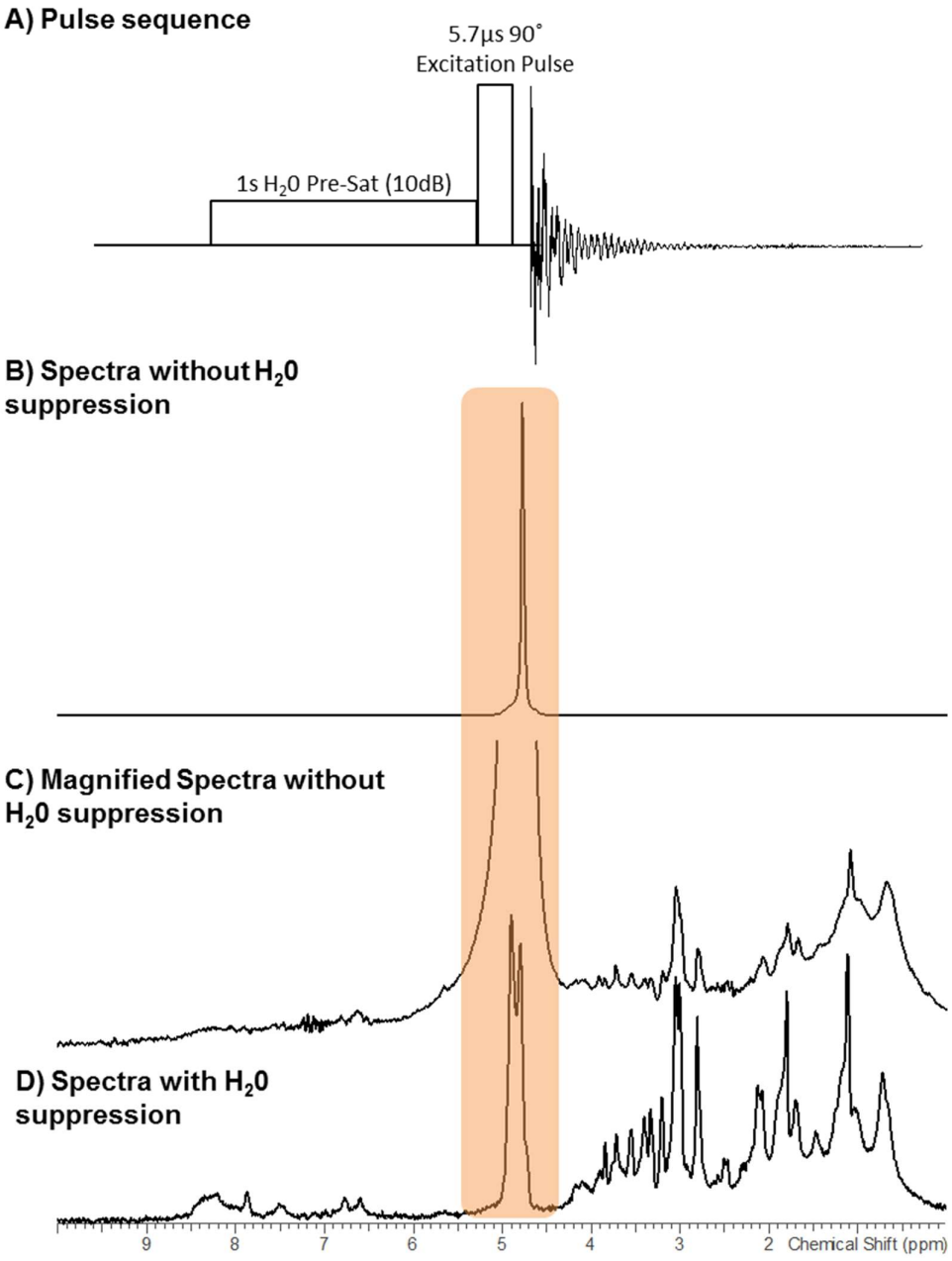

Figure 2.2: Schematic of the pulse and acquire sequence and the effect of water suppression in mouse cortical samples. ***A)*** *The pulse and acquire sequence utilised for the study.* ***B)*** *An example spectrum without water suppression showed predominantly the water resonance.* ***C)*** *The same spectrum magnified shows an elevation in baseline, decreasing the quality of the spectrum.* ***D)*** *An example of a spectrum with water suppression. The water resonance is greatly diminished and the quality of the spectrum vastly improved.*

Data processing and analysis:

Data was processed in Advanced Chemistry Development (ACD, Toronto, Canada) labs. Briefly, raw data was Fourier transformed to 16K points and phased. Integrals were

taken for 8 peaks corresponding to ML9, N-acetyl aspartate (NAA), glutamate/glutamine (Glu/Gln), creatine (Cre), choline (Cho), Taurine, glycine (Gly) and myo-inositol (m-Ins) metabolites. Intensities for each metabolite were standardised to the sum of selected metabolites (m-Ins, NAA, Cho, Cr and Glu/Gln) and displayed with their SEM. This was done to avoid effects of possible variances in standardising to a single metabolite (Forster et al., 2013). These specific metabolites were chosen so spectra would be comparable to those from APPswe/PS1dE9 mice in the literature (Pardon et al., 2016). NMR data was parametric and therefore statistical analysis involved conducting a two-way ANOVA followed by a Tukey's post-hoc where appropriate.

2.4.2 Western immunoblotting:

Tissue preparation

Western blotting was conducted for the purpose of assessing total tau species and $PP2A_C$ activity. While on dry ice, snap frozen tissue was weighed by transferring the frozen tissue to a chilled Eppendorf. Tissue was then homogenised with a pellet homogeniser in a 5x w/v of ice cold radioimmunoprecipitation assay buffer (RIPA buffer: 50 mM Tris-HCl (Sigma, #T5941), 0.1% Triton X-100 (Sigma, X100), 0.25% Na-deoxycholate (Sigma, #D6750), 150 mM NaCl (Sigma, #S3014), 1 mM EDTA (Sigma, #EDS) containing phosphatase inhibitors: 1 mM Na_3VO_4 (Sigma, #S6508) and 1 mM NaF (Sigma, #S7920), and cOmplete protease inhibitor (Roche, # 11697498001). This buffer was chosen as it had previously been documented to successfully enable tau extraction from tissue samples (Planel et al., 2009). Samples were centrifuged at 20,000 RCF for 20 minutes and the resulting supernatant corresponded to the cytosolic fraction. Total protein levels in the cytosolic fraction were measured using the BCA assay (Novagen, #71285) and standardised to a concentration of 2µg/µl in RIPA buffer. Samples were then diluted 1:2 in laemmli buffer (Sigma, #3401), generating a sample solution which was 1µg/µl. To avoid samples from having repeat freeze/thaw cycles, samples were aliquoted into 80µl aliquots and frozen at -80˚C.

Western blot protocol

Tau species and PP2A activity were assessed by western blotting. To denature the samples, they were heated at 100˚C for 5mins. For analysis of PP2A activity, prior to heating at 100˚C, samples were pre-treated with a 2M NaOH solution so that a final concentration of 0.1M NaOH was generated. Subsequently, samples were incubated for 30min at 37 ˚C followed by neutralisation with 2M HCl. This was done to completely demethylate samples which would enable assessment of total PP2A levels. The PP2A catalytic ($PP2A_c$) subunit is

regulated by methylation of its 309L residue which induces its activation. The $PP2A_c$ antibody: 05-421 (Merck) binds $PP2A_c$ at this position and cannot bind if the molecule is methylated, binding only inactive PP2A (Stanevich et al., 2014). Figure 2.3 represents the pre-treatment of samples for total PP2Ac analysis.

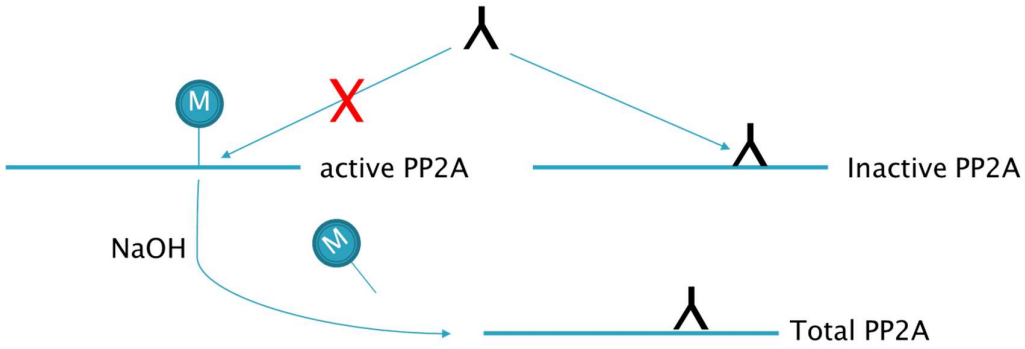

Figure 2.3: Assessment of total and inactive PP2A levels by western blotting. Figure drawn by author.

Following denaturation at 100˚C, 15µg total protein was resolved on either 7.5% criterion gels or 7.5% protean gels at 200V for 40 minutes using SDS-PAGE. For PP2A activity, samples were run on 10% gels. The same number from each group was represented on every blot to account for gel to gel variations. Batches were loaded in a different order to minimise any edge effect might have and where possible, blots were conducted in duplicate although due to insufficient tissue quantity and time constraints meant that this wasn't always possible. While running, Amersham Protran 0.45NC nitrocellulose membranes (GE Healthcare, #10600002) were soaked in transfer buffer for at least 15 minutes prior to the transfer process. Following resolving, samples were transferred to nitrocellulose membranes in ice cold buffer for 30 minutes (criterion) or 1 hour (protean). Nitrocellulose membranes then underwent ponceau staining to determine the effectiveness of the transfer. The ponceau stain was washed using tris buffered saline (TBST) on a see-saw rocker. Non-specific binding was blocked using a solution of 5% w/v milk (CO-OP) in TBST for one hour at room temperature (RT). Primary antibody incubation was conducted overnight at 4˚C in blocking buffer and the primary antibodies used in this chapter are listed in Table 2.3. GADPH (1:40,000, Sigma, #8795) was utilised as a loading control. Following primary antibody incubation, blots were washed in TBST for 3x5min and 3x10min on a see-saw rocker. For secondary antibody incubation, blots were incubated in infrared fluorescent secondary antibodies; IRDye 680CW goat anti-rabbit and IRDye 800CW goat anti-mouse (LI-COR

Biosciences) in blocking buffer for 1h at RT. It had been reported that either a fluorescent TrueBlot antibody or heat stable fractions is required to accurately assess tau concentrations by western blotting (Petry et al., 2014). However, the current protocol which was devoid for both techniques, found that background staining for the tau antibodies utilised in the current thesis were non-existent in mice KO for tau – suggesting that neither technique was required in the current study. Following secondary antibody incubation, blots were washed in TBST for 3x5min and 3x10min on a see-saw rocker and visualised using the Odyssey infrared imaging system (LI-COR Biosciences) using ImageStudio 3.0 (LI-COR). To maintain objectivity, blots were scanned on an identical intensity of 3 (680 channel) and 7 (800 channel).

Table 2.3: Primary antibodies utilised for western blotting

Antibody	Target	Host	Source	Dilution	Lot
Tau46	Total tau	Mouse	Sigma	1:500	T9450
CP13	pS202 tau	Mouse	Peter Davies	1:500	-
PHF1	pS396/404	Mouse	Peter Davies	1:500	-
RD3	3R tau isoforms	Mouse	Millipore	1:1000	05-803
RD4	4R tau isoforms	Mouse	Millipore	1:500	05-804
PP2A	PP2A, subunit C	Mouse	Millipore	1:5000	05-421
GAPDH	GAPDH	Rabbit	Sigma	1:40K	8795

Quantification and statistical analysis:

Blot intensities were measured in ImageStudio 3.0 (Li-Cor, USA) by drawing a rectangular box around each band which was specific for each blot. Depending on antibodies utilised for western blotting, non-specific binding of the antibodies can occur. Despite the antibodies utilised in the thesis being remarkably specific, non-specific binding nonetheless occurs and can be visualised by turning the contrast up on the blots (Figure 2.4A). Note that changing the contrast does not change the band intensities; they remain constant because the blots were scanned on the same intensity. To enable a similar contribution of non-specific binding across samples, the boxes utilised for quantification were kept to the same

height for each antibody across each blot. To do this, a box was drawn for the longest band and was copied for each lane (Figure 2.4B). This was especially important for tau staining which had a spread of binding due to its post-translational modifications. The automatic background subtraction function in ImageStudio 3.0 was utilised to determine the signal.

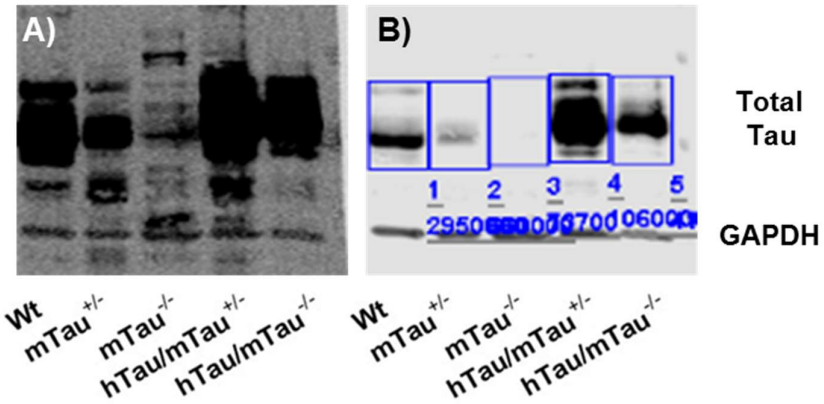

Figure 2.4 A sample Tau46 western blot. ***A)*** *When the contrast is turned up, extremely faint bands are observed in mTau$^{-/-}$ mice. Altering the contrast does not affect band intensities.* ***B)*** *Quantification of a Tau46 western blot. The quantification box was taken from the largest band and copied across all wells to control for contributions from non-specific binding.*

Band intensities were normalised to GAPDH to control for possible variations in loading. The decision to assess tau species individually (opposed to a ratio of total tau) was taken to minimise variation associated with generating a ratio between two separate blots. However, when alterations in total tau levels were significantly different between experimental groups, a ratio of phosphorylated over total tau was also analysed. For the tau isoforms a ratio between 4R:3R tau isoforms was generated. To assess PP2A activity, a ratio between non-NaOH:NaOH treated samples was used to give a ratio of inactive:total PP2A. Intensities were represented as mean ± SEM. Western blot data was normally distributed and a parametric approach was taken for statistical analysis. To account for gel-gel variability, a randomized block trial strategy was taken with the gel batch number as the blocking factor. To assess statistical differences between genotypes, a one-way ANOVA followed by a Tukey's post hoc was conducted. To assess statistical differences following LPS treatment, a two-way ANOVA followed by planned comparison was conducted. Standard SEM's are not appropriate for randomized block trials as the error bar is inflated due to gel-gel variability which the statistical approach evades. Instead the SEM was calculated from the error value from the ANOVA table. For all tau species assessed, mTau$^{-/-}$ were excluded

from the statistical analysis due to the absence of a band in any of the tau species which would violate the assumptions of the ANOVA.

2.4.3 Insoluble tau extraction:

The most common method for the assessment of aggregated tau is through a process known as sarkosyl extraction and involves the solubilisation of tissue samples under detergents of increasing strengths, resulting in separation of the soluble and insoluble protein fractions. The samples are lysed in a lysis buffer and centrifuged at low speed. The supernatant is known as the S1 fraction which corresponds to the cytosolic fraction of the cell and contains both soluble and insoluble tau. The S1 fraction is then incubated with the mild detergent: sarkosyl to solubilise soluble proteins. After incubation, the S1 fractions are ultracentrifuged at high speed to separate the sarkosyl soluble and insoluble fractions. The supernatant corresponds to the former and is termed the S2 fraction. The pellet which corresponds to the insoluble fraction (SI) contains aggregated tau species which can be re-suspended in a high concentration of SDS (Julien et al., 2012). Each fraction can then be probed for total and phosphorylated tau levels by western blotting.

The sarkosyl extraction protocol in the thesis was previously validated in the rtg4510 model (unpublished). Snap-frozen hemi-brains were weighed while being kept on dry ice by switching to a cooled Eppendorf. Hemi-brains were homogenised with a dounce homogeniser in a 11x w/v of ice cold lysis buffer (high salt and sucrose solution: 50mM Tris-HCl (pH 7.5, Sigma, #T2319), 10% sucrose (Fisher, #), 5mM EDTA (Sigma, T9285), 800mM NaCl (Sigma, #S1679), okadaic acid (Merck, #459620), α2-macroglobulin (Sigma, #10602442001) and Halt protease and phosphatase inhibitors (Thermo Scientific, #78440). The homogenates were spun at 6000G for 15 minutes at 4°C and the supernatant collected. The pellet was re-suspended in a 5x w/v of lysis buffer and combined with the previously collected supernatant. The centrifugation and resuspension was to ensure that the samples were fully homogenised. Total protein concentrations were determined with the BCA assay (Novagen #71285) and homogenates were diluted to 5µg/µl in lysis buffer and centrifuged at 14,000G for 15 minutes at 4°C. The supernatant was collected (S1) and corresponds to the cytosolic fraction which is comparable to that achieved from the hippocampal fractions. The pellet was discarded. 150µl of the S1 fraction was aliquoted while the remaining was incubated in 1% sarkosyl for 1h at room temperature. Subsequently, 2.5ml of the supernatant was centrifuged at 160,000G for 30 minutes at 4°C and the resulting supernatant was collected (S2). A wash spin was conducted by adding 2ml of lysis buffer

containing 1% sarkosyl to the pellet and again it was centrifuged at 160,000G for 30min. The pellet was re-suspended in 125µl of solubilisation buffer that was kept at RT (50mM Tris-HCl, 2.3% SDS, 1mM EDTA and Halt protease and phosphatase inhibitors) and corresponded to the SI fraction. All the fractions were snap frozen and stored at -80ºC until use for western blotting. S1 and SI fractions were analysed via western immunoblotting on 4-12% gels. Apart from altering the gel composition, the western blotting protocol was identical to that described above except total protein concentrations were not standardised in the SI fraction. Instead 20µl of each SI sample was loaded onto the gel.

2.4.4 Immunohistochemistry:

Microglial staining

Iba1 staining was performed on 7µm serial coronal slices of paraffin embedded hTau mice brains for microglial examination by a protocol which was previously published albeit with an altered antigen retrieval protocol (Ding et al., 2016). Slides were deparaffinised in xylene followed by rehydrating in descending concentrations of 95%, 70% and 50% industrial methylated spirit (IMS). Antigen retrieval consisted of heating in EDTA buffer (10mM Tris base (Sigma #T1503, 1mM EDTA (Fischer #D/0700/53), 0.05% Tween 20 (Sigma, # P2287) pH9.0) at 100˚C in a water bath for 20 mins. This was done by pre-heating EDTA buffer to 95˚C before the slides were added to the chamber. The 20min started when the temperature of the buffer reached 98˚C. Endogenous peroxidases were quenched in 1% H_2O_2 (Sigma # H1009) for 10 mins. Blocking of non-specific binding was conducted by incubating in 5% goat serum (Vector laboratories #PK-6101) for 30 mins at RT prior to primary antibody incubation of rabbit anti-IBA1 (1:6,000, WAKO #019-19741) for 1h30 mins at RT. Secondary antibody incubation consisted of biotinylated goat anti-rabbit (1:200, Vector Laboratories, #BA-1000) for 1h at RT followed by a standard diaminobenzidine (DAB, Vector Laboratories #SK-4100) procedure. Haematoxylin was used as a counterstain prior to coverslip mounting. Following immunostaining, slides were photographed using a Nanozoomer (Hamamatsu) at a magnification of 20x. For each animal, 6 technical repeats were conducted.

To analyse hippocampal microglial staining, microglia number and soma size were determined by a previously described semi-automated method using Matlab (Ding et al., 2016, Pardon et al., 2016). High resolution images of individual sections were exported from NDP viewer (Hamamatsu) and the hippocampal region was cropped. An overlay was created which was utilised for optimising threshold intensity (to account for variation in staining

strength). Images of each microglial soma were exported and artefacts were manually removed. Automatic analysis of soma/processes was then conducted which determined both the number of microglial soma and their size. The steps involved in the microglial analysis are depicted in Figure 2.5. Soma number and size were parametric. To assess statistical differences between genotypes, a one-way ANOVA followed by a Tukey's post hoc was conducted. To assess statistical differences following LPS treatment, a two-way ANOVA followed by planned comparison was conducted.

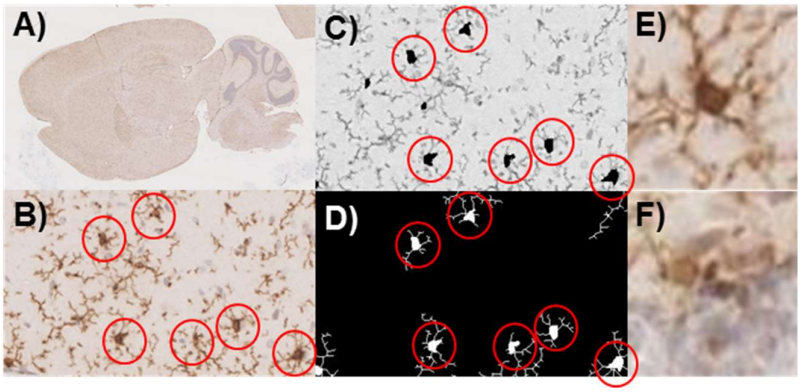

Figure 2.5 Semi-automated quantification of microglial staining. ***A)*** *An exported section of Iba1 staining.* ***B)*** *A hippocampal cropped section which has been enlarged and 7 microglial soma highlighted.* ***C)*** *An overlay created which was utilised to determine the threshold of the automation.* ***D)*** *Final results of the segmentation process results only microglia and their processes are selected.* ***E)*** *Example of an enlarged soma during the manual removal of artefacts.* ***F)*** *Example of an enlarged artefact which was removed from the process.*

Tau staining

To determine tau redistribution and visualise NFT containing neurons, 7µm serial coronal slices of paraffin embedded hTau mice brains were probed with Tau46 and CP13 staining. The protocol for tau staining was previously optimised and published (Rossi et al., 2018). Antibody retrieval consisted of boiling sections in sodium citrate buffer (10mM tri-sodium citrate, pH 6.0, Fischer #S/3320/53) in a similar manner to that for microglial staining. Following antigen retrieval, endogenous peroxidases were quenched by incubating in 3% H_2O_2 in methanol for 5 minutes. Blocking consisted of incubating in Mouse on Mouse (M.O.M.) blocking reagent (Vector, #MKB-2213) for 1h at RT. Following blocking, primary antibody incubation occurred at 4˚C overnight in Tau46 (1:400) or CP13 (1:25) which were diluted in blocking buffer. Secondary antibody incubation consisted of 1:200 biotinylated horse anti-mouse (Vector Laboratories, #BMK-2202) in blocking buffer for 1h at RT. A standard diaminobenzidine (DAB, Vector Laboratories #SK-4100) procedure was conducted and no counterstain was performed. Slides were photographed using a Nanozoomer (Hamamatsu) at a magnification of 20x and tau localisation was assessed qualitatively. Two

technical repeats were conducted per animal and the regions of interest were the hippocampus and the cortex. Significant non-specific staining was observed in Tau46 staining whiched showed staining of axons in mTau$^{-/-}$ genotypes and is given in figure 2.6. The Tau46 antibody has cross-reactivity with microtubule associate protein 2 (MAP2) which is likely the source of axonal staining in mTau$^{-/-}$ genotypes while not showing bands on western blots due to its high molecular weight of ~280 kDa (Kosik et al., 1988).

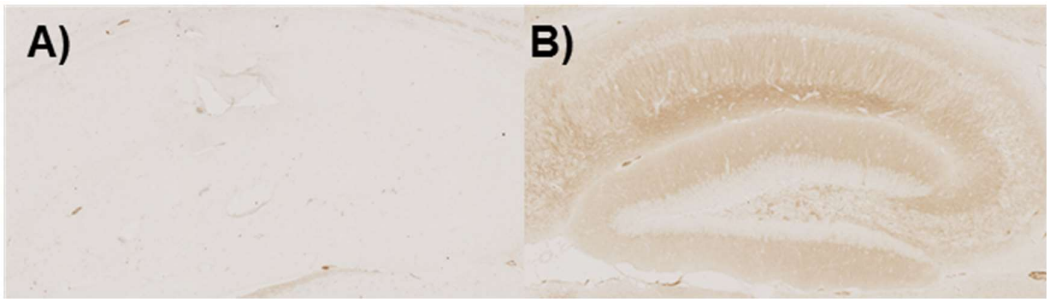

Figure 2.6: Non-specific staining of Tau46 in mTau$^{-/-}$ ***A)*** *No primary antibody reveals no non-specific staining.* ***B)*** *Tau46 staining in mTau$^{-/-}$ mice revealed staining of axons due to its cross-reactivity with MAP2*

2.4.5 Tau aggregation assay:

To verify potential changes in tau aggregation following LPS administration, a HTRF tau aggregation assay (Cisbio) was performed. The protocol was conducted according to the manufacturers' specification. The principle behind the assay is Förster resonance energy transfer (FRET). Briefly, an anti-tau antibody is conjugated to a FRET donor and receiver so that some antibodies are bound to a donor and some a receiver. As the antibody is identical, only one can bind to a single tau protein. If tau is soluble, FRET donors and receivers are not in close proximity and little fluorescence is given. If the tau in the sample is aggregated, the FRET donors and receivers are brought together and release a FRET signal. In the current study 10 µl of S1 fractions from LPS treated mice or a positive control were incubated with 10 µl mix anti-tau-tb and anti-tau-d2 antibodies in a 362 micro-well plate (FRET donor and receiver respectively). Following 2h incubation, the FRET signal was measure on a PHERAstar FS plate reader at a wavelength of 620 nm and 665 nm.

2.4.6 Multiplex analysis of serum cytokine levels:

To verify the inflammatory response following LPS administration, serum IL-1β, IL-6, IL-10, INFγ and TNFα were measured by multiplex analysis (Bio-Rad, custom X-plex). The protocol was conducted according to the manufacturers' specification. Briefly, trunk blood was centrifuged in Li-Heparin serum separating tubes (Sarstedt #41.1503.005) at 3000G for 10 min at 4˚C and the supernatant frozen at -80˚C. Prior to running each multiplex experiment, samples were thawed and centrifuged at 10,000G at 4˚C to remove any

interfering aggregates and diluted 1:2 in sample buffer prior to running the assay. Samples which were below the range of analysis have negligible concentrations and were considered to be void of the cytokine for analysis purposes. Due to the unequal variance generated from this, data was rank transformed prior to running of a two-way ANOVA followed by planned comparisons where appropriate.

Chapter 3 Method optimisation

Introduction

In addition to potentially altering underlying pathologic processes, systemic inflammation has the potential to exacerbate the symptomatic effects of the disease. Systemic infections have been reported to exacerbate cognitive function in AD patients even after the resolution of the original inflammatory insult (Holmes et al., 2003). Inflammation is well known to affect cognitive function by altering the underlying processes involved in learning and memory. For example, IL-1β, TNFα or LPS application to organotypic rat hippocampal slices results in inhibition of LTP (Cunningham et al., 1996). However, assessing cognitive function in rodents following LPS administration is problematic due to the sickness syndrome associated with administration of an immune challenge. This chapter is aimed at validating a contextual fear conditioning (CFC) task which could be utilised in the hTau model to understand the synergism between inflammation and tau pathology on cognitive function without confounding sickness effects. To aid in the development of the task, the dynamics of the LPS-induced sickness syndrome was tracked through measurement of food intake, locomotor activity, respiratory exchange ratio (RER) and calorie expenditure in the Comprehensive Lab Animal Monitoring System (CLAMS™). In validating a learning and memory task which would not be confounded by the LPS sickness syndrome, the author hoped to accurately decipher synergism between cognitive impairments in the hTau model and those which occur in response to systemic inflammation. This would aid in the understanding of the symptomatic contribution of systemic inflammation within AD. Unfortunately, the development of strong allergies to mice while attempting to validate the contextual fear conditioning model meant that the author had to limit exposure in the animal unit. Due to this only minimal *in vivo* work could be conducted for the rest of the PhD and the aim of understanding the symptomatic exacerbation of systemic inflammation in respect to tau pathology was discontinued.

The second objective of this chapter was to validate an *ex vivo* ^{1}H magic angle spinning (MAS) - nuclear magnetic resonance (NMR) spectroscopy technique equivalent to *in vivo* NMR spectroscopy. This was intended to aid in understanding the neuroinflammatory response following LPS administration in a translatable manner. Neuroinflammatory mediators are present at extremely low concentrations (Erickson and Banks, 2011), making quantification of neuroinflammation difficult. Select metabolites assessable by magnetic resonance spectroscopy, the clinical nomenclature for *in vivo* NMR, are thought to relate to

neuroinflammation (Chang et al., 2013). Due to initial issues with breeding in the hTau colony, both the CFC task and NMR were validated in the amyloid APPswe/PS1dE9 model.

3.1 Contextual fear conditioning involving a pre-exposure session:

3.1.1 Contextual fear conditioning overview:

CFC is a form of aversive learning which is commonly utilised in AD models. For a thorough review of CFC see: (Pape and Pare, 2010). Fear conditioning involves the pairing of a neutral stimulus – often referred to as the unconditioned stimulus (US) – with an aversive stimulus which is referred to as the conditioned stimulus (CS). In terms of CFC the US is the context of the conditioning chamber and the US is most commonly a foot shock. In a typical CFC paradigm, a mouse is exposed to a context for a certain period prior to being administered with a foot shock. This causes the animal to associate the foot shock with the context of the conditioning chamber. The delay before the shock is required in order for the animal to form a memory of the context (Wiltgen et al., 2001). This is known as the conditioning trial after which the animal is returned to its home cage. A retention trial is generally held 24h later whereby the animal is returned to the conditioning chamber without the CS. The context acts a cue for the CS and a fear response is elicited which can be measured. Most commonly the fear response measured is known as freezing behaviour. Freezing is an innate behaviour in rodents expressing fear whereby the animal does not move from its position as if frozen in its stance (Curti, 1935). Freezing and immobility measures have a high degree of correlation and measuring the latter enables accurate automatic tracking of the fear response in CFC (Contarino et al., 2002). The stronger the fear memory is expressed in the retention trial, the greater the animal freezes, giving an indication of learning and memory. Following the retention trial, an extinction trial can be held whereby the animal is again exposed to the context without the CS. As the animal has already been in the conditioning chamber without receiving a shock, the contextual fear aversion is weakened as the animal relearns the context is not aversive. A corresponding decrease in freezing levels is observed. Importantly this process is re-learning rather than forgetting and is a measure of cognitive flexibility (Rescorla, 2001). A depiction of a general CFC protocol is given in Figure 3.1.

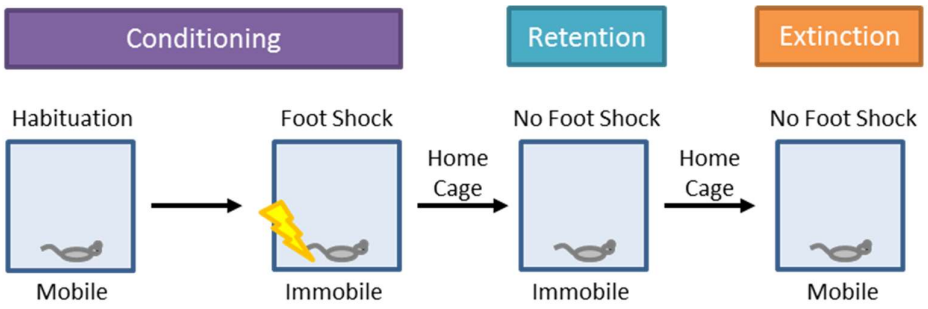

Figure 3.1: A typical CFC protocol: In the conditioning trial, the mouse is given a habituation to form a memory of the context followed by a foot shock. The mouse forms an association with the foot shock and the context so the next time it is introduced to the conditioning chamber it elicits a fear response. Upon placement into the conditioning chamber for a third time the mouse has relearnt that the context is not aversive and spends more time mobile.

The significance of contextual fear in AD models:

The attractiveness of utilising CFC in AD models comes when understanding the underlying memory processes. The task allows assessment of short term memory in the retention trial and cognitive flexibility in the extinction trial – both of which are affected in AD (Albert, 1996, Rescorla, 2001). Even more interesting is considering the neural circuitry involved. While the amygdala is synonymous with associated fear memory, in CFC it is heavily reliant on input from various brain regions (Pape and Pare, 2010). Firstly, the hippocampus is responsible for forming the conjunctive representation, the unified depiction of elements of a context, and damage to the hippocampus impairs CFC while sparing auditory fear tasks (Phillips and LeDoux, 1992, Rudy and O'Reilly, 1999). Secondly, the hippocampus influences the amygdala by projections through prefrontal cortical regions, modulating contextual fear expression (Maren et al., 2013, Giustino and Maren, 2015). Projections from the prelimbic cortex (PL) are involved in retention but not learning nor extinction of contextual fear (Corcoran and Quirk, 2007, Laurent and Westbrook, 2009). On the other hand projections from the infralimbic cortex (IL) but not the PL are involved in extinction of contextual fear through inhibiting the amygdala (Laurent and Westbrook, 2009). This is the real advantage of utilising contextual fear conditioning in AD models, it assesses cognitive domains impaired in AD and the underlying anatomical areas involved in the task are likewise affected in AD (Braak and Braak, 1991, Auld et al., 2002).

Fear conditioning is the most suitable task to assess the effect of immune challenges on hippocampal dependent cognitive function:

LPS and immune challenges in general confound cognition paradigms due to the sickness effects they exert. The LPS-induced sickness syndrome has the ability to negatively affect behavioural models primarily in three ways:

1. The task may not be performed
2. Performance behaviours such as locomotor activity are affected
3. Potential state dependent effects or other unknown behavioural alterations may occur

Although issues arising from the first point are immediately evident, the consequences of the latter two are more severe as inappropriate interpretations of the data are often made. Attempts to circumvent these confounding effects in the literature have taken generally two approaches. The first of which involves utilising tasks which increase the motivation of the animal to perform the task. The Morris Water Maze (MWM) appears ideal for this purpose as the animals have to actively escape to safety. However, an interesting study from (Sparkman et al., 2005a) demonstrated that impairments in the task persisted in mice administered with LPS even when a visible platform version of the task was utilised, indicating performance related behaviours as the source of impairment. A shallow water T-Maze developed by Murray *et al.,* 2012 was a more promising attempt at increasing motivation to assess cognition following LPS administration. The task involved filling a T-Maze with a 2cm depth of water and alternating the arm in which the mouse could escape between trials. To escape, the mouse had to correctly alternate the escape arm (Murray et al., 2012). The advantage that the task has over the MWM is that performance related behaviours do not affect the task. However, the authors report that LPS only managed to impair the task in the ME7 model which is classified as a severe model and ultimately results in death (Murray et al., 2012, Felton et al., 2005). Instead, a more attractive option is to assess cognition after the confounding LPS effects have worn off, with contextual fear conditioning being the prime candidate.

Separate conditioning and retention trials in CFC mean LPS can be administered after conditioning. By allowing for enough time to recover from the LPS-induced sickness syndrome between trials, the task will not be confounded and cognition can be accurately measured. Through a series of experiments, Pugh *et al.*, 1998 determined that LPS impairs cognition in the task, the first of which saw rats administered with LPS immediately after the

conditioning trial demonstrate impairments in contextual fear but not auditory fear conditioning. By utilising a pre-exposure facilitation effect, a phenomenon by which giving an animal a pre-exposure to the context enhances formation of the contextual fear memory (Brown et al., 2011), the impairments were ablated (Pugh et al., 1998). This indicates that deficient knowledge of the context is in fact the source of the impairments in the task rather than confounding LPS effects. Later attempts in mice have shown LPS administered immediately or 24h but not 48h following conditioning to impair contextual fear 3 or 7 days post conditioning (Terrando et al., 2010, Fidalgo et al., 2011). The contextual fear conditioning protocol in this chapter utilises both the contextual pre-exposure effect and an immediate shock deficit and was based off that originally described by Brown *et al.*, 2011. The immediate shock deficit is the phenomenon that if an immediate shock is given during conditioning, the animal does not learn the context (Wiltgen et al., 2001), meaning the animal would need to rely on the context representation previously learnt in the pre-exposure session to form the context aversion (Brown et al., 2011). This protocol was chosen over traditional fear conditioning protocols such as those utilised by Terrando *et al.*, 2010 and Fidalgo *et al.*, 2011 as it allowed for the assessment of short term memory following conditioning.

3.1.2 Study Design

Comprehensive laboratory animal monitoring system (CLAMS™) assessment:

To analyses the dynamics of the LPS-induced, a total of 18, experimentally naïve, 6 month old female APPswe/PS1dE9 mice (n=9/genotype) underwent CLAMS™ testing. Mice were singly housed one week prior to the assessment of metabolic parameters to habituate to single housed conditions. Animals were placed in the chamber at 9am and habituated for 3h followed by a 24h baseline recording starting at 1pm. Following the baseline recording, animals were injected with LPS and the inhibition and recovery of metabolic parameters were tracked for 96 hours. The 96h timeframe was chosen for welfare reasons as placement in the CLAMS™ chamber is a confounding factor as there is no environmental enrichment. Following metabolic testing, the animals were sacrificed immediately. A diagram of the experimental design is given in Figure 3.2.

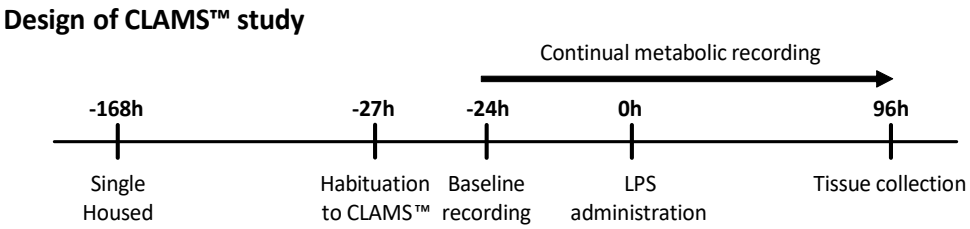

Figure 3.2: Design of the metabolic study: animals were singly housed for one week prior to being placed in the CLAMS™ apparatus. The animals were given a 3h habituation to the chamber before starting the baseline recording at 1pm. After a 24h baseline recording, the animals were administered with LPS and monitored for 96h.

Contextual fear conditioning with a pre-exposure to the context

A total of 56, experimentally naïve, 6 month old female APPswe/PS1De9 mice were utilised for validation of a contextual fear conditioning protocol which would not be affected by the LPS-induced sickness syndrome. Behavioural testing occurred over 5 consecutive days depicted in Figure 3.3. On day one the animals were pre-exposed to the conditioning chamber and administered with LPS. The mice were allowed to recover for two days prior to undergoing conditioning. Retention and extinction of contextual fear memory was assessed on days 4 and 5 respectively. Bodyweight was recorded every day. Cortical samples were utilised for validation of the *ex-vivo* NMR spectroscopy technique.

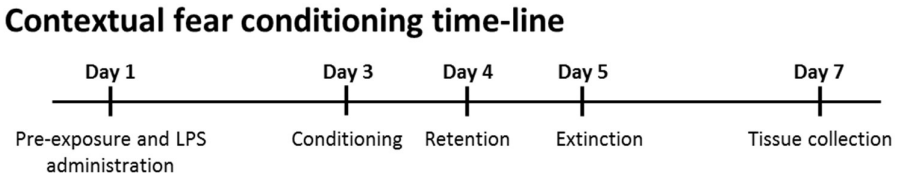

Figure 3.3: CFC protocol: The CFC protocol lasted 5 days, with tissue collection occurring 7 days post-LPS administration.

3.1.3 Results:

3.1.3.1: The LPS-induced sickness syndrome resolves in 48h in both Wt and APPswe/PS1dE9 mice:

Similar bodyweights in APPswe/PS1dE9 mice and their Wt littermates:

To design a task which might be suitable for assessing the cognitive impairing properties of LPS, the persistence of the LPS-induced "sickness" response was measure using CLAMS™. The CLAMS™ apparatus quantifies a variety of metabolic parameters which are affected by the LPS-induced "sickness" syndrome including: food intake, locomotor activity,

calorie expenditure and the RER. Baseline bodyweights in the CLAMS™ study are depicted in Figure 3.4 and were not significantly altered in APPswe/PS1dE9 mice compared to Wt littermates [$t_{(14)}$ = 1.788, p = 0.095].

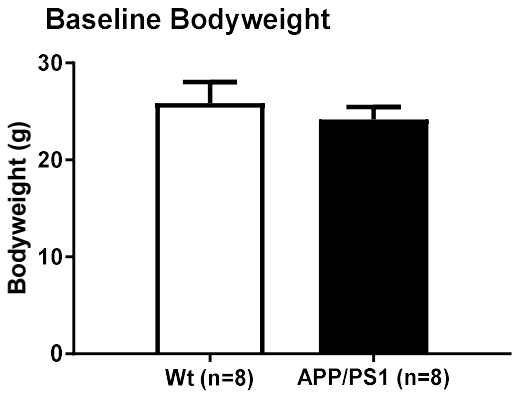

Figure 3.4: Mean ± SEM baseline bodyweights of 6 month old female APPswe/PS1dE9 mice from the CLAMS™ study. APPswe/PS1dE9 and their Wt littermates had similar bodyweights.

Baseline physical and metabolic parameters:

Basal physical and metabolic parameters were assessed in the CLAMS™ for 24 hours prior to LPS administration. To further elucidate differences between APPswe/PS1dE9 mice and their Wt littermates, recordings were split into their light/dark cycle. Food intake is represented in Figure 3.5A and was similar in both genotypes [$F_{(1,13)}$ = 0.85, p = 0.373]. One-way repeated ANOVA analysis revealed there was a significant genotype x light cycle interaction in locomotor activity [$F_{(1,14)}$ = 7.91, p = 0.014] with post hoc analysis indicating APPswe/PS1dE9 mice were more active during the dark cycle (Figure 3.5B, p = 0.006). Both basal RER and calorie expenditure did not differ between genotypes [$F_{(1,14)}$ = 0, p = 0.948] and [$F_{(1,14)}$ = 0.46, p = 0.501] respectively (Figure 3.5C-D).

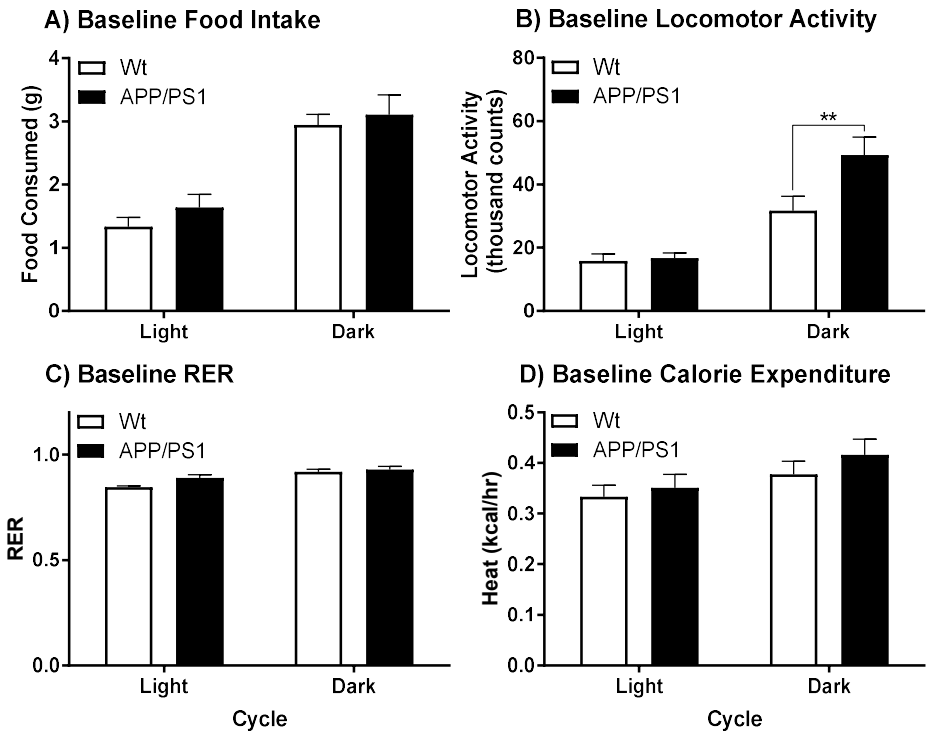

Figure 3.5: Mean ± SEM baseline physical and metabolic recordings pf 6 month female APPswe/PS1dE9 mice (n=8/genotype). ***A)*** *Basal food intake was similar among both genotypes. B) Basal locomotor activity indicated APPswe/PS1dE9 mice as hyperactive.* ***C)*** *Basal RER and* ***D)*** *calorie expenditure were comparable within both genotypes. ** p < 0.01*

Bodyweight returned to baseline by the end of the study:

The recovery of bodyweight following LPS administration is depicted in Figure 3.6 and occurred at a similar rate in both genotypes. One-way repeated ANOVA analysis revealed no significant genotype nor genotype x day interaction [$F_{(1,13)}$ = 0.23, p = 0.684] and [$F_{(3,43)}$ = 2.67, p = 0.06]. In both cases, one sample *t*-tests indicated that bodyweight returned to basal levels within 96h.

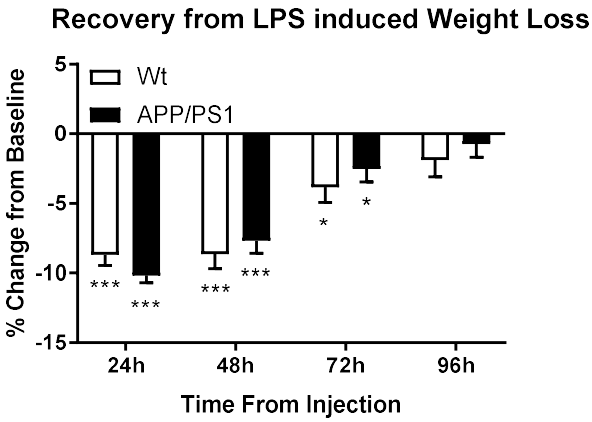

*Figure 3.6: Mean ± SEM bodyweight change following LPS administration in 6 month female APPswe/PS1dE9 mice (n=8/genotype). Both APPswe/PS1dE9 mice and their WT-littermates recover bodyweight to the same extent following LPS administration to basal levels after 96h. One sample t-test: * p < 0.05 and *** p < 0.001 vs. baseline level.*

Food intake returned to baseline within 24h:

The time-course of the LPS-induced suppression in food intake is represented in Figure 3.7A. LPS administration resulted in complete inhibition of eating behaviour in both APPswe/PS1dE9 and Wt littermates for almost 18 hours. To assess whether animals were significantly eating less than at baseline, a one way *t*-test was conducted for each time point – showing that both genotypes returned to baseline within 24h as depicted in Figure 3.7B. One-way repeated measures ANOVA analysis indicated no significant genotype or genotype x time point effects during the recovery [$F_{(1,13)} = 0.13$, $p = 0.722$] and [$F_{(7,91)} = 0.62$, $p = 0.735$] respectively.

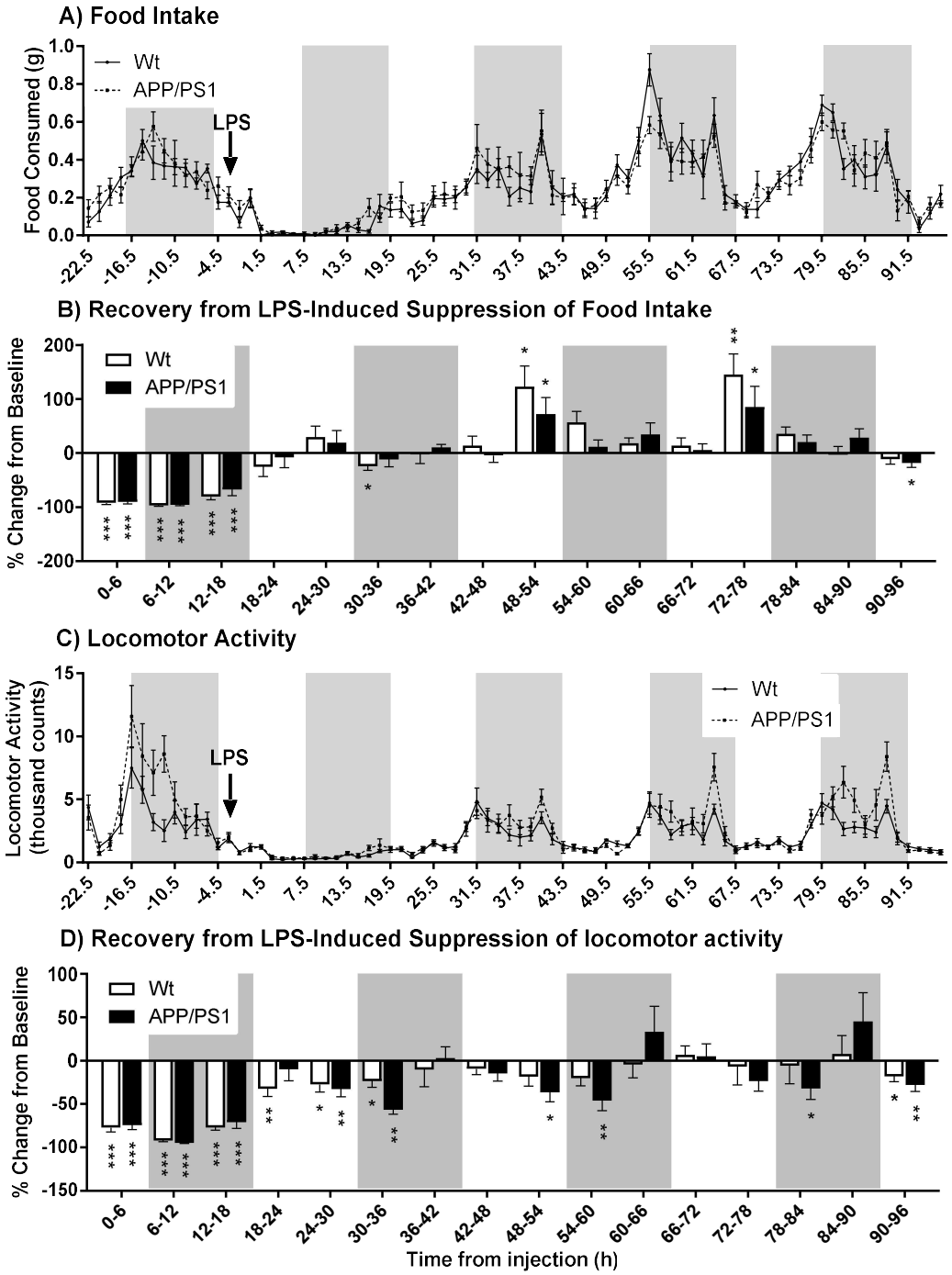

Figure 3.7: Food intake and locomotor activity following LPS administration in 6 month female APPswe/PS1dE9 mice (n=8/genotype). ***A and C)*** *Food intake and locomotor activity time course. Data represented as mean ± SEM for each 1.5h bin and non-shaded/shaded regions represent light and dark cycles respectively. LPS administration is indicated by an arrow.* ***B and D)*** *Recovery from LPS induced suppression. Bars represent mean ± SEM and shaded/non-shaded regions indicating the light and dark cycle respectively. LPS completely suppressed food intake in both genotypes, recovering after 18 hours. Locomotor activity was completely inhibited in both genotypes, recovering within 36h in Wt mice while not fully recovering in APPswe/PS1dE9 mice by the end of the study. One-sample t-test: * p < 0.05, ** p < 0.01 and *** p < 0.001 vs. baseline levels.*

Slower recovery of locomotor activity in APPswe/PS1dE9 mice:

The time-course of the LPS-induced suppression in locomotor activity is represented in Figure 3.7C. LPS administration resulted in almost complete inhibition of locomotor activity in both genotypes which only began to recover at the 30h time-point. Locomotor activity returned to baseline within 48h in Wt mice. Although, APPswe/PS1dE9 mice had regained most activity within the same timeframe, they had not reached baseline levels even by the end of the study as depicted in Figure 3.7D. Despite locomotor activity not returning to baseline levels by the end of the study, it is misleading to suggest the rate of recovery is slower in APPswe/PS1dE9 mice. This is because the recovery rate is identical between both genotypes, but APPswe/PS1dE9 mice have a greater distance to recover due to their hyperactivity (Figure 3.7C). One-way repeated measures ANOVA analysis showed that there was no significant genotype or genotype x bin interaction effect in the recovery of locomotor activity ([$F_{(1,14)}$ = 0, p = 0.973] and [$F_{(7,98)}$ = 1.71, p = 0.116] respectively).

Respiratory exchange ratio returned to baseline within 48h:

The RER time course following LPS administration is plotted in Figure 3.8A. LPS similarly induced a maximal RER suppression of 20% (±1.7) and 20.1% (±1.1) in APPswe/PS1dE9 mice and Wt littermates respectively. RER returned to baseline in 24h for APPswe/PS1dE9 mice and 42h for Wt-littermates as depicted in Figure 3.8B. In both genotypes there was a rebound in RER which was significantly above baseline for a period prior to returning to pre-injection levels. One-way repeated measures ANOVA analysis showed that there were no significant genotype nor a genotype x bin interaction in the recovery of RER following LPS administration [$F_{(1,13)}$ = 1.78, p = 0.211] and [$F_{(15,194)}$ = 1.28, p > 0.224] respectively.

Calorie expenditure returned to baseline within 48h:

The time course of calorie expenditure is plotted in Figure 3.8C. LPS induced a maximal 29.6% (±3.3) and 18.9% (±3.7) reduction in APPswe/PS1dE9 and Wt littermates respectively which persisted for 24h in Wt mice and 48h in APPswe/PS1dE9 mice as depicted in Figure 3.8D. However, one-way repeated measures ANOVA analysis shoed that there was no significant genotype nor genotype x bin interaction effect in the recovery of calorie expenditure [$F_{(1,14)}$ = 0.21, p = 0.65] and [$F_{(15,209)}$ = 1.43, p = 0.135] respectively.

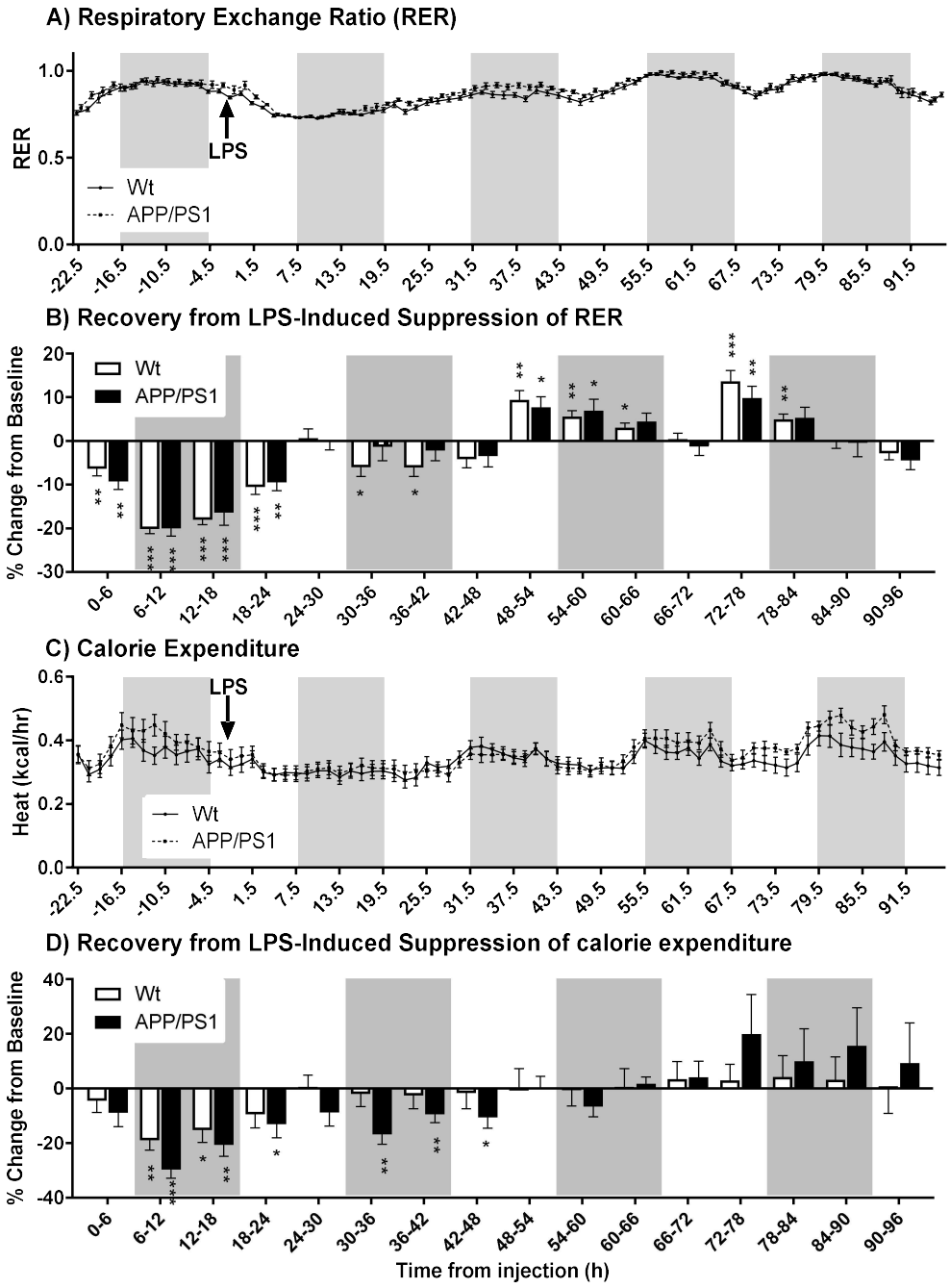

Figure 3.8: Food intake and locomotor activity following LPS administration in 6 month APPswe/PS1dE9 mice (n=8/genotype). ***A and C)*** *RER and calorie expenditure time course. Data represented as mean ± SEM for each 1.5h bin and non-shaded/shaded regions represent light and dark cycles respectively. LPS administration is indicated by an arrow.* ***B and D)*** *Recovery from LPS induced suppression. Bars represent mean ± SEM and shaded/non-shaded regions indicating the light and dark cycle respectively. LPS suppressed RER by about 20% in both genotypes, recovering within 48 hours. Calorie expenditure was inhibited by 30% in APPswe/PS1dE9 mice and 20% in Wt-littermates and recovered in APPswe/PS1dE9 mice within 48 hours compared to 18 hours in their Wt littermates. One-sample t-test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. baseline levels.*

3.1.3.2: Contextual fear conditioning following resolution of the LPS-induced sickness syndrome:

To assess the impact of systemic inflammation on cognitive function in AD, APPswe/PS1dE9 mice were assessed for contextual fear memory following LPS administration. The task was specifically chosen as to not be confounded by the profound sickness behaviours associated with administration of an immune challenge.

Similar immobility in the pre-exposure session:

Immobility during the pre-exposure session was assessed as a measure of underlying locomotor activity in the conditioning chamber. Immobility was assessed during the first 5 minutes and the total length of the pre-exposure session which is shown in Figure 3.9A. Unpaired *t*-test analysis revealed there were no significant differences in immobility during the first 5 minutes of the pre-exposure session between APPswe/PS1dE9 mice and their Wt littermates [$t_{(54)}$ = 0.491, p = 0.635]. Following repeated exposure to the context, both genotypes spent more time immobile. Although unpaired *t*-test analysis revealed there was no significant alterations observed when taking the total pre-exposure session into account (Figure 3.7A), there was a trend for APPswe/PS1dE9 mice to spend less time immobile than their Wt littermates [$t_{(54)}$ = 1.93, p = 0.0548].

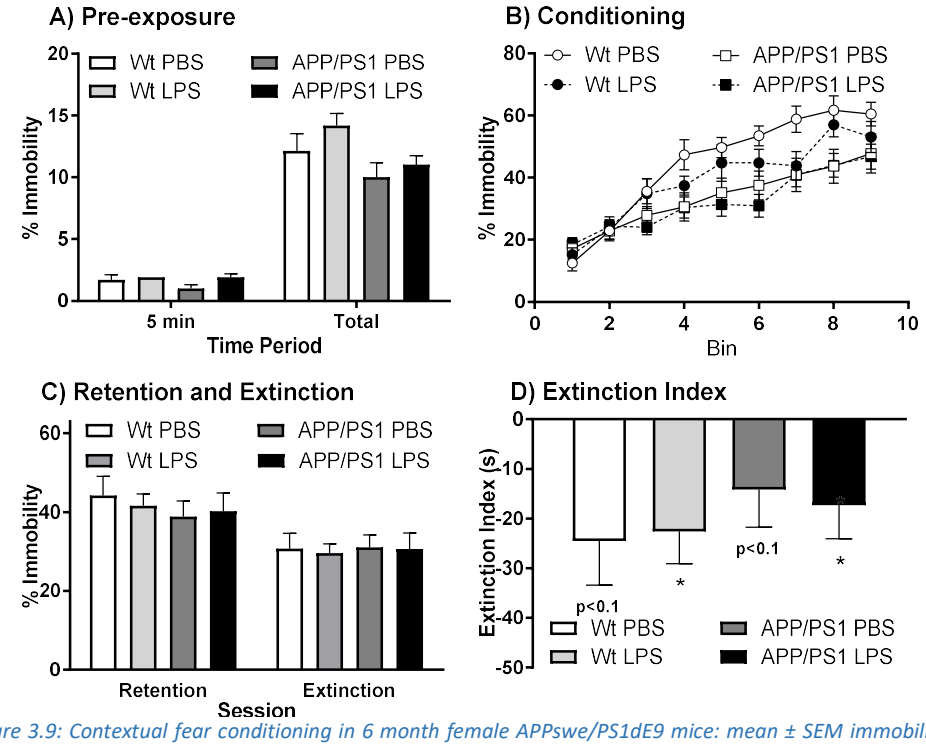

Figure 3.9: Contextual fear conditioning in 6 month female APPswe/PS1dE9 mice: mean ± SEM immobility levels (n=11-16/genotype). ***A)*** *Pre-exposure session: Wt and APPswe/PS1dE9 mice possessed similar locomotor activity during the pre-exposure session.* ***B)*** *Immobility levels during the conditioning session were expressed in 1min bins to highlight the acquisition of contextual fear. APPswe/PS1dE9 mice spent less time immobile over the conditioning trial, likely a result of their hyperactivity which was not affected by LPS.* ***C)*** *Immobility levels during the retention and extinction sessions. APPswe/PS1dE9 mice expressed and extinguished contextual fear to a similar extent as their Wt littermates which was also unaffected by LPS.* ***D)*** *A simple extinction index, the immobility levels during retention – extinction (mean ± SEM), indicated a trend in all groups to extinguish contextual fear. One-sample t-test: * p < 0.05 against retention levels.*

Decreased acquisition of contextual fear in APPswe/PS1dE9 mice:

An immediate shock was given during the conditioning trial in order to induce an immediate shock deficit. Thus, the animal must rely on its memory of the context to form the context aversion. Increasing immobility over each time bin was taken as an index for the acquisition of contextual fear. Similar immobility levels during the first minute of the conditioning trial indicated that the task was not confounded by LPS. Two-way repeated measures ANCOVA analysis revealed LPS failed to significantly impede the acquisition of contextual fear, indicated by no treatment nor treatment x bin interaction effect ([$F_{(1,51]}$ = 1.98, p = 0.165] and [$F_{(8,416]}$ = 1.26, p = 0.26] respectively). However, APPswe/PS1dE9 mice acquired contextual fear to a lesser extent than their Wt littermates, indicated by a significant genotype x time bin interaction [$F_{(8,416)}$ = 5.08, p < 0.001]. Post-hoc analysis revealed from the third bin onwards that APPswe/PS1dE9 were significantly less immobile, which began to converge by the last time bin (Figure 3.9B).

LPS did not affect contextual fear retention or extinction in APPswe/PS1dE9 mice:

Contextual fear was similarly expressed at a level of ~40% in both APPswe/PS1dE9 and their Wt littermates. Two-way ANCOVA analysis revealed that immobility in the retention session was similar among genotypes [$F_{(1,51)}$ = 0.32, p = 0.57] and not affected by LPS administration [$F_{(1,51)}$ = 0.08, p = 0.78] as depicted in Figure 3.9C. Likewise, two-way ANCOVA analysis showed that extinction of contextual fear was unaltered in APPswe/PS1dE9 and Wt mice and was again not affected by LPS administration [$F_{(1,51)}$ = 0.3, p = 0.59] and [$F_{(1,51)}$ = 0.15, p = 0.7] respectively (Figure 3.9D).

3.1.4 Discussion

This section was aimed at validating a cognitive task which would not be confounded by the LPS-induced sickness syndrome and could be utilised in the hTau model. CFC was chosen as the protocol would allow the mice to recover from these confounding effects between trials. In order to accurately design the task, the dynamics of the LPS-induced sickness syndrome was evaluated. The CLAMS™ apparatus provided the ability to non-invasively assess the behavioural suppression associated with LPS administration. Both appetite and locomotor activity parameters give an accurate measurement of the LPS-induced sickness syndrome (Huang et al., 2008). The recovery in appetite following LPS administration was the quickest parameter to recover, returning to baseline levels within 24h in both genotypes. It is important to note that LPS could be affecting appetite at this point but the increasing natural inclination for hunger outweighs the diminishing confounding LPS effects. Locomotor activity, a behaviour not driven by an inherent need, provides a more accurate representation of motivation – the most relevant parameter to a fear conditioning task. While locomotor activity returned to baseline within 48h in Wt-littermates, it didn't return to baseline in APPswe/PS1dE9 mice even by the end of the study. Despite not returning to baseline levels, APPswe/PS1dE9 mice did regain significant levels of locomotor activity within 48h hours, indicating the LPS-induced immobility had passed. Both metabolic parameters, RER and calorie expenditure, returned to basal levels within 48h in both genotypes. A limitation of the CLAMS™ study is that the animals were required to be single house without nesting material or a play tube. Both of these are confounding factors which could alter the stress levels of the animals. Stress is well known to exacerbate inflammation (Munhoz et al., 2006, Espinosa-Oliva et al., 2009) and therefore the recovery times could even be quicker than indicated by the CLAMS™ studies.

Based on the recovery data from the LPS-induced sickness syndrome tracked in the CLAMS^{TM}, a delay of 48h was chosen between LPS administration and continuation in the CFC task. Immobility levels in the first minute of the conditioning trial were similar among PBS and LPS treated animals, suggesting that a two day delay was sufficient for the task to not be confounded. Despite this, LPS failed to affect the acquisition, retention and extinction of contextual fear. This is in contrast to the literature, where the same dose of LPS was found to inhibit contextual fear in a traditional CFC protocol involving LPS administration immediately after conditioning and a retention trial given 7 days following (Fidalgo et al., 2011). The failure of LPS to induce impairments in the current study could be due to issues with the protocol. The protocol relies on an immediate shock deficit during the conditioning session, so that context learning is required during the pre-exposure session. Brown *et al.,* 2011 opted for a 2s, 0.75mA shock whereas the protocol in this chapter utilised a more ethical 0.4mA shock regime. As a lower intensity shock threshold was used compared to that from Brown et al., 2010, a greater number of shocks were utilised. As such, the time between shocks could allow enough time for the animal to learn the context during the conditioning session. Equally possible, the lower threshold shock in itself could fail to produce enough aversion to induce an immediate shock deficit. A learning curve during the conditioning trial in LPS treated animals potentially supports either hypothesis. Indeed, virtually no freezing is observed after giving an immediate shock (Kiernan et al., 1995), although definitive comparisons cannot be made as an immediate shock deficit with a multiple shock conditioning protocol has not been attempted before. In an attempt to avoid the chance to learn the context between shocks, a preliminary study with 5 shocks 30s apart produced similar levels of immobility during retention of contextual fear and again failed to be susceptible to LPS impairments. The failure of this last attempt indicates the 0.4mA shock may indeed be insufficient to induce an immediate shock deficit. At this point the decision was made to not continue with development of the task due to development of allergies meaning exposure to animals had to be limited.

While attempting to find a task which is not directly confounded by the LPS-induced sickness syndrome would be of great interest, there could still be indirect effects. For example, even if no direct effect of the sickness syndrome on a contextual fear conditioning task existed, it still might be indirectly affected. The LPS-induced sickness syndrome is associated with substantial sleep disturbances. Sleep deprivation is well known to impair consolidation of the contextual fear memory, producing impairments in the retention trial of the task (Graves et al., 2003). Therefore, impairments in the task could simply be resultant of

sleep disruption rather than affecting cognition per se. As such, the task is potentially confounded even when appearing devoid of the LPS-induced sickness behaviours. Further exacerbating the issue, common practice is for behavioural studies to be conducted in the morning when the mice are more active. As mice are nocturnal animals, any period the mice are not being conditioned, up until the end of the light cycle, will mostly be spent asleep. This is when consolidation of the contextual fear memory occurs. For example, disruption of sleep in the first 5h after the conditioning trial has been reported to cause impaired memory consolidation in the task while disruption of the 5h thereafter has no affect (Graves et al., 2003). This coincides with the time when the LPS-induced sickness syndrome is at its strongest, suggesting sleep deprivation as a possible source of impairments in contextual fear tasks. Therefore, even in the task which is thought to be least confounded by LPS administration, significant sickness effects could still be affecting the task.

Another approach to understand the symptomatic effect of systemic inflammation on tau pathology would be to use an inflammatory model which is much milder and not affected by the sickness syndrome. The CIA arthritis model would be ideal in this respect as the model experiences mild chronic inflammation without being affected by a sickness syndrome (Paquet et al., 2012, Amdekar et al., 2011, Tsubaki et al., 2015). However, the significant locomotor alterations in the model means the CIA model suffers from similar performance related issues as the LPS-induced sickness syndrome (Hartog et al., 2009). While using food reward learning paradigms instead of those which rely on locomotor activity might circumvent the issue, CIA is a pain model (Fischer et al., 2017) and still might confound these cognitive paradigms. Instead, the ovalbumin asthma model is a preferred approach. Ovalbumin sensitisation in mice is only associated with a brief depression in locomotor activity which is also present in vehicle treated mice, suggesting it is a brief effect of administration rather than inflammation (Domnik et al., 2012). Cognitive impairments following ovalbumin sensitisation have been reported in the MWM as well as decreasing the number of synapses and impairing LTP (Guo et al., 2013). However, as previously stated, the high levels of IL-4 within the model mean it is only suitable for understanding the effect of asthma or other atopic inflammatory conditions and is not suitable for understanding the effect of systemic inflammation in AD. This leaves models of infection, with attenuated bacteria strains the most attractive due to their milder responses. Mice infected with the attenuated bacteria strain; SL3261 *salmonella typhimurium* show only brief reductions in bodyweight and burrowing behaviour which are much less than the duration of the systemic inflammatory response (Püntener et al., 2012). The persistence of systemic inflammation

past the resolution of sickness behaviours makes attenuated infections such as the SL3261 strain of *salmonella typhimurium* ideal for studying the symptomatic effects of inflammation on tau pathology. Highlighting the need for infection with an attenuated infection, cecal ligation and puncture results in significant mortality and pro-longed locomotor activity impairment (Granger et al., 2013). Therefore, the use of attenuated infections appears an attractive avenue for elucidating the symptomatic consequences of the interaction between systemic inflammation and tau pathology.

3.2 ^{1}H Magic Angle Spinning (MAS) - Nuclear Magnetic Resonance (NMR) Spectroscopy analysis of Brain Metabolite Levels:

3.2.1 Introduction:

Alzheimer's disease is notoriously difficult to diagnose, with diagnosis currently relying on behavioural testing, brain structural analysis and requires verification in post-mortem analysis for a definitive diagnosis (Anderson, 2015). While advances are being made in attempts to locate biomarkers to aid in early positive diagnosis of AD, none have made it to become validated tests within the disease (Galasko and Golde, 2013). *In vivo* NMR spectroscopy is a complimentary technique to magnetic resonance imaging (MRI) and allows the measurement of certain metabolites levels non-invasively in subjects. NMR spectroscopy is a research technique aimed at determining the chemical properties of certain nuclei, with ^{1}H NMR spectroscopy the most common *in vivo* due to its high natural abundance. When conducted on tissue samples, whether *in vivo* or otherwise, NMR spectroscopy can be utilised to quantify the relative concentration of particular metabolites. The non-invasive aspect of *in vivo* NMR makes the technique particularly attractive in difficult to diagnose neurological disorders such as AD (Klunk et al., 1992). This section is aimed at validating an *ex vivo* NMR technique which could be utilised to understand neuroinflammatory alterations in hTau mice following LPS administration while being devoid of confounding effects of anaesthesia.

The basis of NMR:

NMR exploits the magnetic properties of certain nuclei to discern information about their surroundings. A detailed description of the basic principles behind NMR can be found in (Levitt, 2001). Nuclei which have a magnetic moment such as ^{1}H, ^{2}H, ^{13}C, ^{14}N and ^{31}P act as magnetic dipoles and can be exploited in NMR spectroscopy. Nuclei with no net magnetic moment such as ^{16}O and ^{12}C cannot undergo NMR analysis. We focus on ^{1}H NMR due to the abundance of protons in organic molecules and the dominant role of ^{1}H NMR *in vivo*. When

placed in an external magnetic field, as a result of their magnetic moment, nuclei precess in a plane perpendicular to the field (B_0). In the presence of B_0, the magnetic moments from ^1H nuclei can align with the field in a lower energy α-state and are said to have +1/2 spin. Conversely, magnetic moments from ^1H nuclei can align against the field in the higher energy β-state and are said to have a -1/2 spin and the difference between both states is known as the Zeeman Effect. The frequency at which a particular ^1H atom spins is known as the Larmor frequency which is a function of the gyromagnetic ratio of a particular atom and the strength of the external magnetic field. At room temperature, slightly more nuclei align in the α-state aligning the net magnetization (M) in direction with B_0. The transient application of a second external magnetic field that is perpendicular to B_0 in the form of a radiofrequency (RF) pulse at the Larmor frequency (B_1) causes M to rotate from alignment with B_0 into the transverse plane. If the direction of B_0 is along the z axis, application of B_1 along the y axis will cause M to rotate into the x axis. The longer the duration of the RF pulse, the greater the angle between B_0 and M increases, with the angle it is rotated to known as the flip angle which is commonly 90˚. In a process known as phase coherence, rotation into the transverse plane causes alignment of magnetic moments and precession around the z axis in the xy plane. The precession in the xy plane induces an electrical current in the coil which can be measured as an oscillation as a function of time. M does not continuously precess and an exponential decay occurs with time in a process known as a free induction decay (FID) which is a function of time. Individual contributions to this signal from different nuclear species can be deciphered using a Fourier transformation.

Depending on the local-environment of a particular ^1H nucleus, changes in the local magnetic field occur, causing nuclei to resonate at different frequencies. Changes in the local magnetic field are both dependent on the molecule which the atom is incorporated in and its position within that molecule. For example, electrons produce a magnetic field which opposes B_0. In a phenomenon known as shielding, ^1H nuclei that are bound to electropositive atoms such as sodium are in the vicinity of a large number of electrons which effectively decreases the strength of B_0. The decrease in external field strength on a particular nucleus affects its Larmor frequency thereby causing a decrease in resonance frequency. Conversely, the binding of a ^1H nucleus to an electropositive nucleus such fluorine increases its susceptibility to the B_0, resulting in an increase in resonance frequency. Furthermore, in a process known as J-coupling, neighbouring ^1H nuclei can affect a given resonance if its environment is different and it is connected by a short chain of chemical

bonds. The effect of J-coupling on a given resonance is minute compared to shielding, resulting in splitting of an NMR peak into for example doublets or triplets.

As the particular resonance of any given atom is dependent on the magnetic strength of the external magnetic field, comparisons between NMR spectrometer are difficult. Instead a chemical shift in parts per million (ppm) is calculated which is the difference from a reference molecule such as tetramethylsilane (TMS) s has high electron shielding (Tiers, 1958) and is normalised to the NMR spectrometer frequency. Therefore the further downfield a nucleus resonates at, the less shielding it has. A summary of the basic principles behind NMR is depicted in Figure 3.10. In relation to *in vivo* NMR, peaks at certain chemical shifts can be attributed to specific metabolites, with amplitude representing their relative abundance.

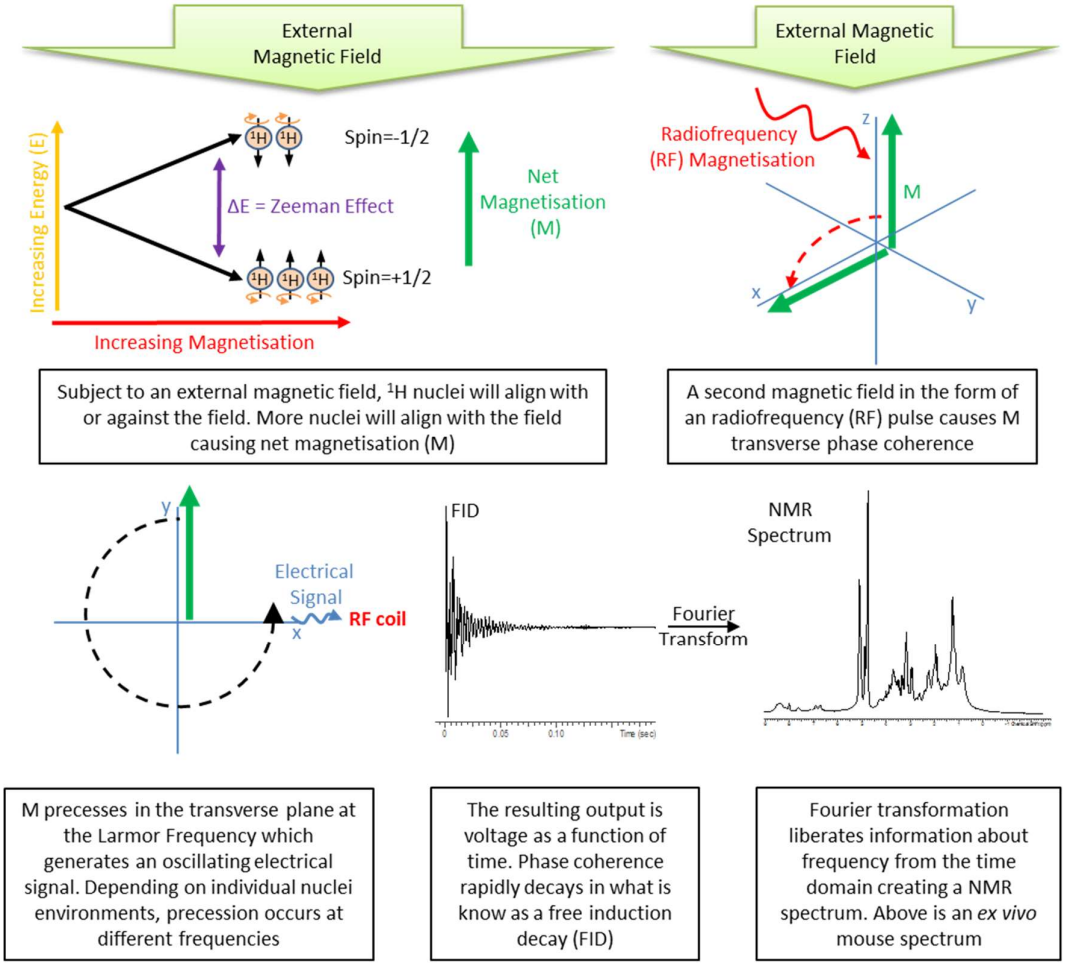

Figure 3.10: The basic principles behind NMR. Figure drawn by author.

Ex-vivo MAS-NMR is a high resolution equivalent of in-vivo NMR:

Two main variants of NMR spectroscopy existed for understanding metabolic changes in tissue: *in vivo* and *in vitro* NMR spectroscopy. The main advantage of *in vivo* NMR spectroscopy is its translatability as the technique can be conducted non-invasively in humans. The major drawback of *in vivo* NMR spectroscopy is poor resolution, resulting in broad peaks due to anisotropic dipolar interactions (Laws et al., 2002, Petroff et al., 1995). Furthermore, *in vivo* NMR in animals requires anaesthesia which can impact certain metabolite concentrations (Zhang et al., 2009b, Pardon et al., 2016). By comparison, *in vitro* NMR does not suffer from low resolution because the sample is extracted into a solution. The rapid random tumbling motion of molecules in liquids means anisotropic dipolar interactions are averaged across the sample (Lohman and MacLean, 1978), leaving isotropic interactions of the chemical shift and J-coupling which provide characteristic information about a specific metabolite resonance (Petroff et al., 1995). Furthermore, anaesthesia is not required for *in vitro* NMR aiding in interpretation of NMR spectra. The major drawback with *in vitro* solution NMR is that it requires the tissue to be extracted, increasing the resolution but decreasing translatability. An additional minor issue is post-mortem degradation of metabolites can occur rapidly.

In order to remove the anisotropic interactions which hinder the resolution of NMR spectra in solids, a technique known as magic angle spinning (MAS) was developed. The high speed mechanical spinning of samples at the so called "magic angle" of 54.74˚ in respect to the external magnetic averages the anisotropic dipole interactions (Andrew, 1981). This allows the acquisition of high resolution spectra in solids with dominant anisotropic nuclear interactions. By adapting this technique to tissue samples, high resolution *ex vivo* MAS NMR spectroscopy can be carried out with the advantages of both *in vivo* and *in vitro* NMR. Firstly, high resolution NMR spectra are achieved, resembling those from *in vitro* NMR (Wilson et al., 2009). Importantly, no tissue extraction is required, meaning similar metabolites, are observed compared to *in vivo* NMR. This feature makes *ex vivo* MAS NMR more translatable compared to its *in vitro* counterpart. Furthermore, the lack of anaesthesia provides an additional advantage over *in vivo* NMR, although the technique is still susceptible to post-mortem degradation of metabolites.

Multiple metabolites assessable by NMR are associated with inflammation:

Monitoring neuroinflammation has been highlighted as a potential use for NMR as metabolites assessable by *in vivo* NMR are associated with the neuroinflammatory response.

Myo-inositol (m-Ins) is the metabolite which has been closest related to inflammation due to its almost exclusive expression in microglial cells and, as such, is seen as a glial marker and a measure of gliosis (Bitsch et al., 1999). Numerous inflammatory conditions including: multiple sclerosis (Srinivasan et al., 2005), human immunodeficiency virus (HIV) infection (Chang et al., 2004) and AD (Huang et al., 2001) have found elevated levels of m-Ins. Both choline (Cho) and creatine (Cre) have also been associated with inflammation due to their higher concentration in glial cells (Brand et al., 1993) and their association with similar inflammatory conditions as m-Ins (Chang et al., 2004, Srinivasan et al., 2005). However, Cre is primarily a marker of cellular energy homeostasis (Andres et al., 2008) and Cho predominately as a marker of cell membrane turnover (Miller et al., 1996) and as such are not normally considered as markers of inflammation. Finally, the 0.9ppm macromolecule resonance is primarily attributed to a thymosin-β4 (Tβ4) in rodent brains (Kauppinen et al., 1992). While Tβ4 has several physiological functions including actin sequestering (Yarmola et al., 2001, Sosne et al., 2007), it posseses potent anti-inflammatory properties (Sosne et al., 2007). The 0.9ppm resonance was found to be upregulated at 4h following a 100 µg/kg LPS administration in Wt but not APPswe/PS1dE9 mice (Pardon et al., 2016) putting it forward as a potential marker of neuroinflammation. This section was aimed at validating *ex vivo* MAS-NMR in cortical APPswe/PS1dE9 samples which could be utilised to understand metabolic changes following LPS administration. A list of the metabolites of interest and their common functions is listed in Table 3.1.

Table 3.1: NMR metabolites of interest

METABOLITE	CHEMICAL SHIFT	BIOLOGICAL FUNCTION
ML9	0.9 ppm	ML9 increases in response to inflammation (Pardon et al., 2016) and is thought to mainly attributed to thymosin-beta 4 (TB4) which possesses anti-inflammatory properties (Kauppinen et al., 1992)
N-ACETYLASPARTATE (NAA)	2.02 ppm	NAA is considered a marker of neuronal viability and injury due to its high predominant expression in neurons (Schuff et al., 2006, Chakraborty et al., 2001)
GLUTAMATE/ GLUTAMINE (GLU/GLN)	2.4 ppm	Glutamate is an important excitatory neurotransmitter. Glutamine is the precursor to glutamate and GABA – the major inhibitory neurotransmitter (Bitsch et al., 1999)
CREATINE (CRE)	3 and 3.9 ppm	Relates to creatine and phosphocreatine. The majority of signal is attributed to the later and is involved in cellular energy homeostasis (Andres et al., 2008)
CHOLINE (CHO)	3.2 ppm	Attributed to multiple choline containing metabolites. Rate limiting component to acetylcholine and a major component of plasma membrane. The latter has highlighted the Cho peak as a marker of cellular membrane turnover (Miller et al., 1996)
TAURINE	3.4 ppm	Numerous functions including as an osmolyte and neuroprotector/modulator (Wu and Prentice, 2010)
GLYCINE (GLY)	3.52 ppm	Inhibitory neurotransmitter (Lopez-Corcuera et al., 2001)
MYOINOSITOL (M-INS)	3.56 ppm	Considered a marker of gliosis due to expression in CNS. Acts as an osmolyte and is involved in PKC signalling (Bitsch et al., 1999)

3.2.2 Results:

To assess metabolites related to inflammation, this chapter aimed to validate a translatable *ex vivo* NMR technique which would be equivalent to *in vivo* MR spectroscopy. ^{1}H MAS-NMR was the ideal method as the technique can analyse whole tissue samples as opposed to extraction required by *in vitro* NMR. A sample spectrum from control mice is given in Figure 3.11. Comparing to MR spectrum from APPswe/PS1dE9 mice in the literature,

there is a high degree of similarity (Pardon et al., 2016). An advantage of ^{1}H MAS-NMR is that the technique is high resolution, providing similar resolution to *in vitro* NMR. Although the resolution was increased compared to *in vivo* MR spectroscopy, demonstrated by sharper peaks, the resolution achieved was much poorer than that which has been reported for the technique (Wilson et al., 2009). Finally, elevated lactate resonances indicate a post-mortem effect is occurring.

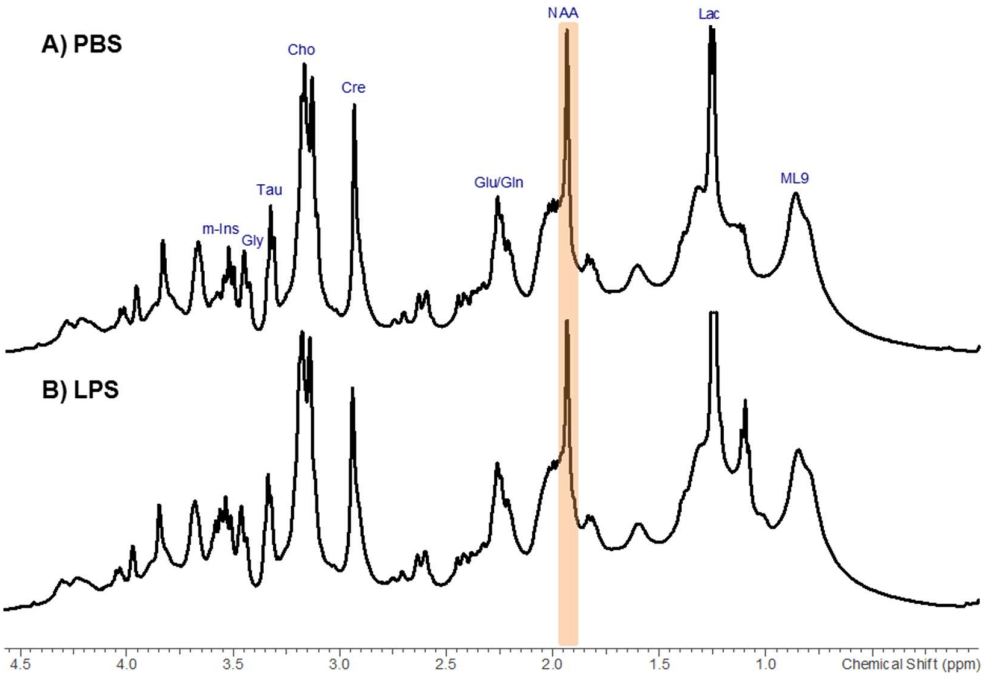

*Figure 3.11: Grouped spectra from cortical samples from **A)** PBS and **B)** LPS treated mice: Spectra were comparable to in vivo NMR but lacked the high resolution achievable by ^{1}H MAS NMR. The 8 metabolite peaks quantified are labelled above each peak. Highlighted is the NAA peak which saw an overall increase following LPS administration. Resonances assigned as denoted in Table 3.1.*

3.2.2.1 Lipopolysaccharide decreases NAA levels:

To quantify relative metabolite concentrations, integrals for each corresponding metabolite was taken and normalised to the sum of selected metabolites and is represented in Figure 3.12. Two-way ANOVA analysis revealed significant overall increases were observed in NAA levels following LPS administration (Figure 3.12B, [$F_{(1,23)}$ = 5.94, p = 0.023]). As well as increases in NAA, there was an overall trend for decrease Glu/Gln levels in APPswe/PS1dE9 mice compared to Wt littermates (Figure 3.12C, [$F_{(1,23)}$ = 3.26, p = 0.085]). There was no significant alterations in either of the inflammatory associated metabolite peaks: ML9 or m-Ins (Figures 3.12A and 3.12H, p > 0.05). Likewise, there were no significant changes in the levels of Cre, Cho, Taurine and Gly (Figure 3.12D-G, p > 0.05).

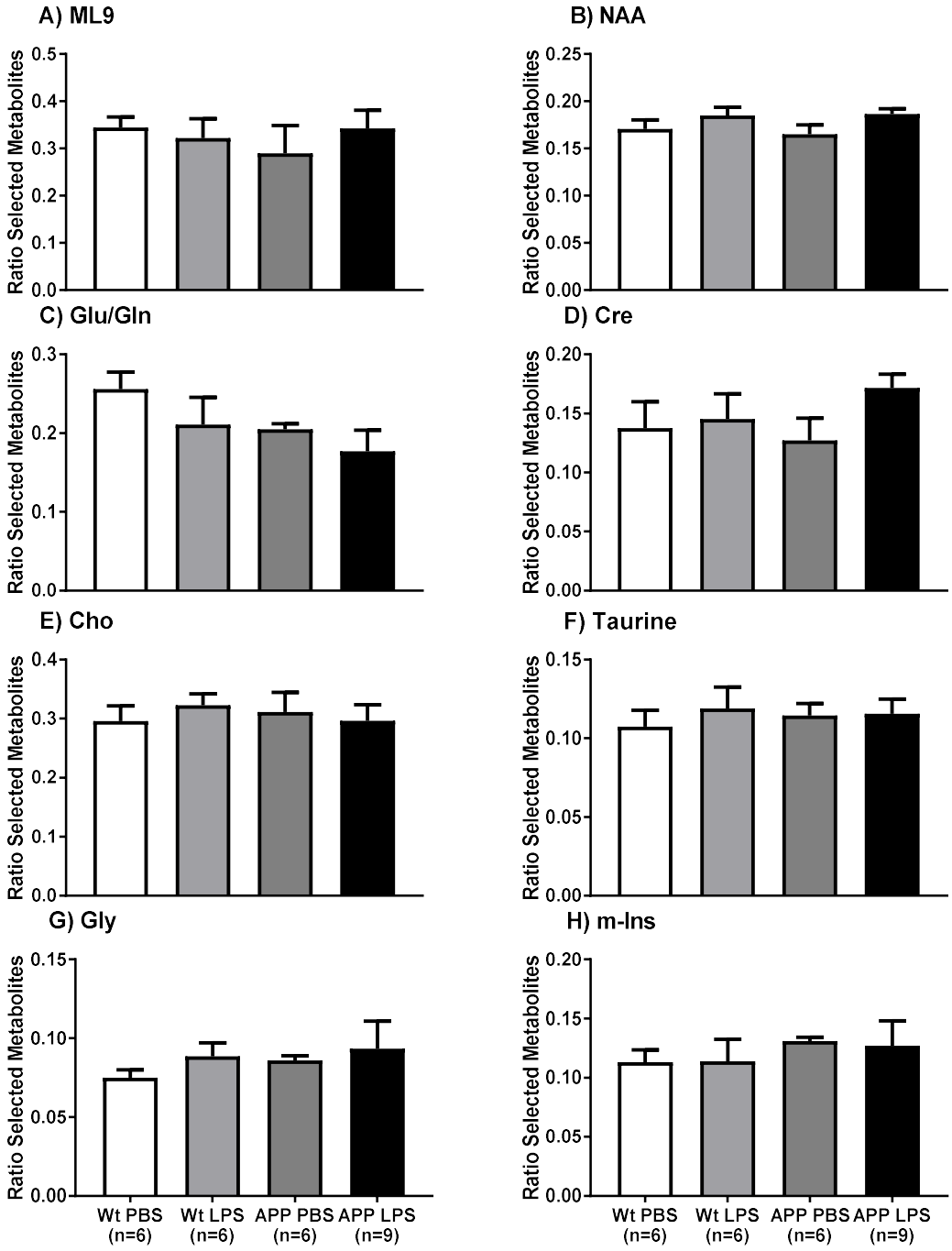

Figure 3.12: Quantification of relative NMR metabolite levels in cortical samples from 6 month female APPswe/PS1dE9 mice (mean ± SEM): ***A)*** *The ML9 peak was unaltered.* ***B)*** *LPS significantly increased NAA levels.* ***C)*** *A trend for decreased Glu/Gln existed in APPswe/PS1dE9 mice.* ***D-H)*** *Cre, Cho, Taurine, Gly and m-Ins levels did not differ.*

3.2.3 Discussion

The final section of this chapter was aimed at validating an *ex vivo* equivalent of *in vivo* NMR spectroscopy which could subsequently be utilised to assess underlying metabolic

levels in hTau mice in a translatable manner. ^{1}H MAS NMR was chosen as the technique would not require tissue extraction, meaning spectra between the two techniques are comparable. The added advantage of ^{1}H MAS NMR is that improved resolution is achievable compared to traditional *in vivo* NMR (Wilson et al., 2009). The technique was optimised using tissue from APPswe/PS1dE9 mice and subsequent validation in the hTau mice 4h following LPS administration was intended. However, a few issues arose during the optimising of the technique. The high resolution spectra which are achievable in with MAS were not achieved due to technical limitations associated with the NMR spectrometer which was used. Instead, the resolution was more akin to that which is observed for *in vivo* NMR spectroscopy. Because of this, accurate quantification of metabolite signals requires modelling, with a process known as LC modelling as the canonical standard (Provencher, 2001). Due to the unforeseen loss of a key collaborator, both the knowledge of how to run LC modelling and access to the software were lost. As an alternative to LC modelling, the current study assigned integrals to determine metabolite levels; a limitation of the study. The combination of reduced resolution of spectra and an accurate method for their analyses meant the decision was taken to not progress the technique to the hTau study.

In the current study 8 key metabolites were quantified under the caveats of these limitations. Only one metabolite was found significantly altered; NAA was found to increase following LPS administration. A similar effect has been reported for LPS when measured with *in vivo* NMR (Moshkin et al., 2014). NAA is generally accepted as a marker of neuronal density and viability (Schuff et al., 2006), making an increase following LPS administration unexpected. However, the association of NAA as a neuronal viability marker is solely due to the observation that it is exclusively expressed in neurons (Moffett et al., 1991). From a functional point of view, *in vivo* NAA synthesis and therefore strength of the NAA NMR signal is dependent on mitochondrial metabolism and ATP production (Heales et al., 1995). As such, changes in the metabolite resonance in NMR could just as likely be due to neuronal metabolism (Bates et al., 1996). LPS is known to induce mitochondrial metabolism (Stetler et al., 2012) and therefore the alterations in NAA in the current study may indicate altered NAA synthesis rather than neuronal viability.

One of the rationale for conducting MAS-NMR spectroscopy was to observe whether there were alterations in the two inflammatory associated metabolites: ML9 and m-Ins – both of which remained unaltered. Due to the neuroinflammatory response following non-septic doses of LPS subsiding within 24h following LPS administration in mice (Biesmans et

al., 2013), lack of changes in the ML9 and m-Ins peaks 7 days following LPS administration is expected. Finally, a trend for decreased glutamate/glutamine levels was observed in APPswe/PS1dE9 mice which is synonymous with cognitive impairments in the model as well as previous reports of decreased glutamate levels in the literature (González-Domínguez et al., 2014). Taken together, these findings indicate *ex vivo* ^{1}H MAS NMR as a viable technique for understanding metabolic alterations in a translatable manner. Despite the shortcomings in the current study, the technique has the potential to be of valuable use in animal models due to its high resolution while avoiding potential anaesthesia effects.

Chapter 4 Validation of the hTau model partially expressing murine tau

4.1 Introduction

This chapter was aimed at validating tau pathology in hTau mice partially expressing mTau. As previously discussed, the hTau model has potential drawbacks. Firstly, there is an isoform ratio of 3R>4R which is not representative of AD (Andorfer et al., 2003). 3R tau isoforms are lacking in the R2 repeat region and the inter-repeat R1-R2 region of tau, the latter has been shown to have double the microtubule binding affinity compared to any other individual repeat region (Goode et al., 2000). Consequently, 3R tau isoforms are considered to have a lesser affinity for microtubules than 4R isoforms, increasing the amount of unbound tau which can undergo aggregation (Goode and Feinstein, 1994, Goode et al., 2000). The lower affinity for microtubules additionally alters microtubule dynamics. For example, 3R tau isoforms have a lower propensity to promote microtubule assembly at a concentration of 0.09µM but were similar at a higher concentration of 0.73µM compared to 4R isoforms (Panda et al., 2003). As physiological concentrations of intracellular tau are generally considered to be between 1-10µM (Chirita and Kuret, 2004), it is likely the altered isoform ratio does not alter microtubule formation. At the more physiologically relevant 0.73µM concentration, 3R tau isoforms were found to stabilise microtubules to a lesser extent than their 4R counterparts (Panda et al., 2003). Therefore a 3R favouring isoform ratio not only affects its affinity for microtubules, but also affects its ability to stabilise microtubules and has been implicated in generation of other tauopathies such as Pick's disease or Down syndrome (Bronner et al., 2005, Shi et al., 2008). Suggestive of the isoform imbalance driving the pathology in hTau mice, only 3R tau isoforms aggregate in the model (Andorfer et al., 2003) compared to both 3R and 4R in AD (Espinoza et al., 2008).

In addition to an isoform imbalance, in house observations have suggested hTau mice to be associated with the development of systemic pathologies. The source of systemic pathologies is likely linked to the KO of the mTau gene rather than introduction of the hTau gene, as $mTau^{-/-}$ mice were similarly affected by systemic pathologies. Although the cause of the systemic pathologies is unknown, KO of tau has been associated with a dysregulation of a number of proteins including microtubule associated protein 1A (MAP1A), histone deacetylase 6 (HDAC6) and Gem GTPase (Ke et al., 2012). HDAC6 is a histone deacetylase which is inhibited by tau through its SE14 domain (Ding et al., 2008). While HDAC6 has many downstream effectors, it has been implicated in a variety of cancers (Kaliszczak et al., 2013, Aldana-Masangkay and Sakamoto, 2011, Putcha et al., 2015). Interestingly, the HDAC6-tau

interaction is dependent on the microtubule binding domain in tau (Ding et al., 2008). Therefore, KO out of murine tau or a shift in isoform ratio could cause disinhibition of HDAC6 leading to the development of systemic pathologies. Although this is one possibility, the development of systemic pathologies could equally be due to some unknown consequence from the KO of mTau. To avert the development systemic pathologies, hTau mice were bred on a heterozygous mTau background: hTau/mTau$^{+/-}$. Unlike the 8c model, we hypothesised that hTau/mTau$^{+/-}$ mice would still develop tau pathology. Furthermore, as mTau consists of solely of 4R tau isoforms (Liu and Götz, 2013), it would help to restore the isoform imbalance. Indeed, previous in-house studies have found hTau/mTau$^{+/-}$ mice do possess tau hyperphosphorylation (Rossi et al., 2018).

4.2 Study design

To validate the hTau/mTau$^{+/-}$ model, three experimental cohorts were utilised. Experimental cohort 3 was utilised to preliminarily determine the effect of heterozygous mTau expression on tau pathology in the hTau model. This was done by assessing tau phosphorylation and isoform ratio in spare cortical tissue from 6 month old male Wt(C57), Wt(mixed), mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice (n=4/group). Mtau$^{+/-}$ and hTau/mTau$^{+/-}$ mice were on a mixed 129SvJ x C57Bl/6 background while their mTau$^{-/-}$ counterparts were on a C57BL/6. Experimental cohort 4 compromised of 20 experimentally naïve 9 month old male Wt, mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice (n=4/genotype). The cohort was utilised to validate tau phosphorylation and aggregation at a later age. Experimental cohort 5 consisted of 177 experimentally naïve 3 month male Wt, mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice. The main objective for this cohort was to assess the effect of a single PBS or 100, 250 and 330µg/kg LPS injection on early tau pathology in Chapter 5 (n=8-9/group). However, PBS treated animals were compared behaviourally and pathologically in this chapter to further validate the hTau/mTau$^{+/-}$ model. This was done through assessing tau phosphorylation, isoform ratio and aggregation. In addition to assessing tau species, behaviour was assessed in the food burrowing and spontaneous alternation tasks to determine whether hTau/mTau$^{+/-}$ mice develop early hippocampal-mediated behavioural alterations (Deacon, 2012, Lalonde, 2002).

4.2.1 Behavioural protocol in experimental cohort 5

The behavioural protocol lasted over three consecutive days and is depicted in Figure 4.1. On the first day the animals were given a practise food burrowing test in their group cage overnight. The following day, the animals were single housed and given the food

burrowing test overnight. The third and final day, the mice underwent LPS treatment and were assessed in the spontaneous alternation task (SA) as a measure spatial working memory and the LPS-induced behavioural suppression. Immediately following, the mice were sacrificed. Bodyweights were recorded on the treatment day and was analysed for statistical significance using a one-way ANOVA approach followed by Tukeys post hoc where appropriate. To further validate the hTau/mTau$^{+/-}$ model, PBS treated animals were compared for tau species and in the spontaneous alternation task. As the food burrowing test and bodyweight measurement occurred prior to LPS treatment all animals in experimental cohort 5 were compared in these measures in the current chapter.

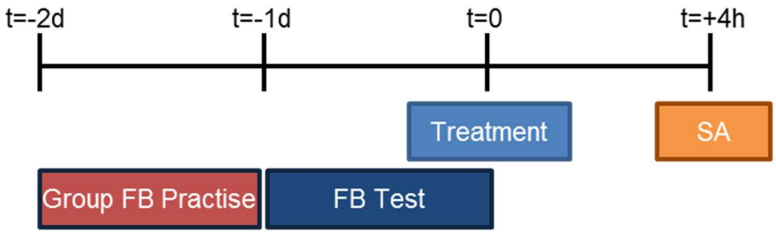

Figure 4.1 The behavioural protocol lasted over 3 days. The mice were given a food burrowing practice while grouped housed on the first day. On the second day, the mice were given a food burrowing test overnight while single housed. The following morning, the mice were treated and underwent a spontaneous alternation test 4h later immediately prior to being culled.

4.2 Results

4.2.1 Knockout of mTau results in systemic pathologies in aged mice

In a separate study, initial attempts at administrating LPS in female mTau$^{-/-}$ and hTau/mTau$^{-/-}$ mice of roughly 18 months of age resulted in a profoundly exaggerated sickness response. The exaggerated sickness response meant abruptly halting the study to ensure the mice did not exceed the severity level. To determine if there was any underlying reason to the exaggerated response, the author conducted necropsies in conjunction with the Named Animal Care & Welfare Officer (NACWO): Sally Wilford. The findings of the necropsies are reported in Table 4.1. The mice were bred under the traditional hTau breeding regime (mTau$^{-/-}$ x hTau/ mTau$^{-/-}$) and therefore the study didn't have any Wt mice to compare to. Although low powered, 75% of mTau$^{-/-}$ mice were affected by a visual systemic abnormality. This was similar to a 70% incidence in hTau/mTau$^{-/-}$ mice. Although limited, this data suggests that knockout of mTau is associated with increased systemic pathologies.

Table 4.1 Number of mice affected by visual systemic pathologies in ~ 18 month old female hTau mice.

Genotype	Systemic Tumour	Spleen abnormality	Systemic cyst	No Systemic abnormalities present
mTau$^{-/-}$ (n=4)	2	1	1	1
hTau/mTau$^{-/-}$ (n=10)	3	1	4	3

Note: *Some animals had more than one incidence of systemic pathologies*

4.2.2 hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice are behaviourally similar

Based on the preliminary tau phenotypes reported in Appendix 1, the decision was taken to assess the effect of LPS-induced systemic inflammation on tau pathology in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice. An added benefit of breeding hTau/mTau$^{+/-}$ mice is the generation in Wt littermates, aiding in accurate behavioural assessment of hTau mice. To further authenticate observations from the preliminary tau phenotyping and further validate the model, PBS treated animals from experimental group 5 were compared. 3 month old male hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice were compared in the food burrowing and spontaneous alternation tasks to assess whether partial murine tau expression alters behavioural abnormalities in the model. As the bodyweight and food burrowing were assessed prior to treatment, all animals from experimental group 5 were compared. One-way ANOVA analysis revealed there were significant differences in bodyweights among genotypes [$F_{(4,171)} = 6.91$, $p < 0.001$]. Tukey's post hoc analysis found that both hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice weighed significantly less than Wt mice but did not differ from one another ($p < 0.05$ and $p > 0.05$ respectively, Figure 4.2A). One-way ANOVA analysis indicated there were significant differences in the amount of food burrowing during the test session among genotypes [$F_{(4,171)} = 5.88$, $p < 0.001$]. Tukey's post hoc analysis highlighted that both hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice burrowed less than their Wt littermates ($p < 0.001$ and $p < 0.05$ respectively), but did not differ from one another ($p > 0.05$, Figure 4.2B). Analysis of PBS treated animals in the spontaneous alteration task by one-way ANOVA approach showed that there were no significant differences in locomotor activity [$F_{(4,40)} = 0.46$, $p > 0.05$] or spatial working memory [$F_{(4,40)} = 1.36$, $p > 0.05$] , although mTau$^{-/-}$ mice failed to alternate above chance levels (Figure 4.2C-D).

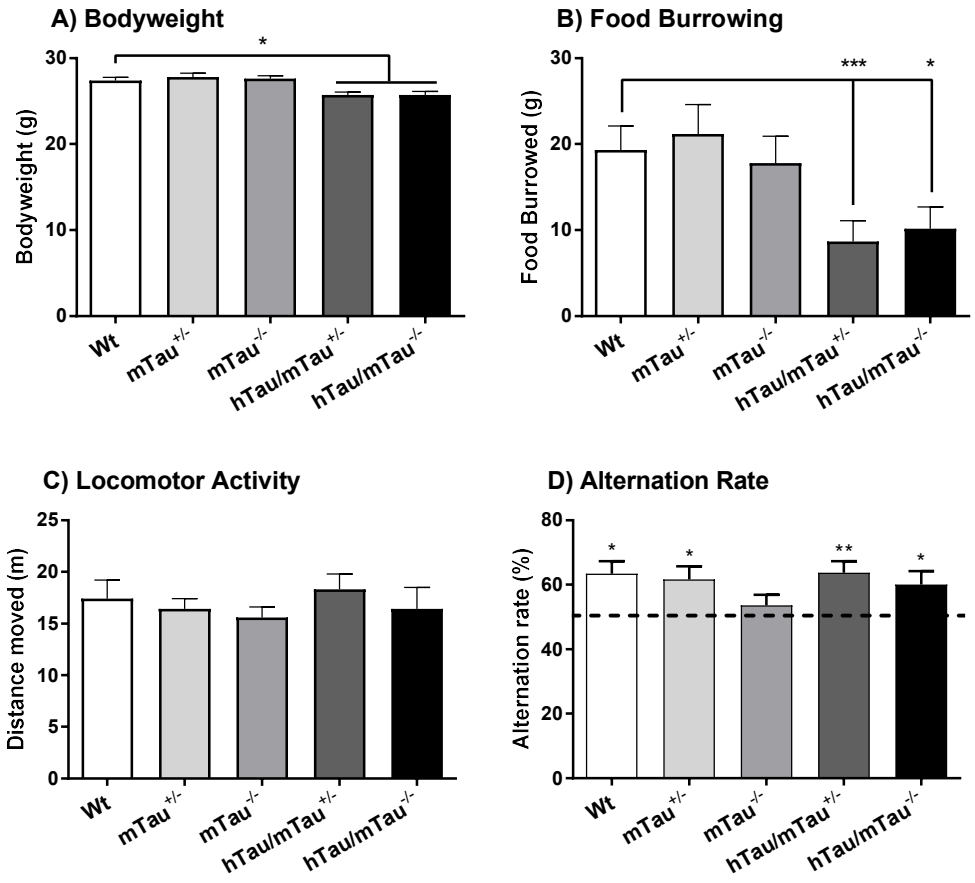

Figure 4.2: Behavioural analysis of 3 month old male hTau/mTau$^{+/-}$ mice. Bars represent mean ± SEM. ***A)*** *Both hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice weighed less than their Wt littermates (n=34-36/genotype).* ***B)*** *Impaired food burrowing behaviour in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice (n=34-36/genotype).* ***C)*** *Similar locomotor activity among genotypes (n=9/genotype).* ***D)*** *Alternation rate in the spontaneous alternation task. Significance represents against chance level (dashed line), with no significant alterations between genotypes (n=9/genotype). One-sample t-test: * p < 0.05, ** p < 0.01 and *** p <0.001 against Wt mice.*

4.2.3 Increased levels of hippocampal tau phosphorylation at 3 months in hTau/mTau$^{+/-}$ mice

To assess tau pathology and isoform ratio in hTau/mTau$^{+/-}$ mice, hippocampal tau species were assessed by western blotting in 3 month old mice. One-way ANOVA analysis showed that there were significant alterations in total tau levels among genotypes [$F_{(3,32)}$ = 9.52, p < 0.001]. While, hTau/mTau$^{+/-}$ mice had slightly elevated levels of total tau compared to hTau/mTau$^{-/-}$ mice, Tukey's post hoc analysis indicated this to not be significant (p > 0.05, Figure 4.3B) despite the heterozygous expression of mTau in the latter. This is in contrast to the preliminary analyses reported in Appendix 1 which reported increased total tau levels. While the latter is what would have been expected, the hTau genotypes in the preliminary

analysis were on different backgrounds which could affect tau expression. Another possibility is that the disparity in total tau levels occurs during the ageing process or is simply resulting from the different brain regions assessed. Both phosphorylated tau epitopes: pS202 [$F_{(3,32)}$ = 8.69, p < 0.001] and pS396/404 [$F_{(3,32)}$ = 10.96, p < 0.001] were significantly altered among genotypes as revealed by one-way ANOVA analysis. Tukeys post hoc analysis found both epitopes to be significantly higher in hTau/mTau$^{+/-}$ mice compared to hTau/mTau$^{-/-}$ (p < 0.01 and p < 0.05 receptively, Figure 4.3C-D). Synonymous with higher levels of phosphorylated tau, hTau/mTau$^{+/-}$ mice had higher molecular weight tau bands (Figure 4.3A). Increased tau phosphorylation while total tau levels remained similar suggests that tau phosphorylation rather than increased tau expression was the source of tau phosphorylation.

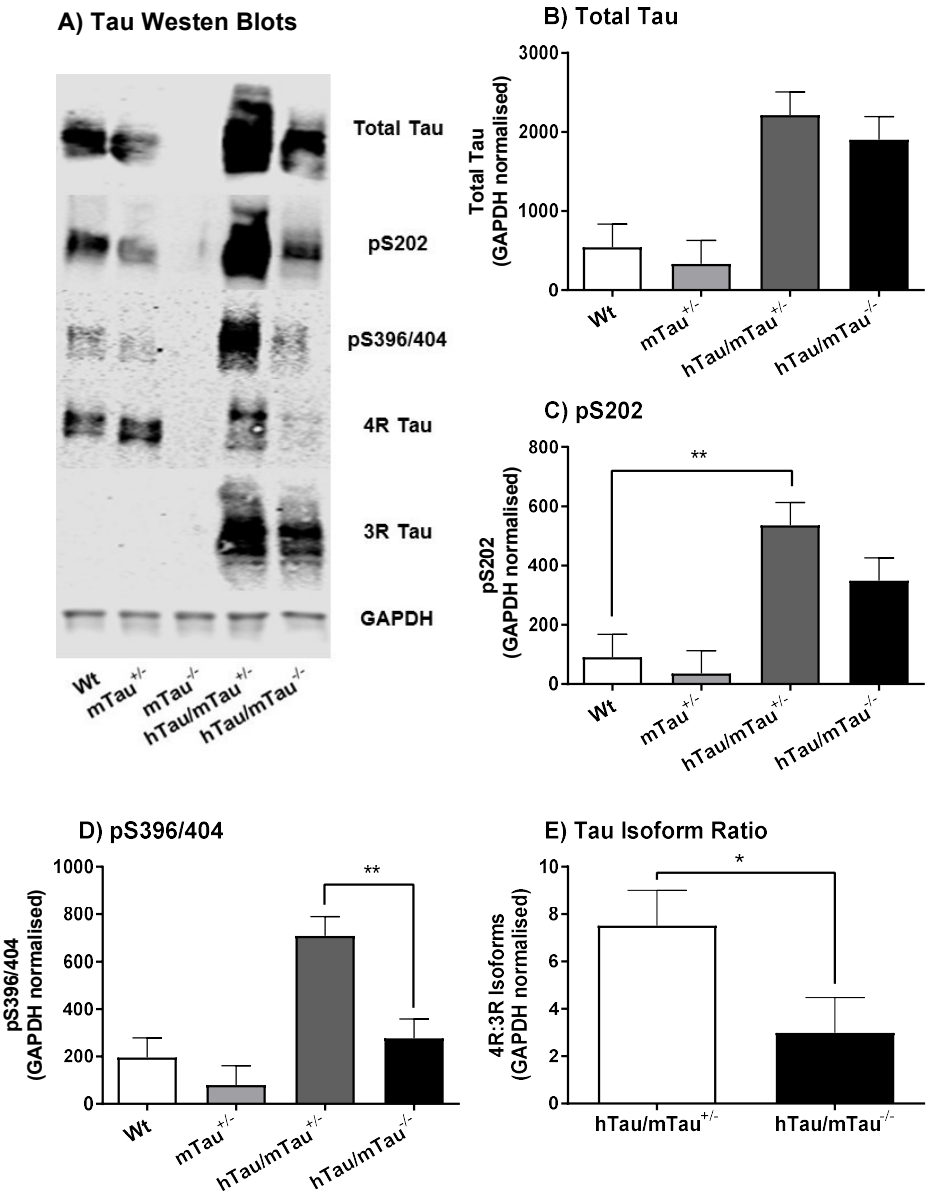

Figure 4.3: Baseline tau levels in 3 month old male hTau mice. Bars represent mean ± SEM (n=9/genotype). ***A)*** *Representative western blots.* ***B)*** *Quantification of total tau levels revealed, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice had similar total tau levels.* ***C-D)*** *hTau/mTau$^{+/-}$ mice had higher levels of phosphorylated tau at pS202 and pS396/404 epitopes compared to hTau/mTau$^{-/-}$ mice.* ***E)*** *hTau/mTau$^{+/-}$ mice had elevated levels of 4R compared 3R tau isoforms. Significance: * $p < 0.05$ and ** $p < 0.01$.*

To verify whether alterations in phosphorylated tau levels in hTau/mTau$^{+/-}$ mice were due to alterations in total tau levels, a ratio between total and phosphorylated tau levels was calculated. One-way ANOVA analysis revealed there were significant alterations in the ratio between pS202:total tau [$F_{(3,32)}$ = 5.47, $p < 0.001$]. Tukey's post hoc analysis revealed there was a significant increase in the pS202:total tau ratio in hTau/mTau$^{+/-}$ mice

compared to hTau/mTau$^{-/-}$ mice ($p < 0.05$, Figure 4.4A). However, there were no significant differences in the ratio between PS396/404: total tau [$F_{(3,32)} = 2.36$, $p = 0.094$], although the ratio tended to be higher in hTau/mTau$^{+/-}$ mice compared to hTau/mTau$^{-/-}$ mice. Interestingly, mTau$^{+/-}$ mice have the highest ratio of pS396/404:total tau which is likely attributed to their low levels of tau (Figure 4.4B). Furthermore, while the differences between hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice in absolute pS396/404 levels appears much larger than that for CP13, the reverse is true when looking at a ratio to total tau. This could indicate that there is a different pattern of phosphorylation between samples with high and low total tau levels. Equally, it could be resulting from a potential issue of skewing data when creating a ratio between two antibodies on different blots. Nevertheless, it suggests increased tau phosphorylation in hTau/mTau$^{+/-}$ mice appears to be due to increased phosphorylation rather than simply resulting from increased tau expression. As anticipated, hTau/mTau$^{+/-}$ mice had elevated levels of 4R:3R tau isoforms compared to hTau/mTau$^{-/-}$ mice as revealed by unpaired *t*-test analysis ([$t_{(16)} = 4.63$, $p = 0.0598$], Figure 4.3E).

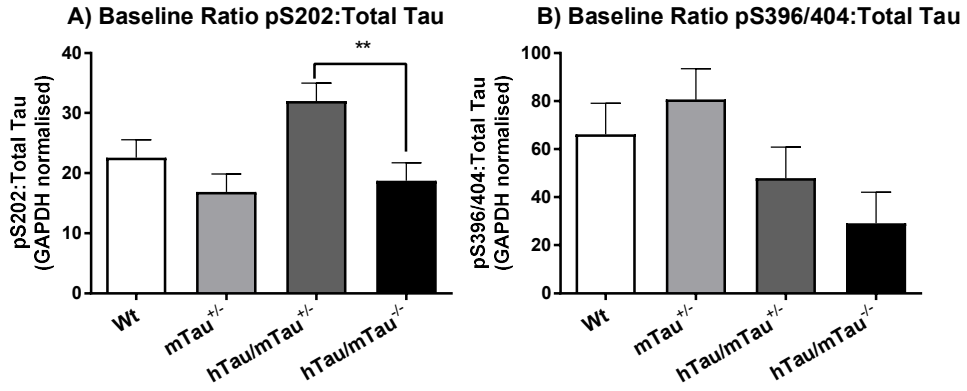

Figure 4.4 Baseline ratio of phosphorylated:total tau levels in 3 month old male hTau mice calculated from Figure 4.3. Bars represent mean ± SEM (n=9/genotype). ***A)*** *hTau/mTau$^{+/-}$ mice had an increase in the pS202:total tau ratio.* ***B)*** *There were no significant alterations in the pS396/404:total tau ratio, although Wt and mTau$^{+/-}$ mice appeared to have the highest ratio. Significance: ** $p < 0.01$.*

4.2.4 Similar PP2A activity in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice

To determine whether increased tau phosphorylation in hTau/mTau$^{+/-}$ resulted from altered PP2A function, hippocampal PP2A$_C$ activity was assessed by western blotting in 3 month old mice. By comparing samples incubated in the absence and presence of NaOH, a ratio between inactive and total PP2Ac is achieved. If the activity of PP2A increases, a decrease in this ratio is expected. One-way ANOVA analysis revealed there was no significant overall genotype effect [$F_{(4,40)} = 0.71$, $p = 0.59$], suggesting alteration in kinase activity rather

than phosphatase activity might be the source of increased tau phosphorylation in htau/mTau$^{+/-}$ (Figure 4.5B). Further validation with a dedicated PP2A assay and assessment of kinases activity is required to understand the dysregulation in tau phosphorylation in the model.

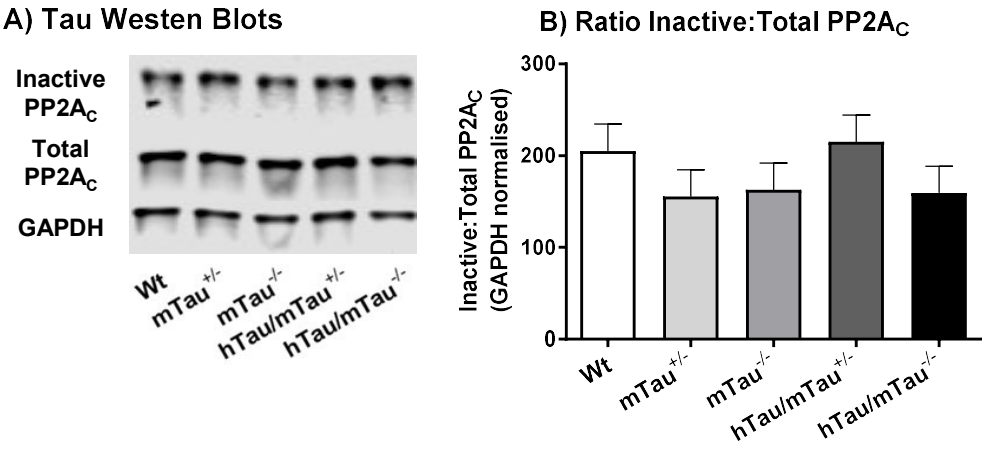

Figure 4.5: Baseline hippocampal PP2A$_C$ activity at 3 months of age revealed a trend for decreased tau phosphorylation in hTau/mTau$^{+/-}$ mice. ***A)*** *Representative blots.* ***B)*** *No significant alterations in PP2A$_C$ activity existed. Bars represent mean ± SEM (n=9/genotype).*

4.2.5 Tau redistribution in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice at 3 months of age

Following tau hyperphosphorylation and dissociation from microtubules, it relocates from the axon to the somatodendritic compartment (Gendron and Petrucelli, 2009). To examine whether hTau/mTau$^{+/-}$ mice have a somatodendritic redistribution of tau, histological examination of pS202 was conducted. A similar pattern of staining was observed to western blotting, whereby the staining intensity compared to western blotting and there was effectively no staining in mTau$^{-/-}$ mice. When tau redistributes to the somatodendritic compartment, a ring of CP13 staining occurs around the cell body (Andorfer et al., 2003). There was mild evidence of tau in the somatodendritic compartment of both Wt and mTau$^{+/-}$ mice. This was greatly increased in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice suggesting higher levels of somatodendritic tau (Figure 4.6). In addition to pS202, sections were probed for total tau. However, there was axonal staining in mTau$^{-/-}$ mice (Figure 2.6) which may be due to the fact that the Tau46 antibody has cross reactivity with MAP2 and therefore Tau46 staining was excluded.

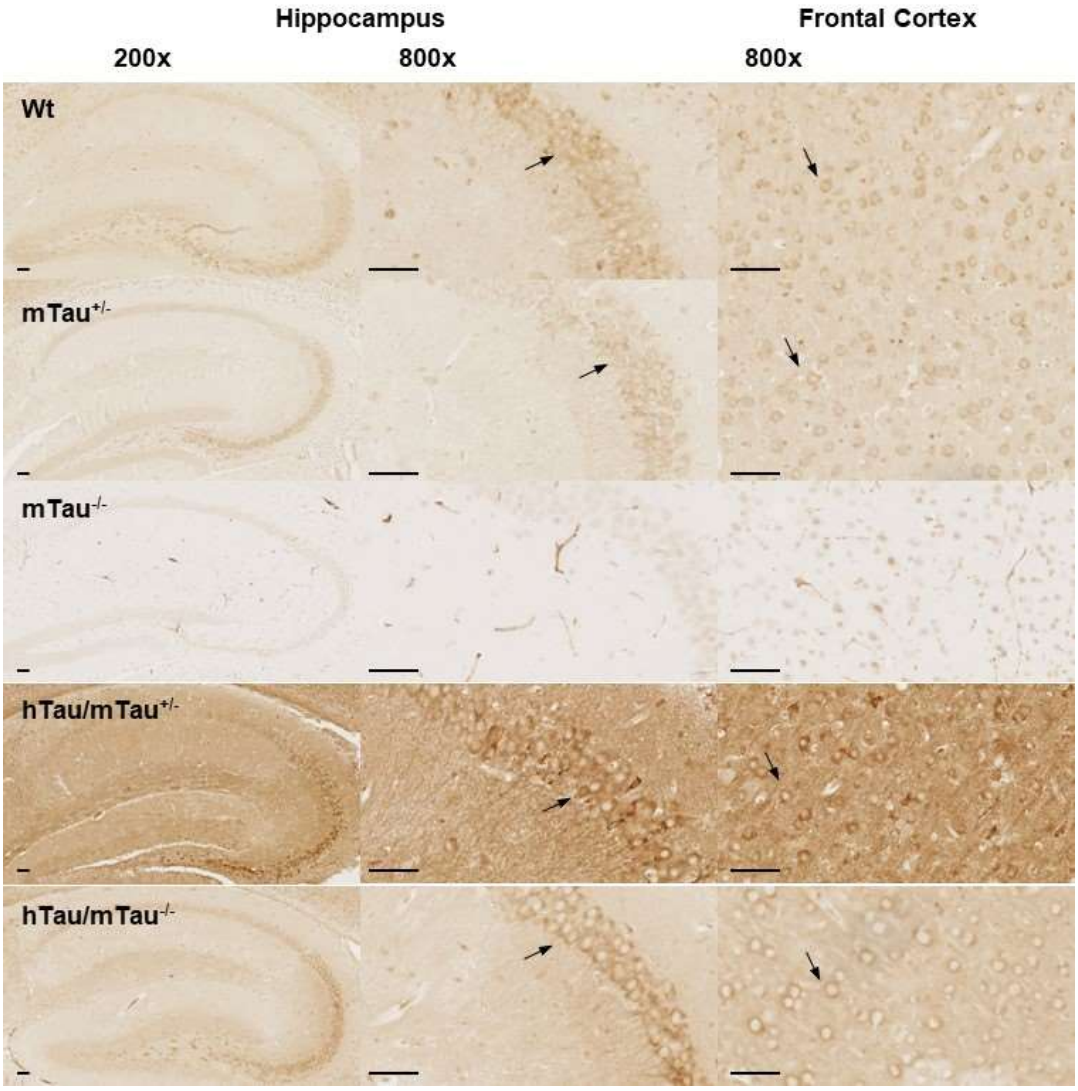

Figure 4.6: Hippocampal tau localisation in 3 month old male hTau mice: Sections stained for pS202 revealed that both hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ demonstrated a high concentration of somatodendritic tau in both hippocampal and frontal cortical regions. To a lesser degree Wt and mTau$^{+/-}$ mice had tau present in the somatodendritic compartment. The black arrow indicates cells with a somatodendritic location of tau. Scale bar represents 50 µm

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4.2.6 Neither hTau/mTau$^{+/-}$ nor hTau/mTau$^{-/-}$ mice exhibit tau aggregation at 3 months of age

To assess whether hTau/mTau$^{+/-}$ mice exhibit tau aggregation, hemi-brains were sarkosyl extracted. The sarkosyl extraction protocol utilises different strengths detergents to isolate sarkosyl insoluble tau which compromises of insoluble tau aggregates (Julien et al., 2012). During the extraction process, 3 separate fractions are generated; S1, S2 and SI. The S1 is the total fraction and is comparable with that which was achieved for hippocampal extractions. The S2 confers to proteins which are soluble while the SI is comprised of insoluble aggregated proteins. Western blotting of the S1 fraction again revealed hTau/mTau$^{+/-}$ mice to have higher levels of ps396/404 tau compared to hTau/mTau$^{-/-}$ mice

while possessing a similar level of total tau (Figure 4.7A). However, western blotting of SI fractions revealed that 3 month old hTau mice do not have aggregated tau. While there did appear to be extremely faint bands in the sarkosyl insoluble fractions, they were also observed in Wt mice and lacked the distinctive shift in molecular weight that is expected with sarkosyl insoluble tau (Figure 4.7B). Instead these extremely weak bands appear to be the result of some residual sarkosyl soluble tau. An example of sarkosyl insoluble tau in the rtg4510 model is given in Figure 4.7C. The faint bands in the SI fractions were confirmed to be soluble tau in the Chapter 5 using the HTRF tau aggregation assay.

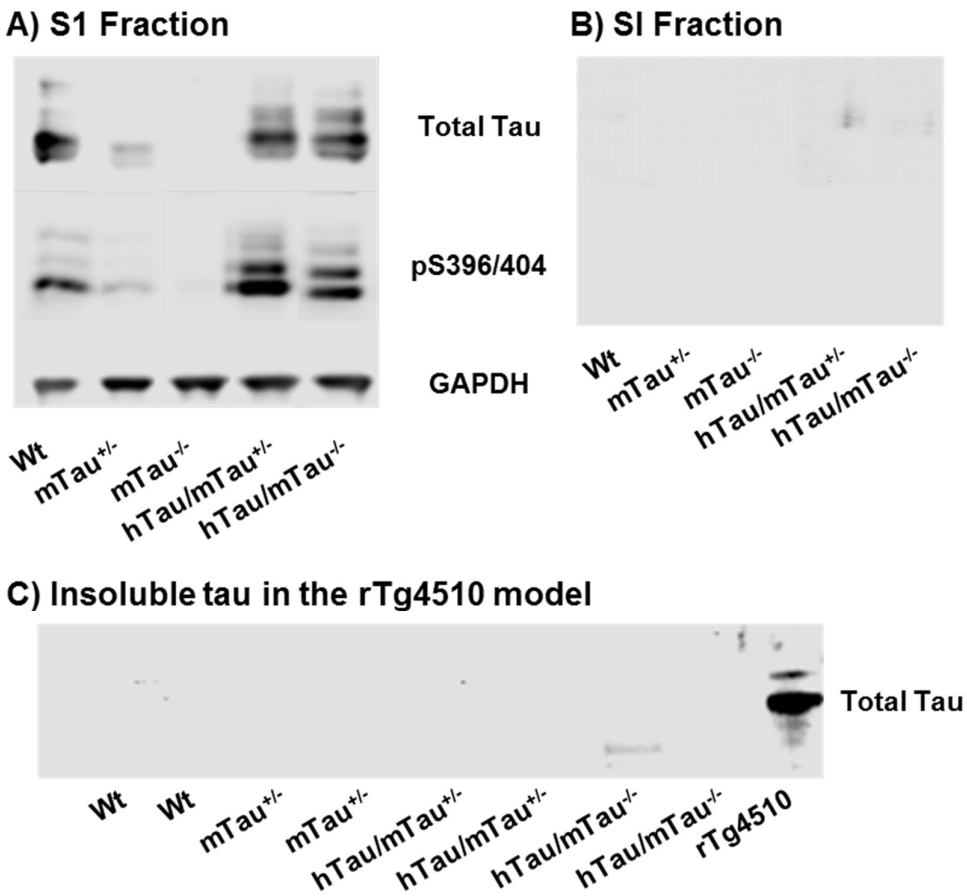

Figure 4.7 Western blots of Sarkosyl extracted 3 month male hTau mice. ***A)*** *Western blotting of the S1 fraction revealed similar findings to hippocampal fractions; that pS396/404 levels were increased and total tau levels were similar in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice.* ***B)*** *Blotting of insoluble fractions revealed there was no aggregated tau at 3 months of age. Extremely faint bands appear to result from contamination with sarkosyl soluble tau.* ***C)*** *Sarkosyl insoluble tau from an aged rTG4510 (>20months) reveals a strong band and is of a higher molecular weight than the weak bands observed in hTau mice.*

4.2.7 Tau phenotype in 9 month old hTau/mTau$^{+/-}$ mice

To determine whether tau aggregation differed in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice, a cohort (n=4/genotype) were aged to 9 months of age and brain samples underwent

sarkosyl extraction. Tau species were determined by western blotting of S1 samples (Figure 4.7A). One-way ANOVA analysis revealed there were significant differences in the levels of total tau between genotypes [$F_{(3,12)}$ = 15.19, p < 0.001]. Tukey's post hoc analysis indicated there were significant differences between Wt and hTau/mTau$^{+/-}$ mice. However, although there was a trend for increased total tau levels in hTau/mTau$^{+/-}$ mice compared to hTau/mTau$^{-/-}$ mice, the effect was non-significant and could be due to the low power (p > 0.05, Figure 4.8B). Nevertheless, there was a trend for increased total tau levels in hTau/mTau$^{+/-}$ mice compared to hTau/mTau$^{-/-}$ mice. One-way ANOVA analysis showed that there was a significant genotype effect for both pS202 and pS396/404; [$F_{(3,12)}$ = 6.86, p < 0.01] and [$F_{(3,12)}$ = 11.28, p < 0.01] respectively. Tukey's post hoc analysis revealed in both cases that Wt and hTau/mTau$^{+/-}$ groups were significantly different but hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice were similar (Figure 4.8C-D). Nevertheless, there was a trend for increased pS202 and pS396/404 in hTau/mTau$^{+/-}$ mice although the later appears to be simply resulting from increases in total tau levels. Supporting this notion, the ratio of pS202:Total tau was elevated in hTau/mTau$^{+/-}$ mice while the ratio of pS396/404:total tau was similar to hTau/mtau$^{+/-}$ mice (4.9A-B). However, due to the low power neither ratio was significant (p > 0.05).

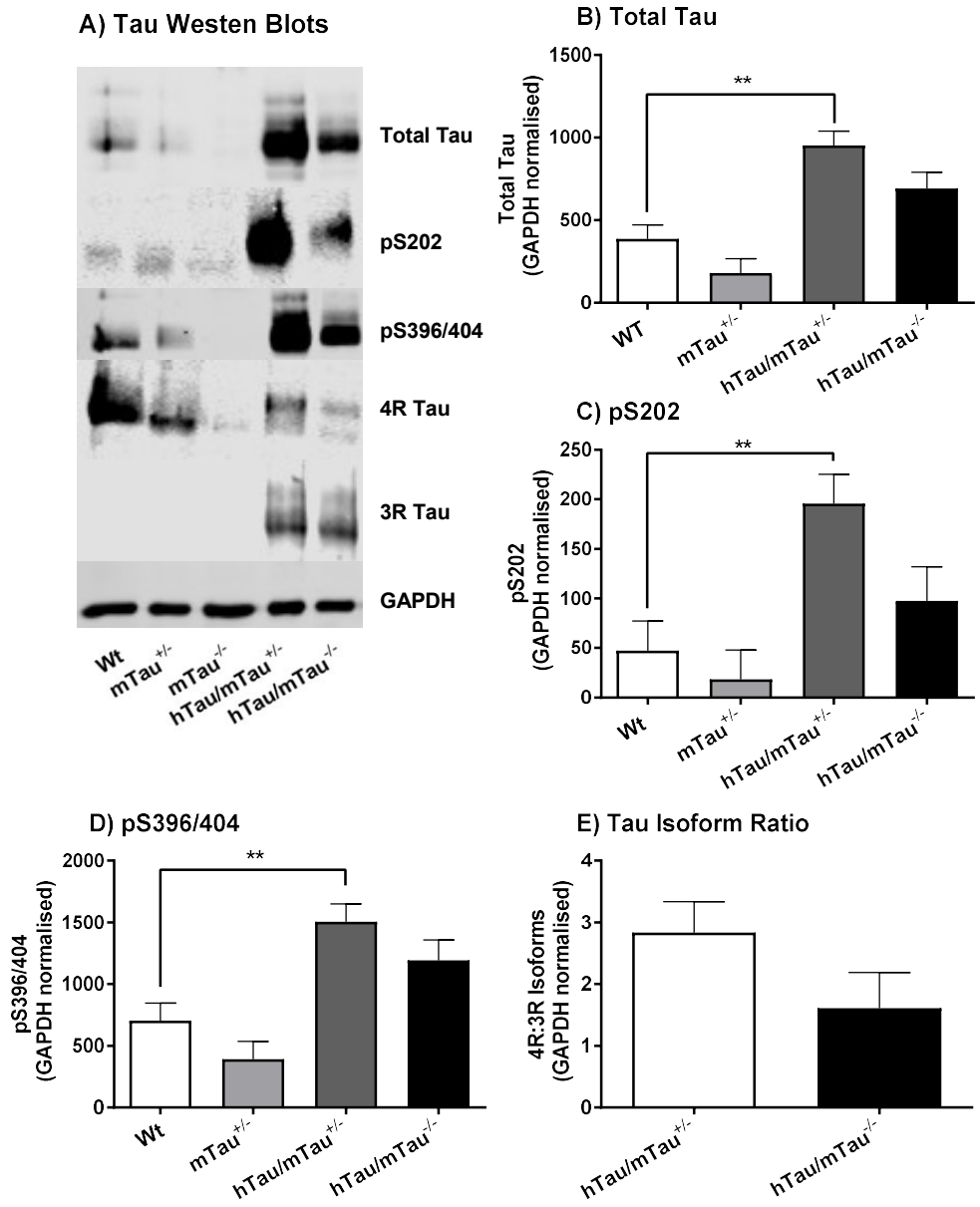

Figure 4.8: Tau levels in S1 fractions from 9 month old male hTau mice. Bars represent mean ± SEM (n=3-4/genotype). ***A)*** *Representative western blots.* ***B)*** *Quantification of total tau levels. Although not significant, hTau/mTau$^{+/-}$ mice tended to have higher levels of tau than hTau/mTau$^{-/-}$ mice.* ***C-D)*** *hTau/mTau$^{+/-}$ mice had higher levels of phosphorylated tau at pS202 and pS396/404 epitopes compared to hTau/mTau$^{-/-}$ mice.* ***E)*** *Although not significant, hTau/mTau$^{+/-}$ mice tended to have elevated levels of 4R compared to 3R tau isoforms. Significance: ** p < 0.01.*

Finally, although there was a trend for an increase in the 4R:3R isoform ratio in hTau/mTau$^{+/-}$ mice, unpaired *t*-test analysis revealed it was insignificant [$t_{(6)}$ = 2.29, p = 0.21]; likely due to the low power (Figure 4.8E).

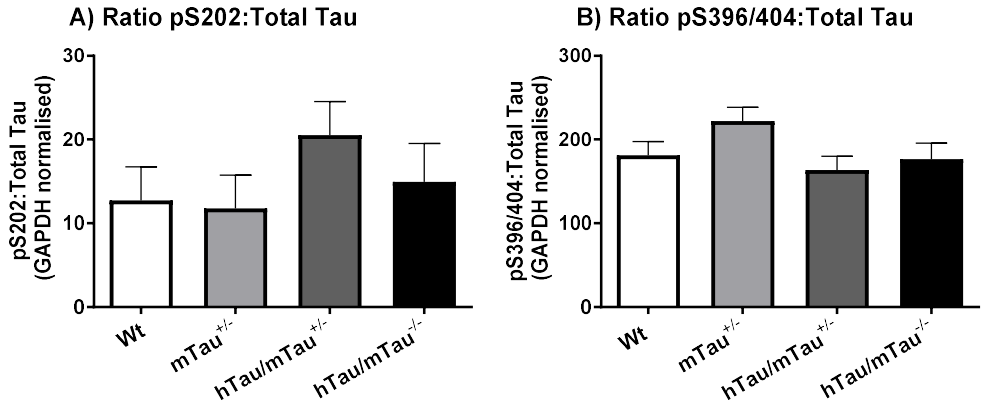

Figure 4.9: Ratio of phosphorylated:total tau levels in S1 fractions from 9 month old male hTau mice. Bars represent mean ± SEM (n=3-4/genotype). ***A)*** *hTau/mTau$^{+/-}$ had an elevated ratio of pS202:total tau while* ***B)*** *pS396/404 remained similar.*

4.2.8 Neither hTau/mTau$^{+/-}$ nor hTau/mTau$^{-/-}$ mice exhibit tau aggregation at 9 months of age

To assess tau aggregation in hTau/mTau$^{+/-}$ mice, SI fractions were analysed by western blotting. Despite reports in the literature showing that 9 month hTau/mTau$^{-/-}$ mice develop aggregated tau (Kelleher et al., 2007), 9-month old hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice failed to exhibit aggregated tau in the SI fraction (Figure 4.10). Again, while there were faint bands in SI fractions, these mirrored expression levels in the S1 fraction and are likely due to contamination of soluble tau. The issue of contamination is not selective to the sarkosyl extraction protocol utilised in the current thesis. An attempt at the sarkosyl protocol from the Noble group which is based off the protocol originally described from Peter Davis (Greenberg and Davies, 1990) found a similar issue (data not shown). Furthermore, increasing the number of wash cycles did not remove the contamination (data not shown). However, as the bands are extremely faint compared to samples positive for aggregated tau Figure 4.7C, the technique is still useful in identifying aggregated tau and is the gold standard for identifying aggregated tau.

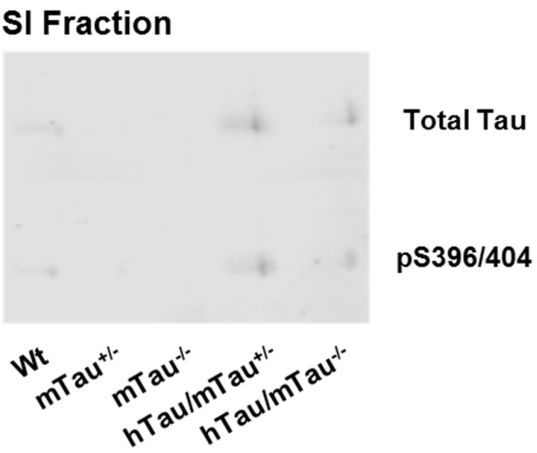

Figure 4.10: Western blot analysis of SI fractions from 9 month old male hTau mice revealed neither hTau/mTau$^{+/-}$ nor hTau/mTau$^{-/-}$ mice had aggregated tau.

4.2.9 Similar hippocampal microglial activation in 3 month hTau mice

To determine whether hTau/mTau$^{+/-}$ mice have an altered neuroinflammatory environment, hippocampal microglial activation was analysed by Iba1 staining. Iba1 stained sections underwent semi-automated analysis to determine the number of hippocampal Iba1 positive cells and the average size of their soma (Ding et al., 2016). Following activation, microglia undergo proliferation which increases the number of cells present (Hovens et al., 2014). In addition to proliferating, activated microglia undergo profound morphological alterations involving shortening of processes and increases in cell body size (Kozlowski and Weimer, 2012). There were no significant differences in the soma density and soma size (Figure 4.11A-C) as revealed by a one-way ANOVA ([$F_{(4,10)}$ = 0.25, p = 0.902] and [$F_{(4,10)}$ = 0.14, p = 0.964] respectively); indicating a similar microglial activation state at 3 months of age.

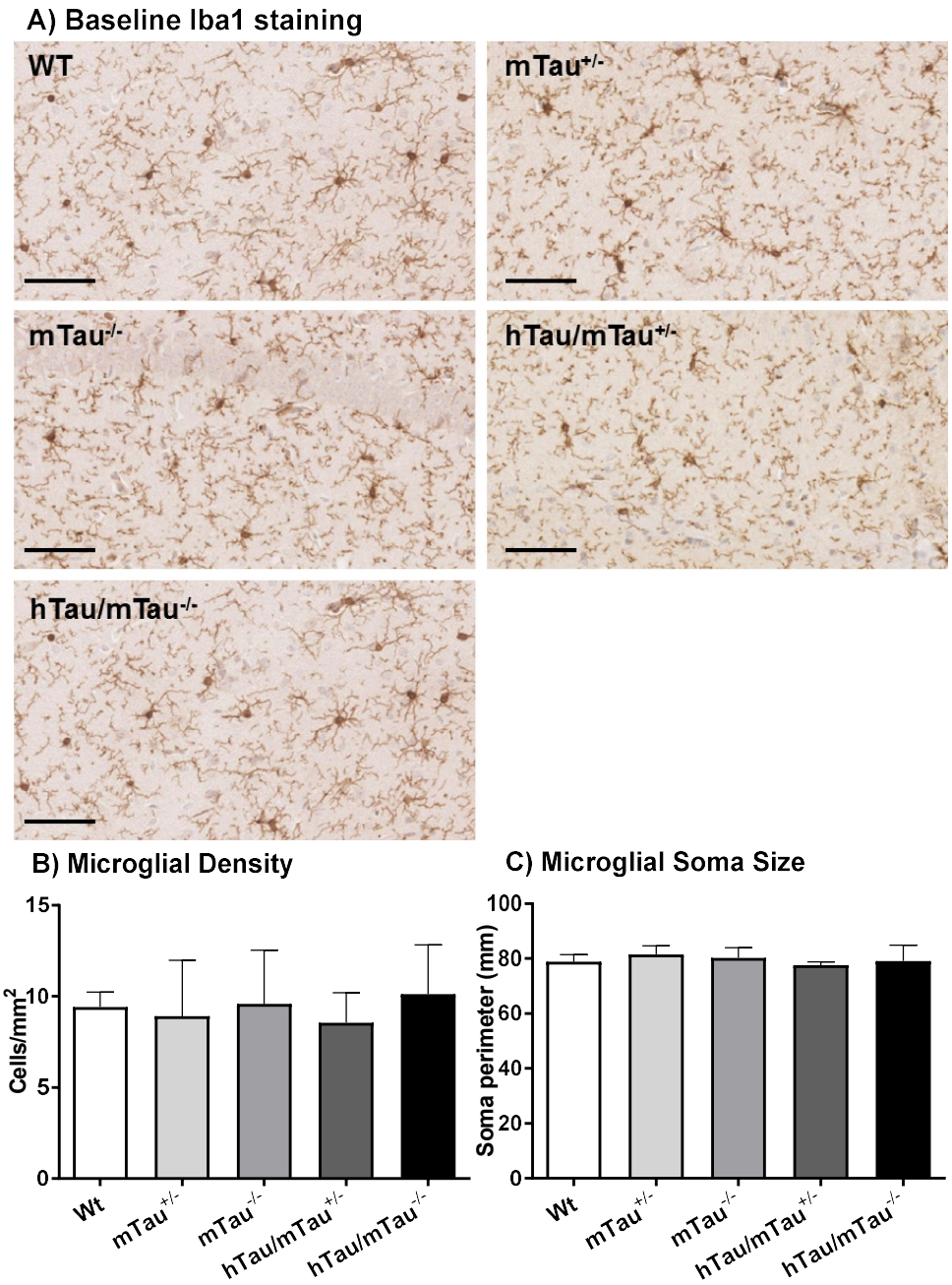

Figure 4.11 Hippocampal Iba1 staining in 3 month male hTau/mTau$^{+/-}$ mice. Bars represent mean ± SEM (n=3/genotype). ***A)*** *Representative sections Iba1 sections. Semi-automated analysis revealed there were no significant alterations in* ***B)*** *microglial density and* ***C)*** *microglial soma size suggesting similar basal microglial activation among genotypes. Scale bar represents 50 µm.*

4.2.10 Comparable hippocampal microglial activation in 9 month hTau mice

To determine whether microglial activation altered during the ageing process, Iba1 staining was conducted on hippocampal sections from 9 month male mice (Figure 4.12A-C).

Similar to the 3 month time-point, one way ANOVA assessment revealed there were no significant differences in the microglia density (Figure 4.12F, [$F_{(4,15)}$ = 0.41, p = 0.797]) or microglial soma size (Figure 4.12G, [$F_{(4,15)}$ = 0.24, p = 0.91]). Together, this suggests similar microglial activation among the genotypes at 9 months of age.

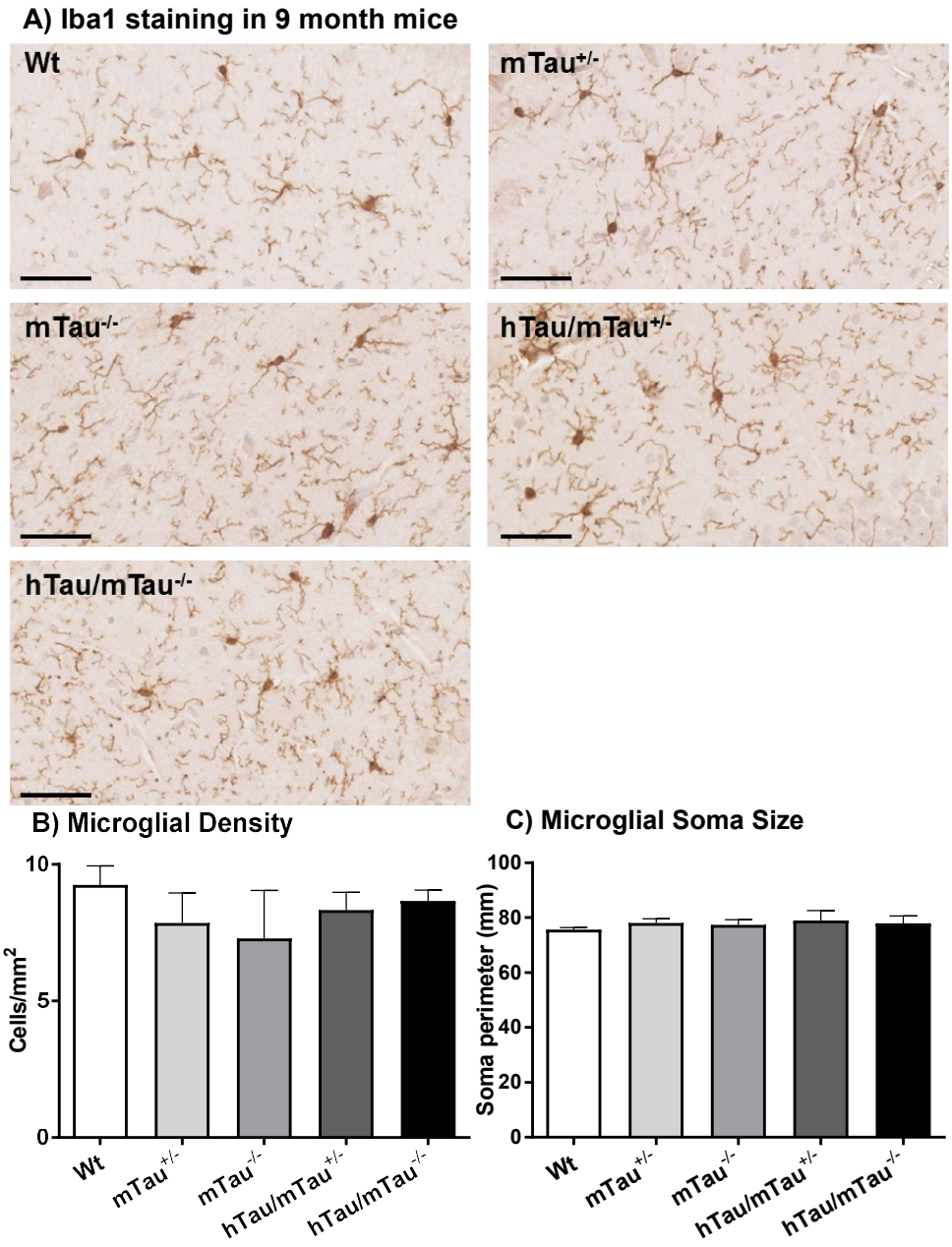

Figure 4.12 Hippocampal Iba1 staining in 9 month male hTau/mTau$^{+/-}$ mice. Bars represent mean ± SEM (n=3-4/genotype). ***A)*** *Representative sections Iba1 sections. Semi-automated analysis revealed there were no significant alterations in* ***B)*** *microglial density and* ***C)*** *microglial soma size suggesting similar basal microglial activation among genotypes. Scale bar represents 50 µm.*

4.3 Discussion

The source of pathology in the hTau mice has been highlighted as one of the major issues of the model. While some of the underlying pathological processes in AD possibly contribute to tau pathology in the model, hTau mice express an isoform imbalance - expressing more 3R tau isoforms compared to 4R (Andorfer et al., 2003). There is roughly an equal isoform ratio in AD (Schmidt et al., 2001) and changes in isoform ratio have been suggested to cause tau pathology in itself (Bronner et al., 2005, Shi et al., 2008). In addition to an isoform imbalance, the knockout of mTau generates systemic pathologies in hTau mice. To address both the issue of isoform ratio imbalance and systemic pathologies linked to removal of murine tau, hTau mice were bred on a heterozygous mTau background. As adult mTau only consists of 4R tau isoforms (Liu and Götz, 2013), the isoform ratio in hTau/mTau$^{+/-}$ mice would return towards what is observed in AD and negate systemic pathologies associated with the KO of mTau. Indeed, preliminary analysis of tau species in 6 month mice by western blotting of cortical suggested hTau/mTau$^{+/-}$ mice to have an altered isoform ratio which is more akin to that which is observed in AD. As hTau mice on an mTau$^{+/+}$ background do not express overt tau pathology, verification of tau pathology was required in hTau/mTau$^{+/-}$ mice. Tau phosphorylation at both pS202 and pS396/404 epitopes were found to be increased in 6 month hTau/mTau$^{+/-}$ mice. However, in the preliminary investigation hTau/mTau$^{+/-}$ mice were found to have increased total tau levels, suggesting increases in total tau as the likely source of increased tau phosphorylation. Nevertheless, the ratio between phosphorylated tau:total tau was similar among hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice, suggesting, at least from the preliminary observations, that hTau/mTau$^{+/-}$ mice have a more relevant isoform ratio while having a similar extent of tau phosphorylation compared to hTau/mTau$^{-/-}$ mice.

There were some limitations from the preliminary tau phenotyping. Both hTau genotypes were on separate backgrounds and the controls for hTau/mTau$^{-/-}$ mice were not Wt littermates. Nonetheless, the study gave the confidence that differences in the isoform ratio between hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice existed and tau phosphorylation remained spared. To verify this, hippocampal samples from 3-month mice were compared. Importantly, the increase in 4R:3R tau isoform ratio compared to hTau/mTau$^{-/-}$ mice was verified in 3 month old hTau/mTau$^{+/-}$ mice when expressed on the same background. Interestingly, when expressed on the same background and at a younger age, total tau levels appeared comparable between hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice. Despite this, tau phosphorylation was increased at both pS202 and pS396/404 epitopes in hTau/mTau$^{+/-}$ mice,

suggesting increased tau phosphorylation in the model. When expressed as a ratio to total tau, the pS202 was significantly elevated in hTau/mTau$^{+/-}$ compared to hTau/mTau$^{-/-}$ mice. Furthermore, assessment by IHC, pS202 revealed significant cellular staining of cell bodies in hTau/mTau$^{+/-}$ mice, suggesting they do indeed develop early tau pathology. Therefore it appears while full expression of mTau results in ablation of pathology (Duff et al., 2000), partial mTau expression causes augmentation. The increase in tau phosphorylation in hTau/mTau$^{+/-}$ mice could be resulting from altering the isoform ratio in the model. In an eloquent study, increasing the ratio of 4R:3R tau using isoform switching antisense oligonucleotides in the hTau model resulted in increased tau phosphorylation (Schoch et al., 2016). Conversely, the partial expression of mTau in the model could in itself result in increased tau phosphorylation. The expression of mTau in the 3xTg model was associated with increased tau phosphorylation and aggregation in the model (Baglietto-Vargas et al., 2014). However, this latter possibility could simple due to increased total tau levels in the study. Despite the development of early tau pathology at 3 months old in both hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice, there was no evidence of aggregated tau, suggesting only mild tau pathology existed at 3 months of age.

In addition to analysis of tau species, hTau/mTau$^{+/-}$ mice were assessed in the spontaneous alternation and food burrowing tasks at 3 months of age to determine whether hTau/mTau$^{+/-}$ mice develop early behavioural abnormalities which are observed in the hTau model (Geiszler et al., 2016). Due to being bred on a Tau KO background, hTau/mTau$^{-/-}$ mice are not bred with Wt littermates. Instead comparisons are made to either aged matched controls (Geiszler et al., 2016, Polydoro et al., 2009) or comparing to younger hTau mice (Andorfer et al., 2003). This makes accurate behavioural and pathological comparisons extremely difficult. By expressing hTau mice on a partial murine tau background, Wt littermates are easily generated, enabling accurate behavioural assessment. To the author's best knowledge, this is the first time Wt-littermates have been utilised for in a study involving hTau mice. Both hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice weighed less than Wt littermates, suggesting a potential similar metabolic phenotype between the two. Previously our group has reported food burrowing as the most prominent behavioural task affected in the hTau model (Geiszler et al., 2016). The food burrowing task is seen as a measure of daily living and is heavily susceptible to hippocampal integrity (Deacon, 2009). In the current study, food burrowing was similarly impaired in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice, indicating similar early hippocampal dependent behavioural impairments between the two. In addition to food burrowing, PBS treated animals were compared in the spontaneous

alternation task to discern whether locomotor activity or spontaneous working memory was altered in hTau/mTau$^{+/-}$ mice; both of which were similar in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice. The only genotype which failed to significantly alternate at more than chance level were mTau$^{-/-}$ mice, again demonstrating that the ablation of tau creates a behavioural phenotype in itself (Geiszler et al., 2016). Finally, by expressing partial murine tau, any loss of function effects resulting from the mTau KO are avoided, leaving solely effects of the pathological tau aggregation process. This is represented by the fact that not one mTau$^{+/-}$ or hTau/mTau$^{+/-}$ mouse over 12 months of age has been found positive for systemic pathologies which affected their mTau KO counterparts.

To determine whether hTau/mTau$^{+/-}$ mice developed aggregated tau at a later age, 9 month old mice were assessed for sarkosyl insoluble tau. Western blot analysis of S1 samples revealed generally similar observations to that from the hippocampal samples from 3 month mice. However the difference in total tau between hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice appeared more pronounced, suggesting the disparity between total tau levels increases with age in the model. Nevertheless, the elevated ratio between pS202 and total tau levels in hTau/mTau$^{+/-}$ mice suggested that increases in tau phosphorylation were not solely due to increases in total tau levels. Surprisingly, at 9 months of age, neither of the genotypes expressed any sarkosyl insoluble tau, despite hTau mice from the same source as the current study (The Jackson Laboratory) having been previously reported to have significant tau aggregation at both 3 and 10 months of age (Kelleher et al., 2007). However, the study reported changes in sarkosyl insoluble tau against 5 week old mice while failing to assess insoluble tau in Wt mice and as such, sarkosyl insoluble tau could be resulting from contamination from the soluble fraction in a similar manner to the faint bands observed in the current study. Supporting this, the increases in sarkosyl insoluble tau in the study identically mimic the increases in total tau in the soluble fraction which. Taken together with the failure to find corresponding increases in tau in the pathogenic MC1 confirmation in the study suggests this to be the case. Therefore, it appears hTau mice purchased from The Jackson Laboratories on the C57BL/6 background have a milder phenotype compared to those originally described by Andorfer *et al.*, 2003 on a mixed Swiss Webster/129/SvJae/C57BL/6 background. The milder phenotype of hTau mice on a C57BL/6 is not selective to tau pathology, with previous in-house studies have suggested a milder behavioural phenotype as well (Geiszler et al., 2016).

Finally, microglial activation was assessed in both 3 month and 9 month hTau mice to determine whether the early tau pathology expressed altered the neuroinflammatory environment. Neither 3 nor 9 month old hTau/mTau$^{+/-}$ or hTau/mTau$^{-/-}$ mice exhibited a significant increase in either the number of microglia or the size of their soma, suggesting a similar neuroinflammatory environment. This is comparable with what has been previously reported where hTau/mTau$^{-/-}$ were found to have similar microglia activation at 6 months of age but significant microglia activation by 12 months (Bhaskar et al., 2010).

Together these results indicate that hTau/mTau$^{+/-}$ mice are an ideal model for assessing early pathological tau changes. They possess a tau isoform ratio more akin to AD and expresses exacerbated tau phosphorylation while expressing similar behavioural perturbations to hTau/mTau$^{-/-}$ mice. The differences in phosphorylation between the models could be resulting from either the altered isoform ratio, the expression of mTau in the model or a combination of both. Importantly, hTau/mTau$^{+/-}$ mice are not affected by undesired effects resulting from ablation of mTau. With the added benefit of generating Wt littermates, hTau/mTau$^{+/-}$ mice are an improvement on the original hTau/mTau$^{-/-}$ model. Further studies need to assess tau aggregation at a later stage in the model and determine whether aggregation in 4R tau isoforms occurs.

Chapter 5 The Effect of LPS on Tau Pathology at 4h

5.1 Introduction

Based on observations from Chapter 4, the hTau/mTau$^{+/-}$ model is viable for assessing early pathological alterations in the tau protein. This chapter was aimed at understanding the effects of mild systemic inflammation on early stage tau pathology in both the improved and established model in hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice respectively. As discussed in Chapter 1 and Barron *et al.,* 2017, systemic inflammation is seen as a key mechanistic inducer of tau pathology, through upregulation of various kinases including CDK-5, GSK-3β and MAPK's which are involved in the phosphorylation of tau. Increases in tau phosphorylation are thought to initiate its dissociation from the microtubules and subsequent pathologic aggregation. Our understanding for the interaction of systemic inflammation and tau pathology relies on experimental models, with its role in humans poorly studied. The majority of these studies utilise either acute or chronic LPS administration to simulate inflammatory responses in mouse models. Rapid LPS tolerance in mice suggests that chronic LPS administration is inappropriate for modelling the effect of pro-inflammatory stimuli on tau pathology (Foster et al., 2007, Fensterheim et al., 2017, Wysocka et al., 2001). The majority of acute systemic LPS studies utilise doses well above 1 mg/kg, a dose which correlates more to sepsis (Thomas et al., 2014, Woodske et al., 2009) rather than the underlying mild inflammation which is observed in AD. Sepsis, or septic shock, is the unregulated inflammatory response to pathogens, or their toxins, which can result in death (Fink, 2014). The same phenomenon occurs in mice when administered with septic doses of LPS (Thomas et al., 2014, Woodske et al., 2009). Furthermore, a single septic LPS dose of 5 mg/kg has been reported to induce spontaneous neurodegeneration and chronic neuroinflammation in surviving mice (Qin et al., 2007), demonstrating the profound effect septic doses of systemic LPS can have within the CNS.

To the best of the author's knowledge, only two studies have assessed the effect of non-septic doses of LPS on tau pathology, both of low power. Roe *et al.,* 2011 found a 100 µg/kg *i.p,* injection to transiently increase tau phosphorylation in Wt mice, although the stress of injection rather than inflammation appears to be the source of phosphorylation. The second study found a 250 µg/kg *i.p,* injection to decrease levels of high molecular pT205 tau 24 hours following administration (Pearson, 2014), indicating a potential beneficial role for mild inflammation on tau phosphorylation. This chapter is aimed at exploring the effect of physiologically relevant levels of inflammation on early tau pathology in the hTau model

to further our understanding of how mild inflammation affects tau pathology in AD. To this end, the effect of three low doses of LPS – 100, 250 and 330 µg/kg were assessed on tau phosphorylation, localisation and aggregation in 3 month old hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice. Both genotypes were utilised in the current study to determine whether altered tau isoform ratio, towards what is observed in AD, would impact the response to systemic inflammation. As systemic inflammation is thought to be an early modulator of tau pathology, 3 month old mice were utilised. At this age, hyperphosphorylation and relocalisation of tau first becomes evident in the hTau model (Andorfer et al., 2003). In order to ascertain a direct role for pro-inflammatory stimuli, tau pathology was assessed during the expected peak cytokine response at 4h following LPS administration (Spulber et al., 2012, Srinivasan et al., 2010, Erickson and Banks, 2011). To determine the dose-dependent inflammatory response induced by LPS, the pro-inflammatory cytokines: IL-1β, IL-6, TNFα and INFγ as well as the anti-inflammatory cytokine: IL-10 were assessed for their serum concentrations by multiplex analysis. Furthermore, to determine susceptibility to the inflammatory response, locomotor activity was taken as a measure of the sickness syndrome in the spontaneous alternation task. The alternation rate in the spontaneous alternation task was taken as a measure of spatial working memory (Hughes, 2004).

5.2 Study design

Experimental cohort 5 was utilised in the current study which comprised of 177 experimentally naïve Wt, mTau$^{+/-}$, mTau$^{-/-}$, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice. Where Chapter 4 compared solely the PBS treated animals of experimental cohort 5, the current chapter compared the cohort as a whole. The study design is depicted in Figure 5.1 and lasted 3 days. On the first day, the animals were given a practise food burrowing test in their group cage overnight. The following day, the animals were single housed and given the food burrowing test overnight. The third and final day, the mice were challenged with PBS, or 100, 250 or 330µg/kg of LPS (*i.v.,* lateral tail vain, n=8-9/group) and were assessed in the spontaneous alternation task (SA) for spatial working memory and the LPS-induced behavioural suppression. Immediately following, the mice were sacrificed.

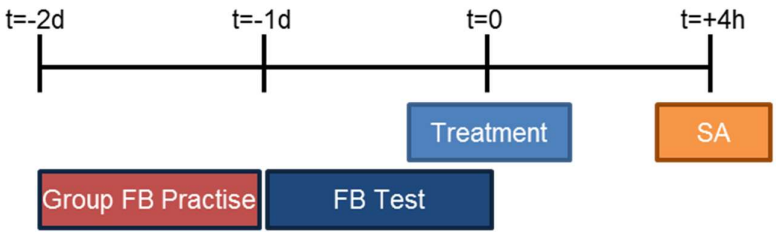

Figure 5.1 Behavioural assessment was conducted over 3 days. The mice were given a food burrowing practice while grouped housed on the first day. On the second day, the mice were given a food burrowing test overnight while single housed. The following morning, the mice were challenged and underwent a spontaneous alternation test 4h later immediately prior to being culled.

5.3 Results

5.3.1 LPS confounds the spontaneous alternation task

LPS suppressed locomotor activity in all genotypes, indicated by an overall significant treatment effect following two-way ANOVA analysis [$F_{(3,157)}$ = 28.26, p < 0.001]. There was no genotype x treatment interaction [$F_{(12,157)}$ = 1.29, p = 0.229], signifying similar behavioural suppression following LPS administration among genotypes. Interestingly, the response appeared to be dose-dependent except in both genotypes heterozygous for murine tau (Figure 5.2). Due to the LPS-induced sickness syndrome being present, the spatial working memory aspect of the task was confounded and not included in the thesis.

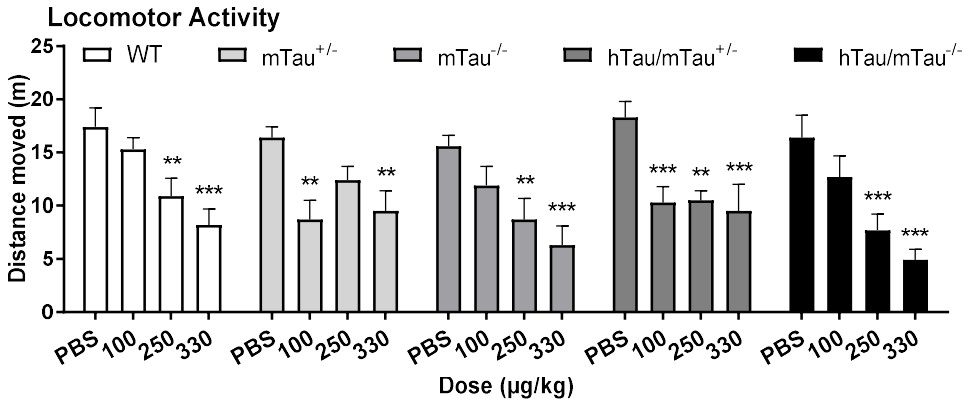

*Figure 5.2: LPS-induced locomotor suppression in the spontaneous alternation task. Bars represent mean ± SEM (n=8-9/group) and significance is against vehicle treated mice for each genotype. All mice were dose-dependently inhibited by LPS 4 hours following i.v. administration. Significance vs. vehicle: ** p < 0.01 and *** p <0.001.*

5.3.2 LPS induces a systemic pro-inflammatory response

To assess the underlying pro-inflammatory response induced by LPS, serum concentrations of pro-inflammatory cytokines were measured. To enable parametric assessment of cytokine levels, the data was rank transformed for statistical analysis. In PBS treated animals, serum concentrations of IL-1β, IL-6, INFγ and TNFα were effectively non-existent. Following LPS administration, there was a marked upregulation in all four cytokines, indicated by overall significant treatment effects; IL-1β: [$F_{(3,139)} = 42.7$, $p < 0.001$], IL-6: [$F_{(3,139)} = 44.89$, $p < 0.001$], INFγ: [$F_{(3,139)} = 42.85$, $p < 0.001$] and TNFα: [$F_{(3,139)} = 45.83$, $p < 0.001$]. Corresponding with reports in the literature following LPS administration (Erickson and Banks, 2011), IL-6 was the cytokine with the highest concentration following LPS administration (Figure 5.3A-D). While assessing pro-inflammatory cytokines following LPS administration is important, equally vital is the assessment of anti-inflammatory mediators to truly gauge the nature of the inflammatory response. The serum concentration of the prototypical anti-inflammatory cytokine: IL-10 was additionally measured by multiplexing. Similar to the pro-inflammatory cytokines, IL-10 concentration in PBS-treated animals was essentially non-existent. Following LPS administration, there was an increase in IL-10 concentration in all genotypes, indicated by a significant overall treatment effect [$F_{(3,139)} = 61.5$, $p < 0.001$]. Interestingly, hTau/mTau$^{+/-}$ mice appeared to have decreased IL-10 induction as the dose of LPS was escalated (Figure 5.3E).

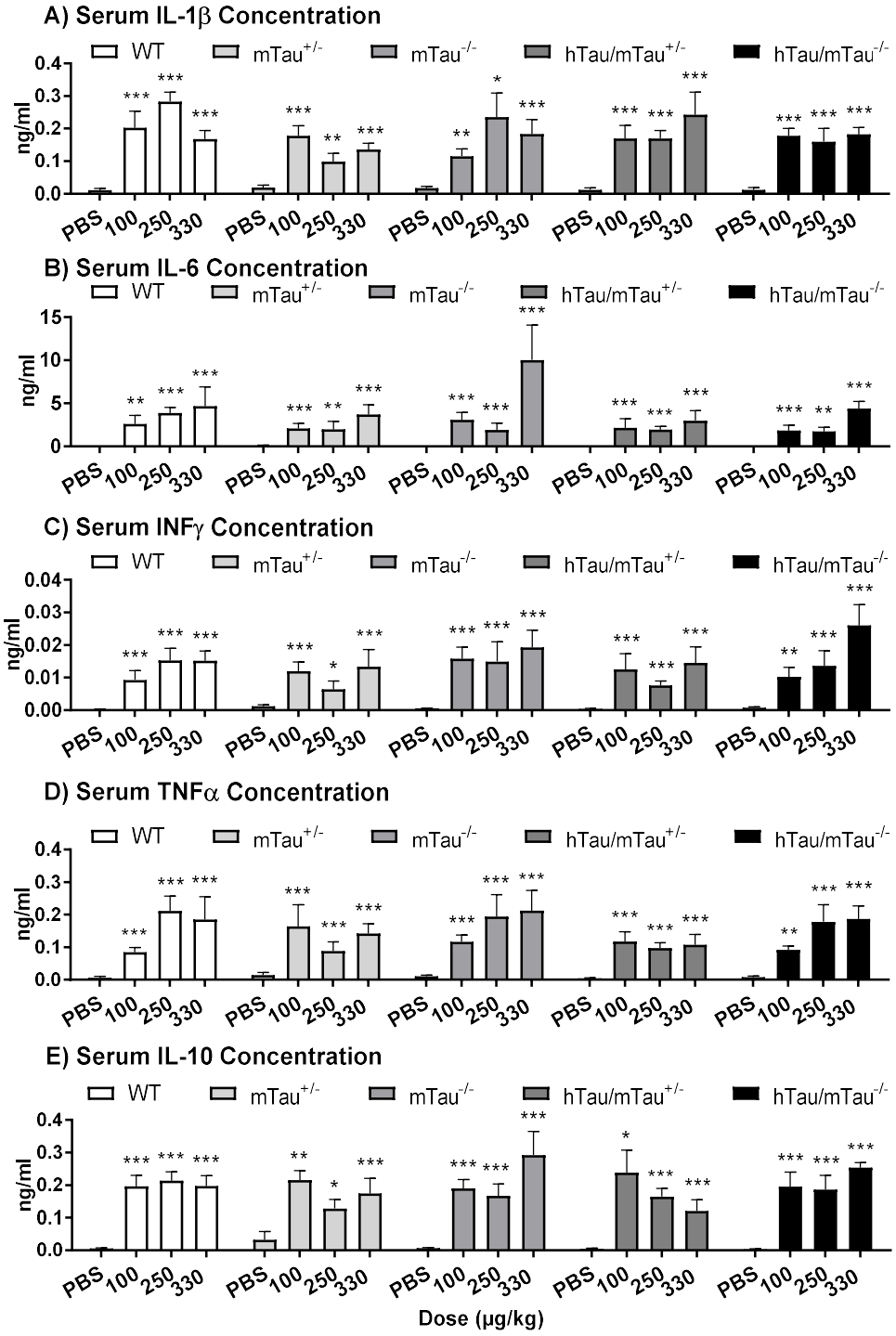

*Figure 5.3: Serum inflammatory cytokine concentrations 4h following LPS administration in 3 month old male hTau mice. Bars represent mean ± SEM (n=6-8/group). LPS induced marked increases in **A)** IL-1β, **B)** IL-6, **C)** INFγ, **D)** TNFα and **E)** IL-10 serum concentrations. Significance vs. vehicle: * p < 0.05, ** p < 0.01 and *** p < 0.001.*

Systemic IL-6 production has been reported to be an important meditor in the LPS-induced sickness syndrome (Harden et al., 2006, Bluthé et al., 2000). A Pearson's product moment correlation was conducted between the concentration of circulating IL-6 and locomotor activity from the spontaneous alternation trial to determine the contribution of systemic inflammation to sickness behaviour. As with the cytokine data, the concentration of IL-6 was rank transformed to run a parametric analysis. Systemic IL-6 concentration was found to have a moderate negative correlation with distance moved in hTau/mTau$^{-/-}$ mice ($r = -0.449$, $p < 0.05$). This compares to a strong negative correlation in Wt, mTau$^{+/-}$, mTau$^{-/-}$ and hTau/mTau$^{-/-}$ mice ($r = -0.677$, $p < 0.001$, $r = -0.699$, $p < 0.001$, $r = -0.668$, $p < 0.001$ and $r = -0.525$, $p < 0.001$ respectively, Figure 5.4). Together this suggests IL-6 highly correlates with locomotor activity following LPS administration irrespective of genotype.

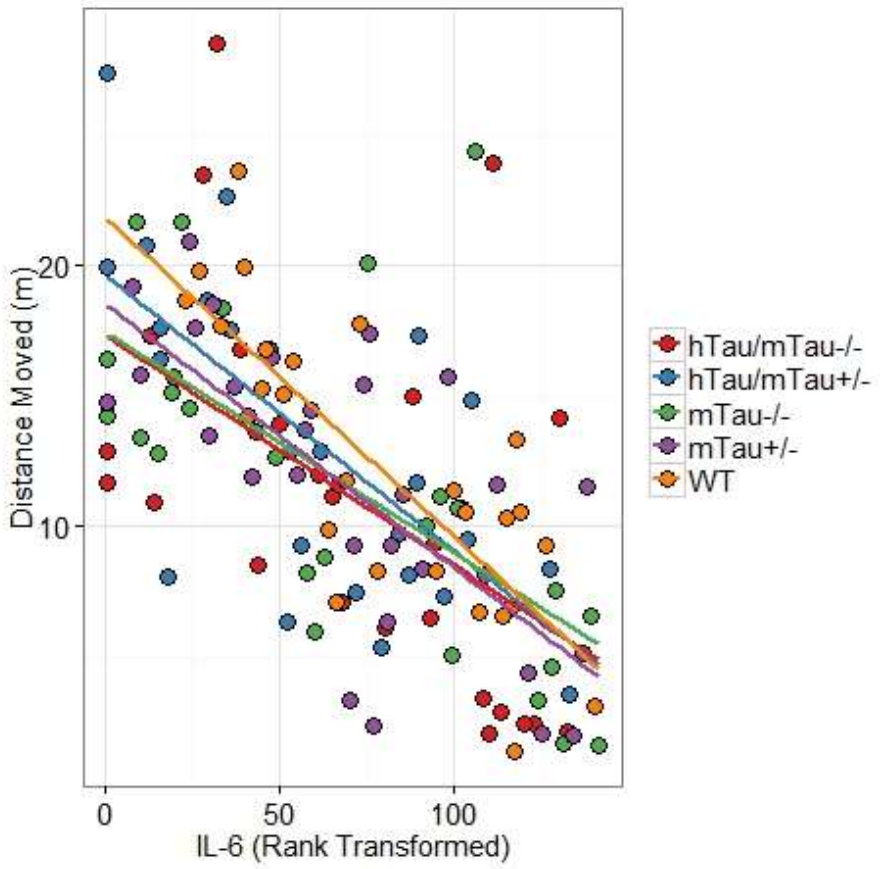

Figure 5.4 Pearson's product moment correlation between distance moved in the spontaneous alternation task and IL-6 concentration (rank transformed) showed a strong correlation (n=34-36/genotype).

5.3.3 LPS induced microglial activation

Hippocampal sections underwent Iba1 staining to determine whether the acute response to LPS induced microglia activation. Representative sections from Wt, hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice show a shortening of processes and enlarging of soma size following LPS administration (Figure 5.5A). For representative sections from all genotypes see Appendix 8.3. Two-way ANOVA analysis revealed LPS failed to significantly affect the number of microglia present (Figure 5.4B, [$F_{(3,55)}$ = 1.03, p = 0.392]), suggesting LPS did not induce microglia proliferation. However, two-way ANOVA analysis indicated that LPS significantly enlarged the soma size of microglia (Figure 5.5B, [$F_{(3,55)}$ = 4.63, p < 0.01]. Post hoc analysis revealed that the lower two doses overall significantly affected the soma size. The failure for the highest dose to overall affect the microglia soma size was the non-significant decrease in this group among Wt mice (Figure 5.5C). Nevertheless, the increase in soma size indicates that systemically administered LPS is inducing a neuroinflammatory response in the current study.

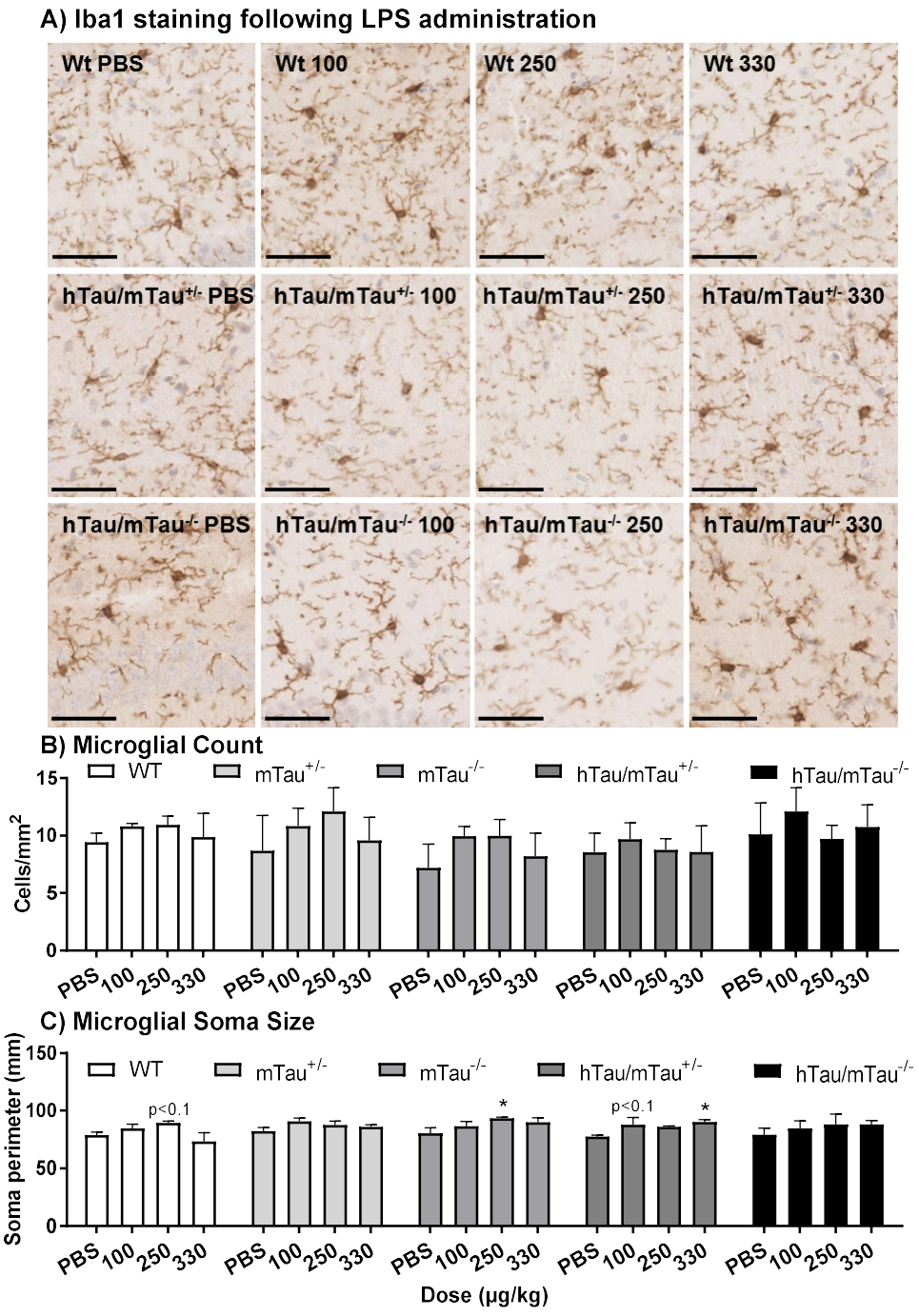

Figure 5.5 Iba1 staining 4h following LPS administration in 3 month old male hTau mice. Bars represent mean ± SEM (n=3/genotype). ***A)*** *Representative sections reveal a shortening of processes and enlarging of microglia soma size.* ***B)*** *LPS did not affect the number of microglia present.* ***C)*** *LPS significantly affected the size of microglia soma. Significance represents against vehicle treated mice * p < 0.05.*

5.3.4 LPS decreases hippocampal tau phosphorylation in hTau mice

Inflammation has been suggested to exacerbate early stage tau pathology through inducing tau hyperphosphorylation. To assess the effects of low doses of LPS administration on tau phosphorylation, hippocampal extracts were probed for pS202 and pS396/404 tau. Surprisingly, LPS administration was associated with decreased phosphorylation at the pS202 epitope with all genotypes appearing to have decreased levels following LPS administration. Two-way ANOVA analysis revealed there was a significant overall treatment effect in pS202 protein levels [$F_{(3,136)} = 5.32$, $p < 0.01$]. Post hoc analysis showed that all three doses significantly decreased pS202 levels in hTau/mTau$^{+/-}$ mice ($p < 0.05$-$p < 0.0001$). This compared to only 100 µg/kg in hTau/mTau$^{-/-}$ mice ($p < 0.05$, Figure 5.6B). Similar to pS202 levels, pS396/404 levels decreased following LPS administration. Two-way ANOVA analysis indicated that there was an overall significant treatment effect in pS396/404 following LPS administration [$F_{(3,136)} = 2.53$, $p < 0.05$]. Post hoc analysis revealed decreases in pS396/404 levels occurred following all three doses in hTau/mTau$^{+/-}$ mice ($p < 0.01$ – $p < 0.001$) but not hTau/mTau$^{-/-}$ mice ($p > 0.05$, Figure 5.6C). There was no alterations in total tau levels following LPS administration [$F_{(3,136)} = 0.12$, $p = 0.95$] suggesting de-phosphorylation rather than clearance as the source of the decreased tau phosphorylation (Figure 5.6D). Together, these results suggest that inflammation induced by a low dose of LPS can cause tau dephosphorylation.

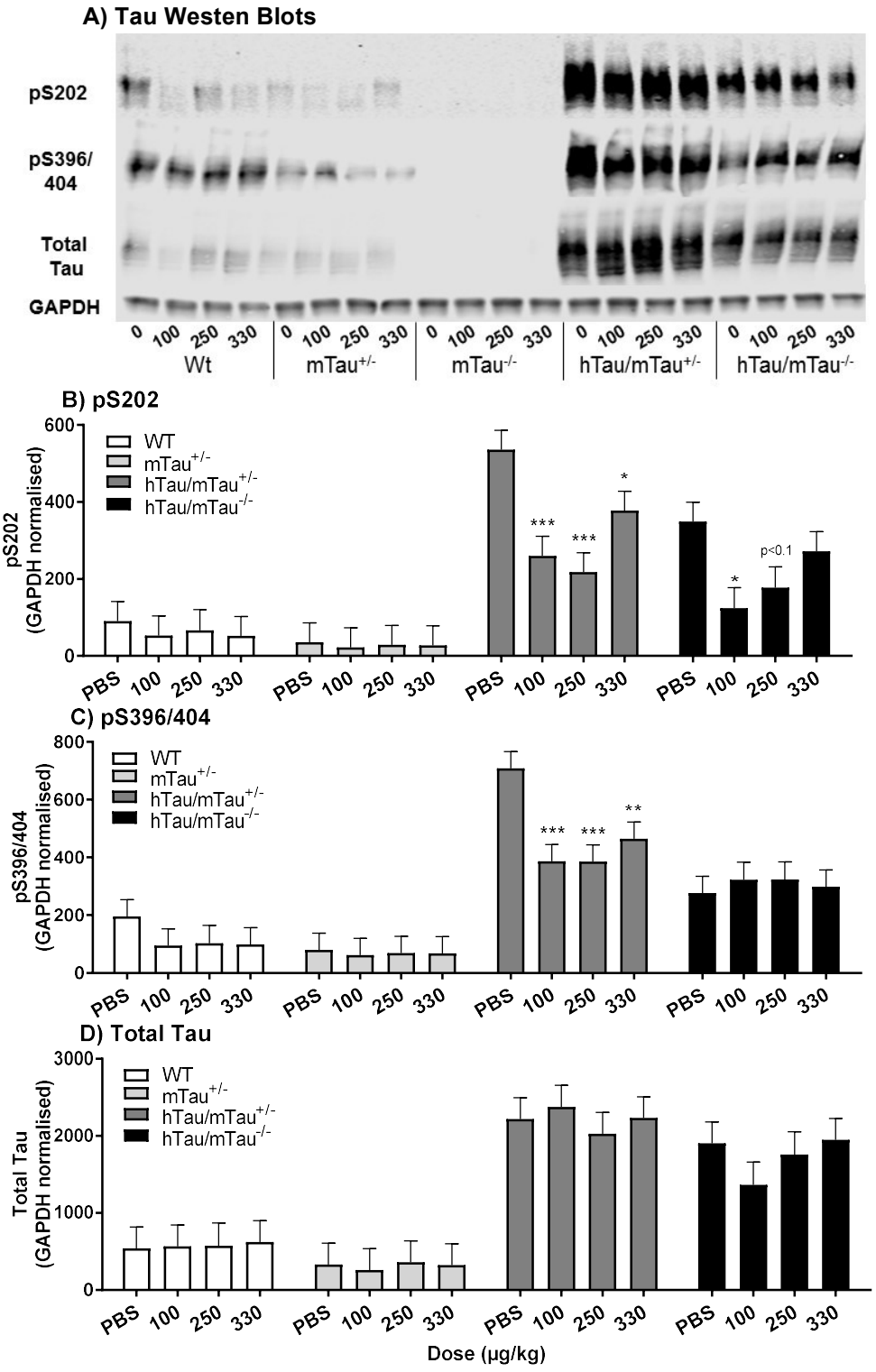

Figure 5.6: Tau phosphorylation 4h following LPS administration in 3 month old male hTau mice. Bars represents mean ± SEM (n=8-9/group). ***A)*** *Representative tau western blots. LPS decreased tau phosphorylation at the* ***B)*** *pS202 and* ***C)*** *pS396/404 epitopes.* ***D)*** *LPS did not affect total tau levels. Significance vs Vehicle: * p < 0.05, ** p < 0.01 and *** p < 0.001.*

5.3.5 LPS did not affect hippocampal PP2A activity

PP2A is the most common phosphatase in the brain and the most important tau phosphatase (Qian et al., 2010) which we hypothesise is the most likely mechanism for tau dephosphorylation following LPS administration. PP2A activity was assessed by western blotting to determine whether its activity was upregulated following LPS administration. Although a trend for decreased ratio of inactive:total PP2A in both hTau genotypes existed, LPS failed to significantly affect the measure ([$F_{(3,172)}$ = 0.48, p = 0.7], Figure 5.7B). Further validation of these findings is required in a dedicated PP2A activity. Unfortunately due to an unexpected freezer defrost, the samples which were intended for this purpose became unviable.

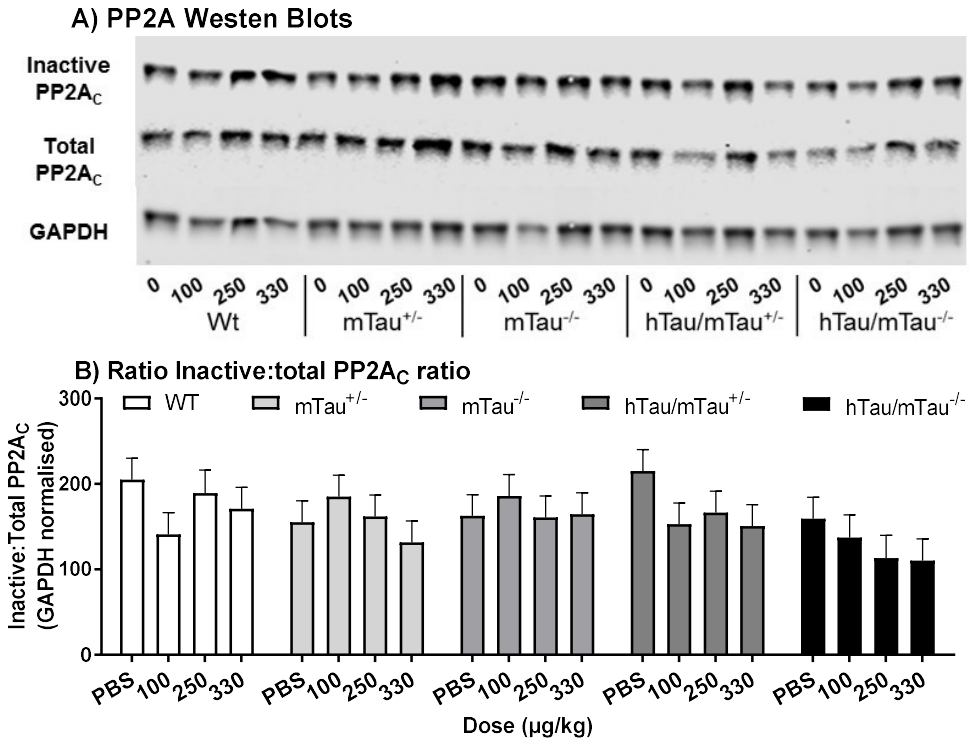

Figure 5.7: PP2A activity 4h following LPS administration in 3 month old male hTau mice (n=8-9/group). Bars represents mean ± SEM. ***A)*** *Representative PP2A western blots.* ***B)*** *There was a trend for increased PP2A activity following LPS administration in both hTau genotypes.*

5.2.6 LPS induced tau dephosphorylation in hemi-brain samples

Finally, to determine whether LPS administration was associated with alterations in tau aggregation, sarkosyl insoluble tau was assessed. S1 samples were assessed for pS396/404 and total tau by western blotting to assess whether alterations in tau phosphorylation were global. Analysis of pS396/404 by western blotting showed that tau

phosphorylation was likewise reduced in S1 fractions following LPS administration. Two-way ANOVA analysis revealed there was an overall genotype effect [$F_{(3,68)}$ = 7.23, p < 0.001]. Post-hoc analysis showed that all three doses significantly reduced pS396/404 in Wt and hTau/mTau$^{+/-}$ mice (p < 0.05, Figure 5.8B). Furthermore, there was a trend for a genotype x treatment interaction in total tau levels following two-way ANOVA analysis [$F_{(6,65)}$ = 2.01, p = 0.0798]. Post hoc analysis demonstrated that all three doses decreased total tau levels solely in Wt mice (p < 0.05, Figure 5.8C). To determine whether the decrease in tau phosphorylation among Wt mice was due to decreased total tau levels, a ratio between pS396/404:total tau was calculated. Two-way ANOVA analysis revealed there was a significant genotype x treatment interaction in the pS396/404:total tau ratio [$F_{(6,65)}$ = 2.17, p = 0.0595]. Post hoc analysis revealed that all three doses significantly reduced the pS396/404:total tau ratio in hTau/mTau$^{+/-}$ suggesting tau dephosphorylation to be the cause of decreased tau phosphorylation within the genotype (p < 0.001). The pS396/404:total tau ratio remained constant in Wt mice suggesting tau clearance to be the source of decreased tau phosphorylation within the model (p > 0.05, Figure 5.8D). The disparity between this and the effect of LPS on hippocampal tau in Wt type mice could either be due to different brain regions involved are due to the difference in extraction protocols. Nevertheless, it suggests that LPS induced that tau dephosphorylation is not selective to the hippocampus in the current study.

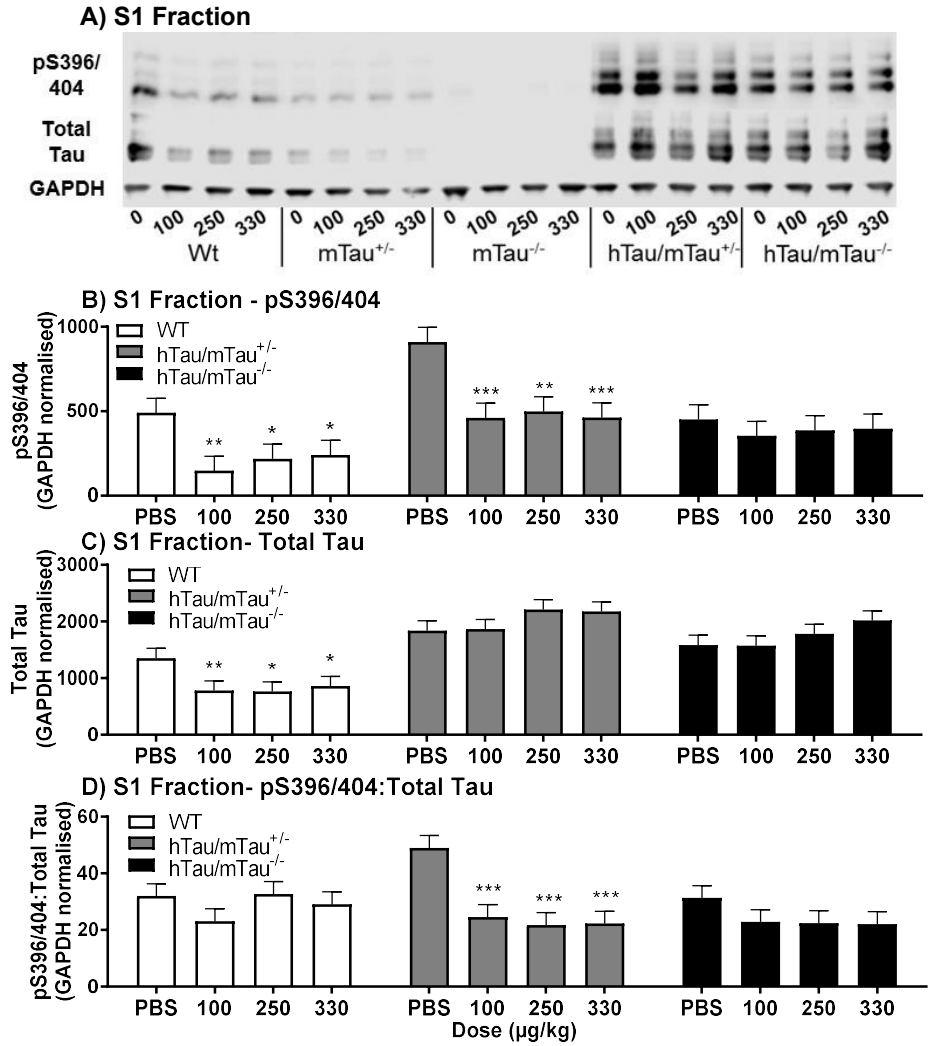

Figure 5.8: LPS induced tau dephosphorylation in S1 fractions 4h following LPS administration in 3 month old male mice. Sarkosl extracts following LPS administration with bars representing mean ± SEM. (n=6/group) ***A)*** *Representative blots from S1 fractions.* ***B)*** *Quantification of pS396/404 levels in s1 fractions revealed a decrease in Wt and hTau/mTau$^{+/-}$ mice* ***C)*** *LPS induced a decrease in total tau levels in Wt mice while not affecting both hTau genotypes.* ***D)*** *The ratio between pS396/404:total tau was decreased in hTau/mTau$^{+/-}$ mice but was unaltered in Wt mice. Significance vs. vehicle: * P < 0.05 and ** p < 0.01.*

5.3.7 LPS did not induce tau aggregation

While 3 month hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ do not express aggregated tau, there is the possibility that LPS could induce tau aggregation despite a reduction in phosphorylation. In a similar manner, the diabetes treatment: metformin reduces tau phosphorylation at multiple tau epitopes in the P301S model, but increases tau aggregation (Barini et al., 2016). However, all doses of LPS failed to induce tau aggregation in any genotype. While there appeared to be faint bands of total tau in sarkosyl insoluble fractions

(Figure 5.9A), the absence of phosphorylated tau suggested them to be contamination from the soluble fraction. This was confirmed in the HTRF tau aggregation assay (Figure 5.9B).

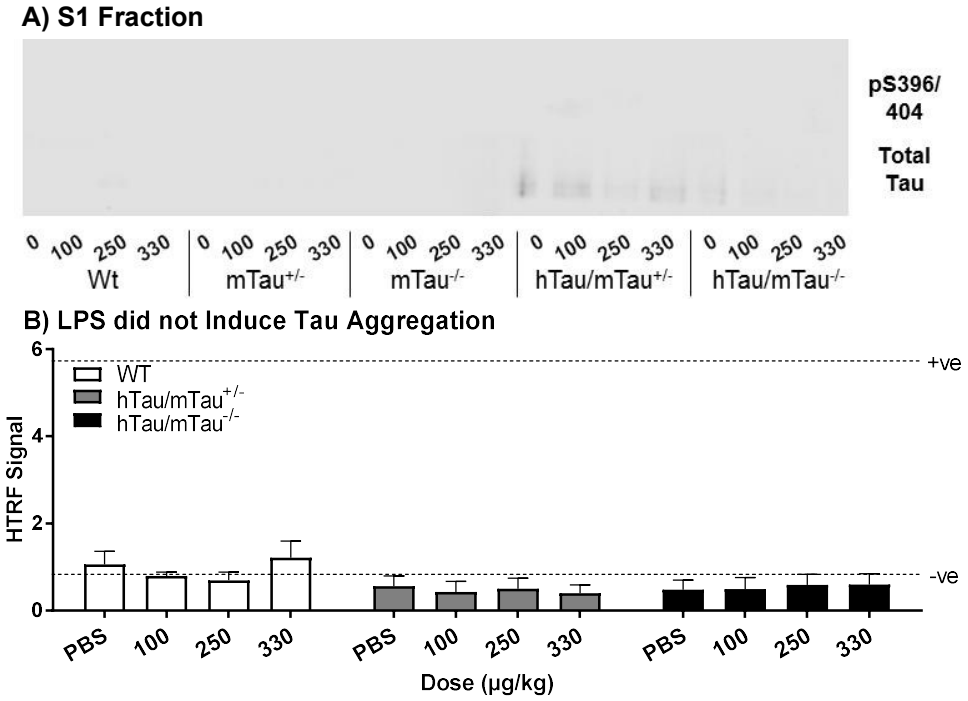

Figure 5.9: Analysis of Aggregated tau 4h following LPS administration in 3 month old male hTau mice. ***A)*** *Western blotting of SI samples indicated LPS did not induce tau aggregation.* ***B)*** *S1 fractions analysed in HTRF tau aggregation assay. Bars represent mean ± SEM (n=6/group). Bottom dotted line represents average signal from 4 mTau$^{-/-}$ mice and top line represents positive control included with assay. In comparison, aged rtg4510 mice produce a signal of over 25 (data not shown).*

5.4 Discussion

The effect of systemic inflammation induced by LPS was assessed in both hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ genotypes to determine whether altered tau phosphorylation or isoform ratio impacted on any pathological alterations. A 4h time-point was chosen as this is when many pro-inflammatory mediators peak (Spulber et al., 2012, Srinivasan et al., 2010, Erickson and Banks, 2011) and would simulate the direct effect of pro-inflammation stimuli on tau pathology. Indeed, LPS dose-dependently inhibited locomotor activity to similar levels in all genotypes, indicating similar behavioural suppression among genotypes and that spatial working memory assessment was confounded in the task. This corresponded with increased serum concentrations of IL-1β, IL-6, INFγ and TNFα, all of which were markedly elevated 4h after LPS administration and demonstrate a significant pro-inflammatory response at the time of assessment.

Furthermore, there was a strong correlation between IL-6 and locomotor activity suggesting the extent of inflammation was related to the sickness behaviours assessed. This is consistent with the literature where systemic LPS administration is associated with an increase in circulating cytokines and induction of the LPS-induced sickness syndrome (Skelly et al., 2013, Leone et al., 2012). As well as inducing a systemic inflammatory response, significant microglial activation suggests there was crosstalk with the CNS, a phenomenon well reported following peripheral LPS administration (Erickson and Banks, 2011, Bhaskar et al., 2010). Furthermore, similar microglial activation in response to LPS among genotypes in the current study suggests that hTau mice do not exhibit increased susceptibility to neuroinflammation at least at 3 months of age. Without overt tau aggregation at this age, the lack of increased susceptibility to microglial activation could be expected.

Based on animal models involving LPS administration, systemic inflammation has been suggested to exacerbate tau pathology through inducing its phosphorylation (Bhaskar et al., 2010, Kitazawa et al., 2005, Lee et al., 2010). However, these studies have either utilised doses which are more akin to sepsis or utilised chronic LPS treatment regimens which are likely to involve a tolerant response. Despite this, there is strong aetiological evidence for inflammation as an exacerbating force in AD. Based on both factors, we hypothesised that lower doses of LPS would similarly exacerbate tau pathology through inducing tau phosphorylation. In the current study, 3 increasing doses of LPS were administered to assess whether hTau/mTau$^{+/-}$ or hTau/mTau$^{-/-}$ mice were susceptible to LPS-induced tau pathological alterations. Unexpectedly, there were reduced levels of hippocampal pS202 tau after administration of all doses of LPS in hTau/mTau$^{+/-}$ mice, compared to only 100 µg/kg affecting pS202 in hTau/mTau$^{-/-}$ mice. Interestingly, there were no associated decreases in total tau levels, suggesting tau dephosphorylation rather than clearance as the source of decreased tau levels. The CP13 antibody which is targeted to pS202 has been reported to be associated with earlier stages of the disease (Espinoza et al., 2008) and phosphorylation in this region has been related with decreasing the affinity of tau to microtubules (Schneider et al., 1999, Sengupta et al., 1998) indicating mild inflammation potentially could be beneficially modulating the binding of tau to microtubules. Interestingly, the effect of tau phosphorylation appears to be dose dependent, especially in hTau/mTau$^{-/-}$ mice, with increasing doses of LPS step-wise returning pS202 levels to baseline. Even in hTau/mTau$^{+/-}$ mice, 330 µg/kg produced the lowest depression of pS202 levels. While only significant effects on hippocampal pS202 levels were observed in both hTau genotypes, a

similar trend was observed in both Wt and mTau$^{+/-}$ genotypes, with lack of significance likely due to the overall decrease in pS202 levels indicating a non-selective response.

In addition to assessing pS202, the levels of pS396/404 were assessed following LPS administration. The pS396/404 epitope is associated with later stages of AD (Augustinack et al., 2002) and phosphorylation in this region is reported to promote tau aggregation (Liu et al., 2007). All doses of LPS resulted in dephosphorylation at the pS396/404 epitope in what appeared to be a selective in hTau/mTau$^{+/-}$ mice. The hTau/mTau$^{+/-}$ selective response on pS396/404 could be due to a few possibilities. Firstly, the altered isoform ratio could affect the phosphorylation dynamics following LPS administration. Increasing the expression of 4R tau in the hTau model has been reported to increase the level of tau phosphorylation (Schoch et al., 2016). Although the authors did not assess the underlying mechanism for increased phosphorylation, it demonstrates there is a dysregulation of the molecules which regulate tau phosphorylation. Therefore, an altered tau isoform ratio in hTau/mTau$^{+/-}$ mice could cause it to be differential affected by molecules which induce its dephosphorylation following LPS-induced inflammation. Secondly, the expression of mTau could be resulting in increased susceptibility to tau dephosphorylation in the model irrespective of the altered isoform ratio. The development of systemic pathologies in hTau/mTau$^{-/-}$ but not hTau/mTau$^{+/-}$ mice indicates that partial expression of mTau restores dysregulation due to its removal. This suggests there are underlying cellular alterations between the models which could alter its susceptibility to LPS-induced dephosphorylation. Indeed, mTau expression in the 3xTg model has been shown to increase tau phosphorylation (Baglietto-Vargas et al., 2014) indicating mTau expression alters the underlying regulation of tau phosphorylation. However, this latter possibility could simple due to increased tau levels in the model. Finally, the increased tau phosphorylation in the hTau/mTau$^{+/-}$ model could result in increased susceptibility to LPS-induced tau dephosphorylation due to their increased phosphorylation burden.

While the current study was limited to a maximum dose of 330 µg/kg due to licence restrictions, a 1 mg/kg LPS administration in 2 month hTau/mTau$^{-/-}$ was reported to increase phosphorylation at both pS202 and pS396/404 epitopes 24h following administration (Bhaskar et al., 2010). The potential discrepancies between the effects on tau phosphorylation could be due to differences in age between the studies. However, this is unlikely as a 1 month age gap is relatively small, and any increase in age would make the animal even more susceptible to LPS-induced inflammation (Mouton et al., 2012, Kohman et

al., 2010). Likewise, the diverging findings between the two studies could be due to different time-points. Again this is unlikely due to 4h being the height of the inflammatory response (Erickson and Banks, 2011). Instead, the diminishing response as the dose is increased in the current study suggests the dose to be responsible for the discrepancy between the studies. Together, these results suggest the effect of LPS on tau phosphorylation is a spectrum. At low doses, tau dephosphorylation occurs and as the dose increases, the response flips to tau phosphorylation at septic doses of LPS.

Tau dephosphorylation could be resultant of either decreased kinase activation or increased phosphatase activation. We hypothesised that alterations in phosphatase activity was the source for tau dephosphorylation in the current study, with PP2A the likely culprit. PP2A accounts for about 1% of cellular content within the brain and is the predominant phosphatase which affects tau phosphorylation (Qian et al., 2010). LPS has been shown to induce PP2A activation *in vitro* in endothelial cells (Chuang et al., 2015, Menden et al., 2013). Furthermore LPS and superoxide, one of the products of LPS-induced inflammation (Pei et al., 2007), have been shown to induce PP2A activation in hippocampal slices (Zhang et al., 2014, Sheth et al., 2009) indicating LPS can directly and indirectly induce PP2A activation within the CNS. To asses PP2A activity in the current study, western blot analysis of the PP2A catalytic subunit were conducted after incubation with or without NaOH, giving a measure of inactive PP2A:total PP2A. Despite no significant alterations in this measure, there was a trend for a decrease in both hTau genotypes. As assessing PP2A activation by western blotting is not the method with greatest sensitivity, further analysis in a dedicated PP2A assay is required.

The effect of LPS-induced systemic inflammation was not limited to the hippocampus, with decreases in pS396/404 observed in the S1 fractions from hTau/mTau$^{+/-}$ hemibrains. This indicates tau dephosphorylation following LPS administration is not selective to the hippocampus where tau pathology is the strongest in the model (Andorfer et al., 2003). While LPS failed to significantly affect hippocampal pS396/404 tau levels in Wt mice, there was a significant reduction in the S1 fractions from hemibrains. The mechanism behind this appears to be different compared to hTau/mTau$^{+/-}$ mice in that reductions in pS396/404 appear to be due to a decrease in total tau levels. Interestingly, the reduction in total tau levels was selective to Wt mice, with LPS not affecting total tau levels in both hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice, suggesting potential defects in tau clearance due to the expression of the human tau gene. The two predominant processes for tau clearance are

proteasome and autophagy pathways (Lee et al., 2013), both of which may be defective in AD (Nixon and Yang, 2011, Keller et al., 2000). Further research is required in the hTau model to decipher whether either of the two clearance mechanisms is impaired under the current experimental conditions.

To assess whether LPS affected tau pathology past phosphorylation, sarkosyl insoluble fractions were assessed. One limitation of the current study was that neither hTau genotypes exhibited overt tau aggregation. As such, only induction of tau aggregation could be measured, a scenario less than likely due to the decrease in tau phosphorylation induced by LPS. Nevertheless, the possibility exists that tau aggregation can be induced despite decreased levels of phosphorylated tau. For example, metformin was reported to exacerbate tau aggregation despite reducing tau phosphorylation in the P301S model (Barini et al., 2016). However, there was no evidence of sarkosyl insoluble tau in any genotypes following LPS administration which was confirmed in the HTRF tau aggregation assay. Future studies are required at an age where hTau mice exhibit tau aggregation to determine whether decreases in tau phosphorylation are associated with decreased levels of tau aggregation.

The objective of this chapter was to assess the pro-inflammatory effects of mild systemic inflammation induced by low doses of LPS on tau pathology. It is difficult to determine an exact figure as to what level of systemic inflammation would be relevant to AD because of two limitations in the current study: assessment at an early age and assessment in mice. Ageing is well known to increase the susceptibility to inflammation (Gomez et al., 2007, Starr et al., 2015). Although less studied, the mouse immune system is less immune responsive compared to humans (Copeland et al., 2005, Zschaler et al., 2014). Together, this suggests that the extent of systemic inflammation needs to be higher in the current study compared to what is observed in AD patients. However, it is clear from the concentrations of pro-inflammatory mediators that the extent of systemic inflammation was too high to replicate the underlying inflammatory processes in AD. The average concentrations for IL-1β, IL-6, INFγ and TNFα in the current study at the lowest dose were 170, 2389, 11 and 114 pg/ml. While there is some inter-assay variability between studies, the four cytokines are in the low pg range in serum from AD patients (Wu et al., 2015, Choi et al., 2008, Holmes et al., 2009, Dursun et al., Chao et al., 1994, Yasutake et al., 2006, Singh and Guthikonda, 1997).

One possibility for the systemic inflammatory response being too strong is the concentration of LPS utilised in the current study was too high. As discussed in Chapter 1,

LPS was chosen as an immune challenge for its relevance to AD. LPS has been reported to be upregulated in serum of AD patients at a concentration of 60 pg/ml (Zhang et al., 2009c). While there are limited studies assessing the circulating concentration of LPS following systemic administration in mice, one study reported a peak 370 and 190 ng/ml concentration following an interperitoneal injection of either 1 or 0.5 mg/kg LPS respectively (Huang et al., 2007). If the concentration in the current study would follow the similar pattern, the circulating concentration will approximately lie between 37-122 ng/ml, much greater than that observed in the Zhang *et al.*, 2009c study. However, humans are more sensitive than mice to LPS. One study found that to induce a similar IL-6 response in humans and mice found the dose needed to be 250 times greater in the latter to induce a similar response (Copeland et al., 2005). Based on the Zhang, Huang and Copeland studies, this would suggest the doses of LPS in the current study are still too high and a dose of 40 μg/kg would be more physiologically relevant. However, a dose of 40 μg/kg assumes that the serotypes and potency of LPS are the same between the current study and those found elevated in AD patients, an assumption which is highly unlikely.

While the dose was potentially too strong to to mimic the underlying systemic inflammatory conditions, LPS could in itself be unsuitable for modelling mild inflammation. Supporting this, administration of a sub-pyrogenic dose of 1 μg/kg in mice found IL-6 and IL-1β concentrations to peak at around 7400 pg/ml and 10 pg/ml (Teeling et al., 2007). The increased IL-6 concentration compared to the current study could be resulting from assessment at a different time point, differences in the route of administration (*i.p.*) or LPS serotype used. Instead, inflammatory models which produce mild chronic inflammation such as the CIA model of rheumatoid arthritis and the high fat diet model of Type 2 diabetes could be seen as more appropriate. The circulating concentrations of IL-1β, IL-6 and TNFα in the CIA model were found to be 65, 50 and 100 pg/ml 35 days following induction of arthritis (Tsubaki et al., 2015). Similarly, the concentrations in mice fed a high fat diet are around 100, 50 and 150 pg/ml respectively (Kim et al., 2012). While these levels are still higher than what is observed in AD patients, under the caveats of being a mouse model and the need to assess early tau pathology at an early age, the slightly higher concentration could be more representative of what is observed in AD. Furthermore, both models induce chronic systemic inflammation which provides added relevance to systemic inflammation observed in AD.

To date, only one study has assessed the effect of CIA-induced systemic inflammation on tau pathology. Consistent with the results in this chapter, CIA induction in the P301S model was associated with a decrease in axonal pS202/pT205 tau (Lang et al., 2017). Furthermore, the authors report a decrease in the number of Gallyas silver positive cells suggesting a beneficial effect of chronic mild inflammation on aggregated tau. While the authors failed to assess the mechanism behind the decrease in tau phosphorylation, CIA induction was associated with an increase in pS202/pT205 tau which was engulfed by microglia, suggesting microglial phagocytosis as one mechanism for decreased tau propagation in the study. Together, this study is adding to a growing body of evidence that systemic inflammation can have a beneficial effect on tau pathology. Interestingly, the Lang *et al.*, 2017 study found P301S mice to have an increased incidence and severity of arthritis, suggesting tau pathology enhances rheumatoid arthritis. This highlights an issue with epidemiological evidence, is systemic inflammation associated with rheumatoid arthritis causing AD (Azizieh et al., 2017) or is systemic inflammation associated with AD causing rheumatoid arthritis (Swardfager et al., 2010).

While only one study has attempted to understand the effect of CIA induced inflammation on tau pathology, a multitude of studies have assessed the effects of high fat diet on tau pathology. Only one study found a detrimental role of a high fat diet in the THY-Tau22 model on tau phosphorylation and could be due to increased insulin signalling which was selective in the genotype (Leboucher et al., 2013). Indeed, insulin has been shown to induce tau phosphorylation in primary cortical neurons (Lesort and Johnson, 2000). This again demonstrates the issue of using a model where systemic inflammation is the secondary pathology and the primary pathology can in itself affect tau phosphorylation. Furthermore, the Leboucher *et al.*, 2013 study reported that despite affecting tau phosphorylation, the high fat diet failed to affect tau aggregation. Moreover, the majority of studies failed to find an effect of high fat diet on tau phosphorylation in both Wt mice and models expressing tau pathology (Moroz et al., 2008, Becker et al., 2012, McNeilly et al., 2012, Knight et al., 2014). Finally, one study reported found Wt mice or mice expressing the ApoE4 allele and fed with a high fat diet saw a reduction in tau phosphorylation multiple sites (To et al., 2011). Despite Type-2 diabetes models being less than ideal for understanding systemic inflammatory alterations in AD, systemic inflammation induced by a high fat diet supports a role for mild inflammation not being a detrimental force on tau pathology.

This study was aimed at elucidating the effect of acute LPS administration on tau pathology which would confer to mild inflammation rather than the septic doses traditionally utilised in the literature. Especially at the lower doses, LPS resulted in rapid dephosphorylation at epitopes implicated in its microtubule affinity and propensity to aggregate. Furthermore, the proximity in tau dephosphorylation and LPS administration suggests a direct role for pro-inflammatory stimuli in reducing tau phosphorylation. To date, only two studies have assessed the effect of LPS on tau phosphorylation following an acute low dose of LPS, both of which were low powered and only involved Wt mice. The first, found multiple tau phosphorylation epitopes transiently increased following a 100 μg/kg LPS administration (Roe et al., 2011). However, there appears to be an injection effect on tau phosphorylation in the study and the lack of sham controls in the study make conclusions difficult. The second study found a 250 μg/kg LPS injection decrease the ratio of high molecular pT205:total tau 24 hours following administration (Pearson, 2014), supporting a role for low dose LPS reducing tau phosphorylation. Although, the extent of inflammation in the current study was not mild, it does suggest that tau phosphorylation induced by LPS only occurs at septic concentrations. Supporting a role for mild inflammation producing a beneficial effect on tau pathology, systemic inflammation induced by the CIA model not only reduces levels of tau phosphorylation but also stimulates clearance of its aggregates. Therefore, pro-inflammatory stimuli might not be as detrimental as originally thought, producing both beneficial and detrimental effects on tau pathology depending on its magnitude.

Chapter 6 The Effect of LPS on Tau Pathology 24h Following Administration

6.1 Introduction

Chapter 5 aimed to clarify the role of systemic inflammation in AD by inducing systemic inflammation with an acute low *i.v.* dose of LPS and assessment of tau pathology during the period of maximal pro-inflammatory response at 4h. This time-point was chosen to ensure any alterations in tau pathology were a direct result of the pro-inflammatory response which might be continually occurring during the chronic inflammation present in AD. Contrary to studies which have utilised higher doses, systemic inflammation was found to cause tau dephosphorylation at both pS202 and pS396/404 epitopes, suggesting a positive role for inflammation on tau pathology. This chapter was aimed at furthering this observation by assessing tau pathology 24h following LPS administration. This would give the opportunity slower effects of systemic inflammation on tau pathology to manifest and enable a holistic view of the effect of inflammation on tau pathology.

The LPS-induced acute inflammatory response is a dynamic balance between pro- and anti-inflammatory mediators and can be classified into two phases; the pro-inflammatory phase and the resolution phase. The pro-inflammatory phase compises of mediators that fall into two categories – those which are pre-synthesised and released immediately and those which require *de novo* synthesis. A few mediators such as TNFα are pre-synthesised (Gordon and Galli, 1991) and LPS induces their rapid release causing a peak in its serum concentration 2h following administration (Qin et al., 2007, Biesmans et al., 2013, Erickson and Banks, 2011, Srinivasan et al., 2010), although elevated concentrations are observed past this peak due to the additional *de novo* synthesis (Gordon and Galli, 1991). However, most pro-inflammatory mediators, such as IL-6, require *de novo* synthesis following LPS administration and peak in serum concentration 4-6 hours post-LPS administration (Biesmans et al., 2013, Srinivasan et al., 2010, Erickson and Banks, 2011). This is the point at which the pro-inflammatory response is at its greatest with the expression of pro-inflammatory mediators returning to negligible levels within 24h (Qin et al., 2007, Biesmans et al., 2013, Erickson and Banks, 2011, Srinivasan et al., 2010). The second phase of the LPS-induced inflammatory response is anti-inflammatory and results in the resolution of the noxious pro-inflammatory response. Despite the resolution phase occurring subsequently to the pro-inflammatory phase, many of its mediators actually peak in their

serum concentration at a similar time as pro-inflammatory mediators which require *de novo* synthesis. For example, the prototypical anti-inflammatory cytokine: IL-10 peaks in its serum concentration around 4h, but unlike pro-inflammatory mediators, remains elevated past 24h (Qin et al., 2007, Biesmans et al., 2013, Erickson and Banks, 2011, Srinivasan et al., 2010). In an interesting attempt to summarise the dynamics of the LPS-induced inflammatory response, Srinivasan *et al.,* 2010 averaged relative changes in 23 cytokines following a 3.3 mg/kg LPS injection in mice. The authors report that average changes in pro-inflammatory mediators peaked at 4h post administration and had almost returned to basal levels by the last time-point at 18h. However, the average changes in anti-inflammatory mediators peaked within 2h, but were found to remain highly elevated until 18h. The initial anti-inflammatory response is important for curtailing the pro-inflammatory phase and initiating its resolution. The overall nature of the inflammatory response is dictated as the balance between pro- and anti-inflammatory responses. During the initial phase, the induction of pro-inflammatory mediators outweighs the induction of anti-inflammatory mediators. As the time progresses to around 24h post-administration, the concentration of pro-inflammatory mediators rapidly drops, while the concentration of anti-inflammatory mediators remains relatively stable (Sugimoto et al., 2016, Srinivasan et al., 2010). This tips the response to anti-inflammatory and resolves the pro-inflammatory response. Figure 6.1 represents the hypothetical temporal concentration of pro-inflammatory cytokines following LPS administration based off Srinivasan *et al.*, 2010.

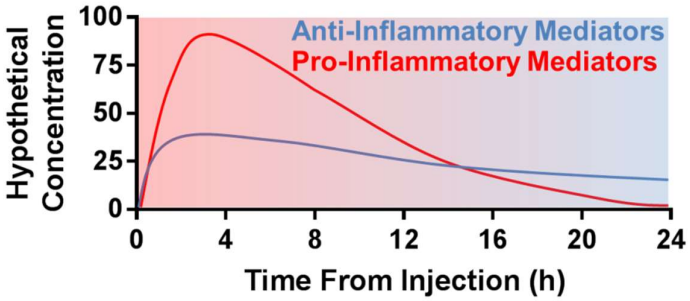

Figure 6.1: A summary of the hypothetical average inflammatory responses following acute LPS administration. The initial phase of the response is pro-inflammatory and is represented by a background red colour. As the concentration of pro-inflammatory mediators diminishes and the concentration of anti-inflammatory mediators remains stable, the inflammatory response is resolved. Figure drawn by author and based off Srinivasan et al., 2010.

The current chapter was aimed at understanding whether the effects of LPS on tau pathology persisted until after the expected resolution of the pro-inflammatory response. This chapter focussed on hTau/mTau$^{+/-}$ mice due to their altered isoform ratio and greatest susceptibility to LPS-induced tau dephosphorylation. Furthermore, only the 250 µg/kg dose

was utilised in the current chapter because LPS produced the strongest effects on tau phosphorylation at this dose. A 24h period between LPS administration and culling was chosen as this would allow the pro-inflammatory response to subside while permitting lasting pathological alterations to persist and enable slower effects of inflammation on tau pathology to occur. In addition to assessment of tau pathological features, food burrowing was assessed following LPS administration to assess whether LPS affects daily activity which is dependent of hippocampal integrity (Deacon, 2006). Furthermore, the spontaneous alternation task was conducted to assess whether the sickness syndrome persisted and spatial working memory (Hughes, 2004).

6.2 Study design

Experimental cohort 6 was utilised for the current protocol which comprised of 59, experimentally naïve, 3 month old male Wt, mTau$^{+/-}$ and hTau/mTau$^{+/-}$ mice. The behavioural protocol was adapted from Chapter 5 group to include a baseline assessment and lasted 9 days and is depicted in Figure 6.2. Due to the differences in protocols, the data was separated into two chapters. The mice were given a grouped food burrowing practise from day 1 to 4 where a 50g food jar was placed within the cage on day 1 and removed on day 4 without refilling. The extended food burrowing practice was chosen to improve habituation to the food jar compared with the 4h time point and has been found to increase burrowing behaviour in house. Following the practise, mice were singly housed and a baseline food burrowing test was conducted. The following morning, mice were returned to their home cage and given a spontaneous alternation baseline assessment. On the 8th day, mice were administered with either PBS or 250 µg/kg LPS (*i.v.*) and given a food burrowing test overnight. Only Wt, mTau$^{+/-}$ and hTau/mTau$^{+/-}$ mice were utilised for this section (n=9-10/group). 24h following treatment, mice were given a spontaneous alternation test immediately followed by culling. Bodyweights were recorded on the treatment and culling days and were analysed for statistical significance using a two-way repeated measures ANOVA approach followed by planned comparisons where appropriate. Due to worsening of mice allergies, the last behavioural batch was conducted by Dr David Watson and Mrs Clare Spicer.

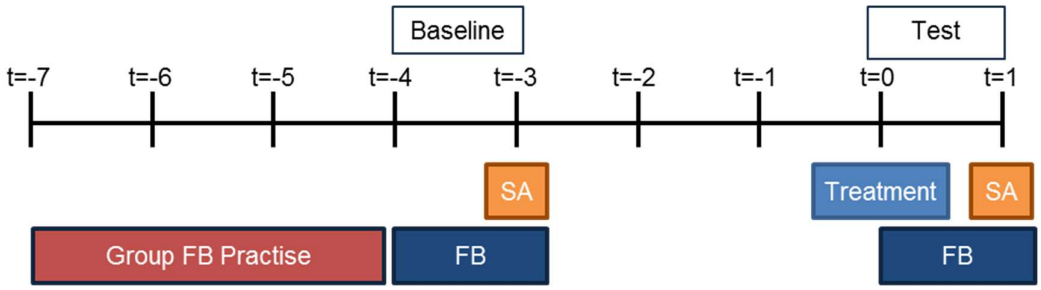

Figure 6.2: Timeline of study: Mice were given a group food burrowing (FB) practice for 4 days followed by a single housed baseline FB test overnight. The following morning, mice were returned to their home cage and given a baseline spontaneous alternation (SA) test. At t=0, mice were treated with either PBS or 250 µg/kg LPS and given an overnight FB test. 24h following treatment, mice were given a SA test followed by culling.

6.3 Results

6.3.1 LPS-induced behavioural suppression persists 24h following administration

As expected, two way repeated measures ANOVA analysis revealed that LPS induced a reduction in bodyweight 24h following LPS administration, indicated by an overall treatment effect [$F_{(1,58)}$ = 193.13, p < 0.001], but no signficant genotype x treatment interaction was observed [$F_{(2,57)}$ = 0.61, p = 0.0918]. Post hoc analysis showed that all genotypes significantly weighed less following LPS administration (p < 0.001, Figure 6.3A). Again demonstrating impaired burrowing behaviour, hTau/mTau$^{+/-}$ mice burrowed less food during the baseline session. Two way repeated measures ANOVA analysis revealed that food burrowing was impaired following LPS administration, indicated by a significant treatment x trial interaction [$F_{(1,58)}$ = 48.02, p < 0.001] but no genotype x treatment x trial interaction [$F_{(2,57)}$ = 0.15, p = 0.861]. Again, post hoc analysis revealed that all genotypes significantly burrowed less food following LPS administration (Figure 6.3B). As with the initial phenotype in Chapter 4, hTau/mtau$^{+/-}$ mice burrowed less than Wt and mTau$^{+/-}$ mice. Interestingly, there was an increase in performance among PBS treated hTau/mtau$^{+/-}$ mice between the baseline and test sessions. This is likely due to the baseline session acting as an additional practice session and enabling hTau/mtau$^{+/-}$ mice to increase their performance in the test session. This phenomenon was not observed in Wt and mtau$^{+/-}$ mice, possibly due to their higher baseline performance in the task. Two way repeated measures ANOVA analysis showed that there was a significant overall effect of session [$F_{(1,58)}$ = 122.07, p < 0.001] on locomotor activity in the spontaneous alteration task, with post-hoc analysis revealing all groups were more immobile in the test session (P < 0.05). The decrease in locomotor activity between sessions is expected as the maze is less novel. LPS further depressed locomotor activity, indicated by a session x treatment interaction [$F_{(1,58)}$ = 8.07, p = 0.0064], signifying

animals were still recovering from the LPS-induced sickness syndrome (Figure 6.3C). There were no significant differences in the alternation rate following LPS administration and all groups significantly alternated above chance level (Figure 6.3D, $p < 0.05$)

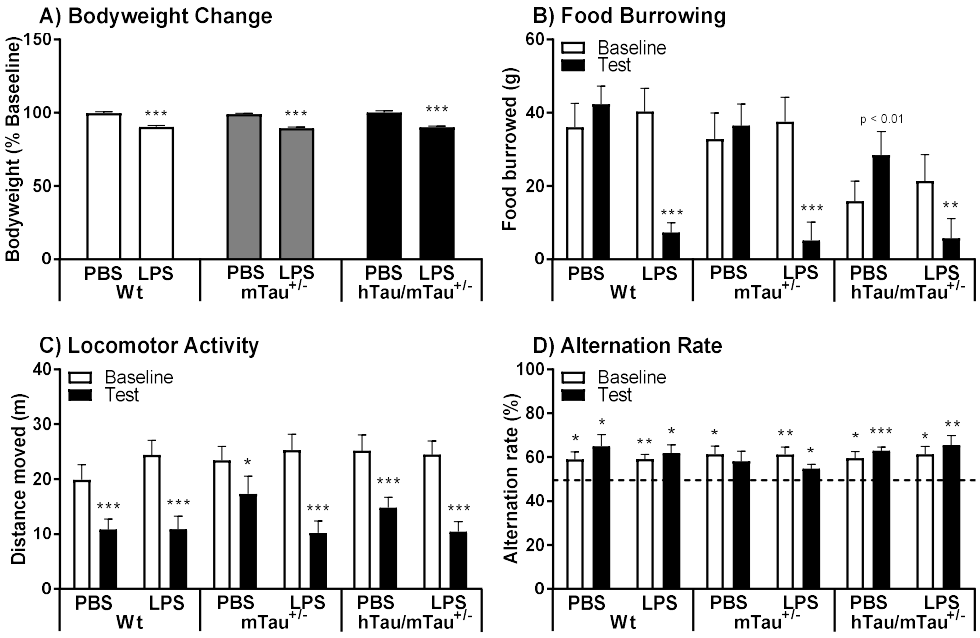

Figure 6.3: LPS-induced behavioural suppression persisted at 24h following administration in 3 month old male hTau/mTau$^{+/-}$ mice. Bars represent mean ±SEM. ***A)*** *LPS similarly decreased bodyweights in all genotypes.* ***B)*** *hTau/mtau$^{+/-}$ mice were impaired in the food burrowing task. LPS strongly inhibited food burrowing in all genotypes.* ***C)*** *While all groups were less mobile in the second session of the spontaneous alternation task, LPS further suppressed locomotor activity, indicating impairments in the food burrowing tasks were due to the LPS-induced sickness syndrome.* ***D)*** *LPS failed to affect spatial working memory with all groups significantly alternating. A-C significance vs. vehicle, D One-sample t-test: * p < 0.05, ** p < 0.01 and *** p <0.001 against vehicle (n=9-10/group).*

6.3.2 The LPS-induced inflammatory response subsided 24h following administration

To assess whether the inflammatory response had resolved 24h following administration, concentrations of select pro-inflammatory and anti-inflammatory cytokines in serum were measured by multiplex analysis. Two-way ANOVA analysis revealed that at 24h there was no overall significant effect of LPS on the concentration of IL-1β ([$F_{(1,56)}$) = 3.12, p = 0.0832], Figure 6.4A). However, there was a significant treatment effect in serum IL-6 concentrations [$F_{(1,51)}$ = 156.43, p < 0.001] but no genotype x treatment interaction [$F_{(2,51)}$ = 0.28, p = 0.754]. Post hoc analysis revealed all genotypes had significantly higher concentrations of IL-6 in serum 24h following LPS administration (p < 0.001, Figure 6.4B). Compared to IL-6 concentrations at 4h, there is an order of magnitude difference between

the two, indicating at 24h IL-6 induction was almost completely ablated. There was no significant treatment effect on either INFγ [$F_{(1,51)}$ = 0.26, p = 0.612] and TNFα [$F_{(1,51)}$ = 2.89, p = 0.0] levels ar 24h following LPS administration (Figure 6.4C-D). This is opposed to circulating IL-10 concentrations, which remained elevated at 24h post-injection indicated by a significant treatment effect [$F_{(1,51)}$ = 185.89, p < 0.001]. There was no signficant genotype x treatment interaction on circulating IL-10 concentration [$F_{(2,51)}$ = 1.93, p = 0.156] with post hoc analysis revealing all three genotypes having elevated levels (p < 0.001, Figure 6.4E). Unlike IL-6, IL-10 concentrations remained relatively comparable to their 4h time-point suggesting the inflammatory response had shifted to anti-inflammatory.

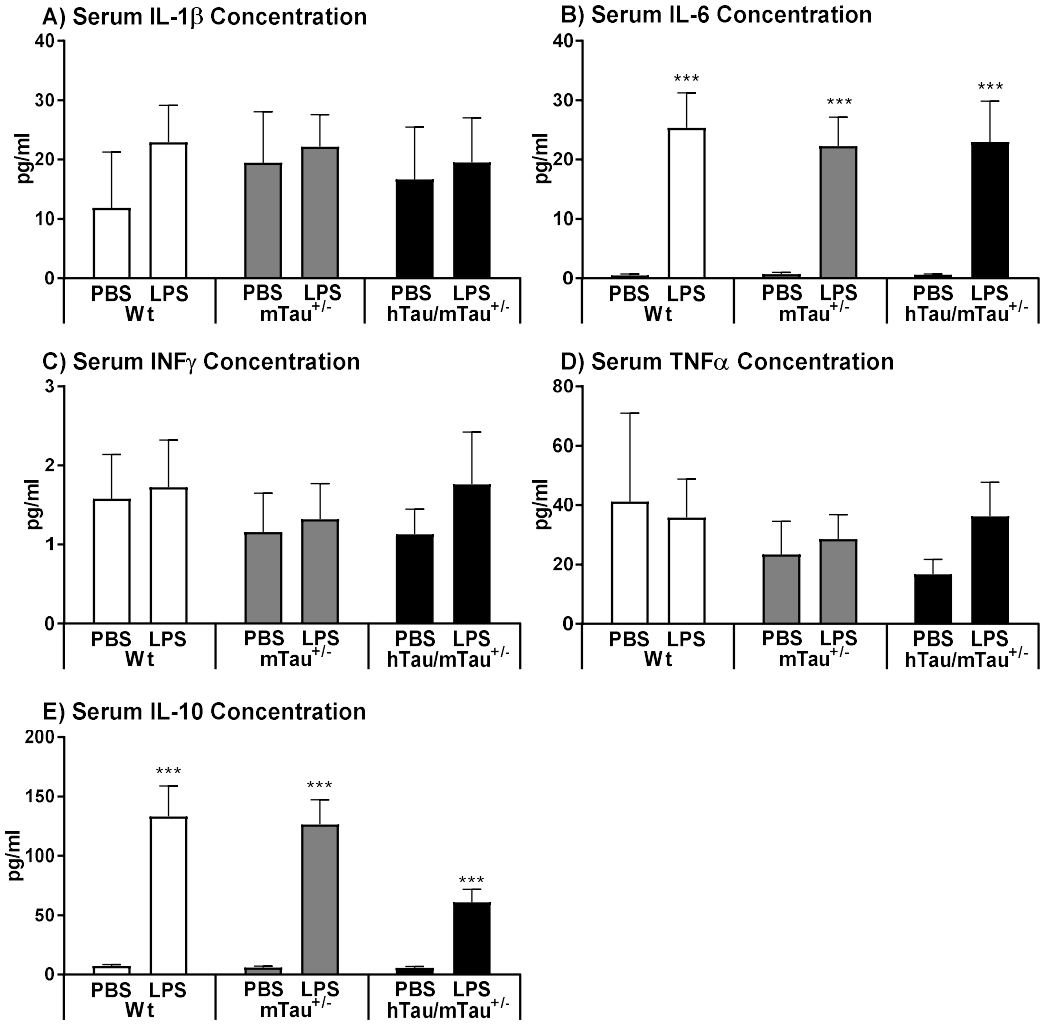

*Figure 6.4: The pro-inflammatory response had subsided by 24h in 3 month old male hTau/mTau$^{+/-}$ mice. Bars represent mean ± SEM (n=9-10/group). Out of the pro-inflammatory cytokines assessed only IL-6 remained significantly elevated at 24h, albeit at a greatly reduced concentration compared to its levels at 4h. In comparison, IL-10 concentrations remained significantly elevated, indicating a shift to the anti-inflammatory response. Significance vs. vehicle; *** p < 0.001.*

To determine whether the LPS-induced sickness syndrome could be the source of the impairments in the food burrowing task, a Pearson's product moment correlation between food burrowed in the test session and IL-6 concentration was conducted. Systemic IL-6 concentration was found to have a strong negative association with the amount of food burrowed in the test session in Wt, mTau$^{+/-}$ and hTau/mTau$^{+/-}$ mice ($r = -0.704$, $p < 0.001$, $r = -0.611$, $p < 0.01$ and $r = -0.834$, $p < 0.001$ respectively). This suggests that indeed the confounding rather than cognitive effects of LPS are the source of the impairment in the food burrowing task (Figure 6.5).

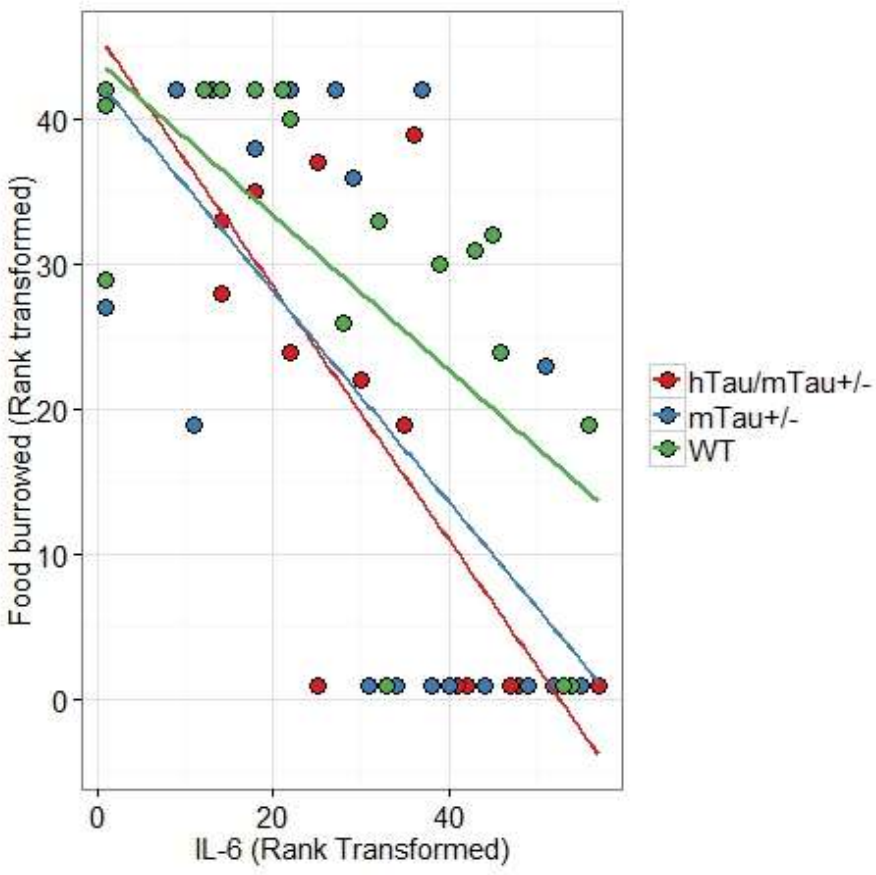

Figure 6.5 Pearson's product moment correlation between food burrowed in the test session (rank transformed) and IL-6 (rank transformed) showed a strong correlation (n=19-20/genotype).

6.3.3 Significant microglial activation 24h following LPS administration

To determine whether the central inflammatory response following systemic LPS administration persisted 24h following administration, Iba1 quantification of microglia was carried out (Figure 6.6A). Similar to what was observed at 4h, there was no significant

treatment effect in the number of microglia [$F_{(1,21)}$ = 1.34, p = 0.263] as revealed by two-way ANOVA analysis, although there was a trend towards an increase in microglial number following LPS administration (Figure 6.6B). However, two way ANOVA analysis indicated there was an overall treatment effect in the in the size of microglial soma [$F_{(1,21)}$ = 18.03, p = 0.001]. Post hoc analysis showed that both Wt and hTau/mTau$^{+/-}$ mice exhibited a significant enlargement in microglial soma size (Figure 6.6C, p < 0.05), which again suggested crosstalk between the peripheral and central inflammatory response.

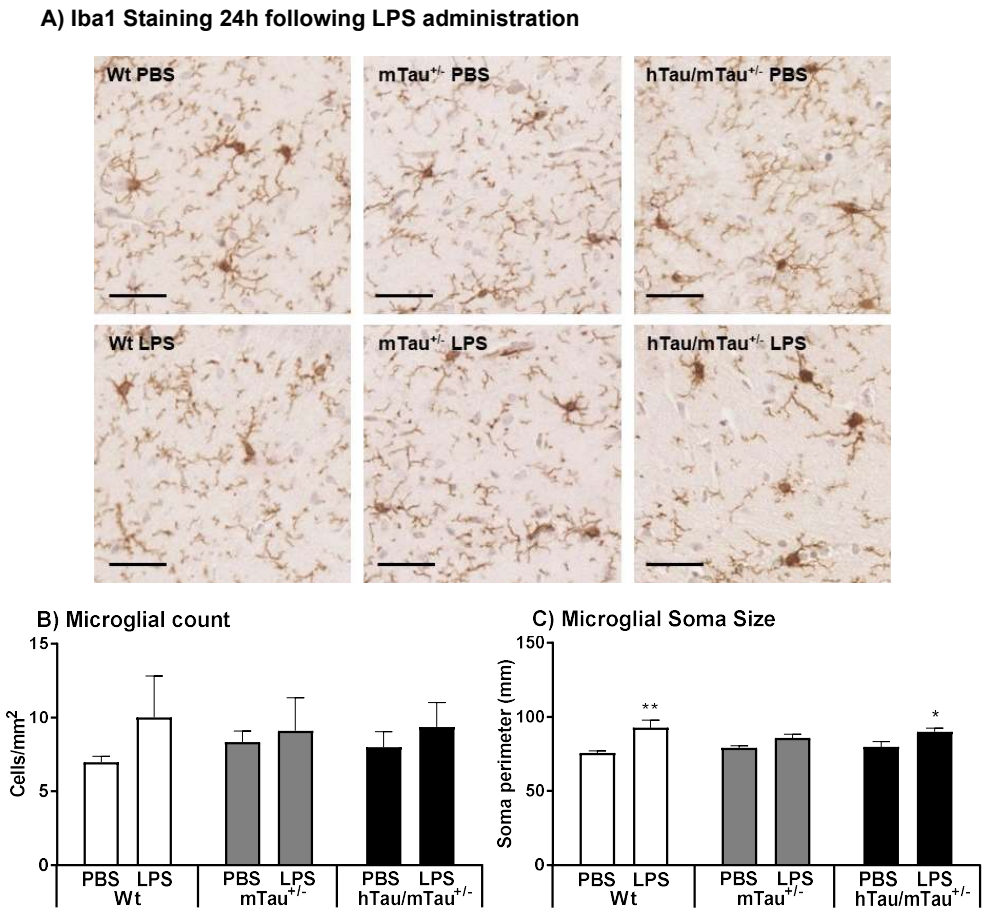

Figure 6.6: LPS resulted in significant microglial activation 24h following administration in 3 month old male hTau/mTau$^{+/-}$ mice. Bars represent mean ± SEM (n=3-4/genotype). ***A)*** *Immunohistochemistry sections of Iba1 staining.* ***B)*** *There was a trend for an increase in the number of microglial cells following LPS administration.* ***C)*** *LPS resulted in increased Soma size indicating significant microglial activation had occurred. Significance vs. vehicle; * p < 0.05 and ** p < 0.01. Scale bar represents 50 µm.*

6.3.4 LPS-induced tau dephosphorylating persist until 24h following LPS administration

The primary objective of this chapter was to determine whether the tau dephosphorylating effects of mild inflammation persisted until after the resolution of the

systemic inflammatory response. To this end, hippocampal extracts were assessed for phosphorylated tau by western blotting. Two way ANOVA analysis revealed that there was a significant overall effect of treatment on levels of tau phosphorylated at the pS202 epitope [$F_{(1,58)}$ = 4.8, p < 0.05]. Furthermore, there was a trend for a genotype x treatment interaction [$F_{(2,57)}$ = 2.81, p = 0.0697]. Post hoc analysis found pS202 levels were significantly decreased in hTau/mTau$^{+/-}$ mice following LPS administration (Figure 6.7B, p < 0.01). Two way ANOVA analysis demonstrated that there was a significant overall treatment effect in the level of pS396/404 tau [$F_{(1,58)}$ = 6.75, p < 0.05]. Again, there was a trend for a genotype x treatment interaction [$F_{(2,57)}$ = 2.74, p = 0.0744]. Post hoc analysis revealed decreased levels of pS396/404 in hTau/mTau$^{+/-}$ mice treated with LPS (Figure 6.7C, p < 0.01). Finally, two way ANOVA analysis showed that there was no significant effect of treatment on total tau levels [$F_{(1,53)}$ = 0.07, p = 0.0791], indicating again that reduced levels of pS202 and pS396/404 are resulting from tau dephosphorylation rather than tau clearance (Figure 6.7D). Together, these results suggest that tau dephosphorylating effects of LPS persist even after resolution of the systemic pro-inflammatory response.

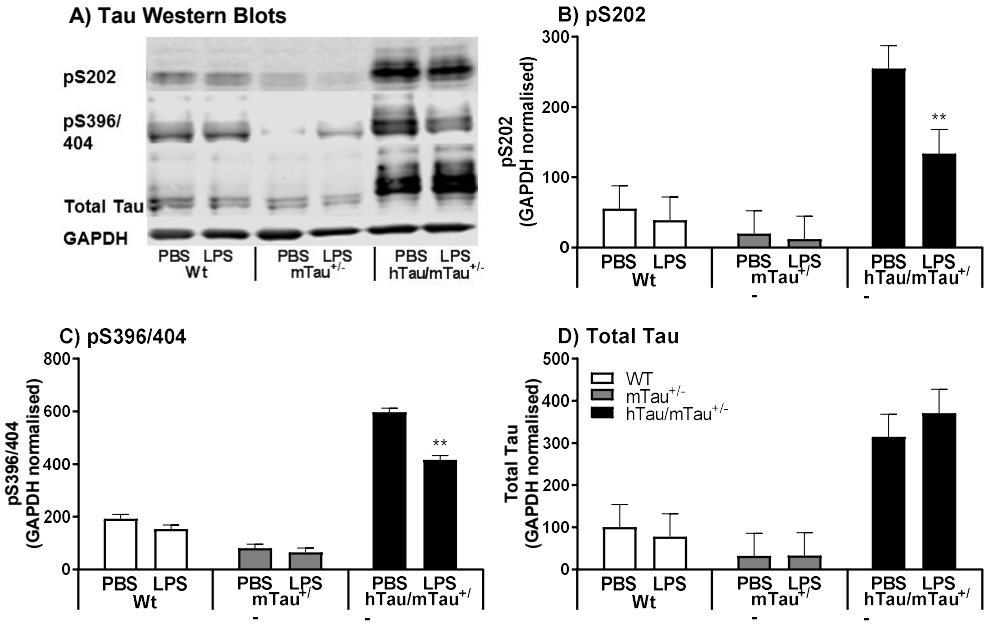

Figure 6.7: LPS-induced tau dephosphorylation persisted 24h following LPS administration in 3 month old male hTau/mTau$^{+/-}$ mice. Bars represent mean ± SEM (n=9-10/group). ***A)*** *Representative tau western blots. LPS reduced both* ***B)*** *pS202 and* ***C)*** *pS396/404 levels while not affecting D) total tau levels. Significance vs.vehicle; ** p < 0.01.*

6.3.5 LPS did not affect hippocampal PP2A activity at 24h

To assess whether PP2A was the source of tau dephosphorylation, inactive and total $PP2A_C$ were assessed by western blotting. Two way ANOVA analysis revealed that there was no significant overall genotype effect in the ratio of inactive $PP2A_C$:total $PP2a_C$ [$F_{(1,58)} = 0.25$, p = 0.619]. However the trend of increased PP2A activation following LPS administration which was observed in Chapter 5 was completely obliterated (Figure 6.8B). This could support that the pro-inflammatory response is required for PP2A induction and, that as the inflammatory response is resolved, PP2A levels return to basal levels. Again, further validation of this is required in a dedicated PP2A assay.

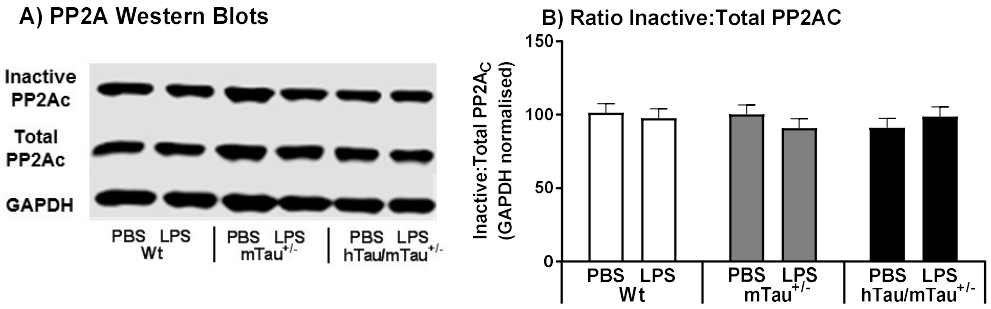

Figure 6.8: LPS did not affect PP2A activation 24h following administration in 3 month old male hTau/mTau$^{+/-}$ mice. Bars represent mean ± SEM (n=9-10/group). ***A)*** *Representative $PP2A_C$ blots.* ***B)*** *LPS failed to induce PP2A activation.*

6.3.6 LPS did not induce tau aggregation

To determine whether LPS induced tau aggregation at 24h, sarkosyl insoluble fractions were assessed by western blotting. S1 fractions were positive for both total and pS396/404 tau (Figure 6.9A). Despite not inducing tau aggregation at 4h, tau aggregation is likely a slower process compared to its phosphorylation and could require a later time point. However, as with the 4h time point, LPS failed to induce tau aggregation in all genotypes as indicated by lack of sarkosyl insoluble tau (Figure 6.9B).

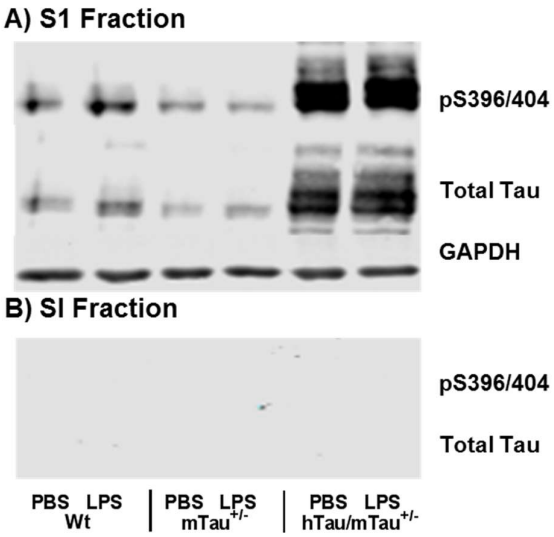

*Figure 6.9: LPS did not induce tau aggregation 24h following administration in 3 month old male hTau/mTau$^{+/-}$ mice. Representative western blots of **A)** S1 and **B)** SI fractions.*

6.4 Discussion

The previous chapter was aimed at understanding the effects of acute mild inflammation on tau pathology. LPS was utilised as a model of systemic inflammation because not only is it found at increased concentration in circulation of AD patients (Zhang et al., 2009c), but acute systemic LPS stimulation in mice creates a similar microglial phenotype to those observed in AD (Friedman et al., 2018). While prolonged inflammation is more akin to the underlying inflammation which is observed in AD, LPS is unsuitable for assessing the effect of chronic inflammation on tau pathology due to the rapid development of tolerance (Frankenberger et al., 1995, Medvedev et al., 2002, Ziegler-Heitbrock, 1995) which is not apparent in AD (Magaki et al., 2007, Rosenberg et al., 2009, Vida et al., 2017, Ciaramella et al., 2010). As chronic underlying inflammation in AD results in the sustained upregulation of pro-inflammatory inflammatory mediators, it is important to assess the effect on tau pathology during the pro-inflammatory phase. In the previous chapter, LPS-induced systemic inflammation was found to cause tau dephosphorylation at epitopes associated with affecting its propensity to bind to microtubules and to aggregate during the pro-inflammatory phase.

The current chapter was aimed at furthering this observation by assessing whether the tau dephosphorylating effects of systemic inflammation persisted until after the resolution of the pro-inflammatory response. The 24h time-point was chosen as it would enable resolution of the pro-inflammatory response and allow for the assessment of any non-immediate effects that the inflammatory response might have on tau pathology.

Indicating that the pro-inflammatory response had recovered in the current study, almost all pro-inflammatory cytokines assessed had returned to baseline 24h following LPS administration. Only IL-6 remained slightly elevated at 24h with concentrations albeit at around 500x lower concentration compared to at their 4h concentrations. In comparison, IL-10 concentrations were only slightly reduced in comparison to their concentrations at 4h. Taken together, the pro-inflammatory response by in large appears to have resolved 24h following LPS administration. Despite the apparent resolution of the pro-inflammatory response, the behavioural parameters measured appeared to be confounded. For example, the food burrowing task was inhibited in all three genotypes, with the complete inhibition observed indicative of impairments due to sickness effects rather than hippocampal suppression. The majority of burrowing in the task has been reported to occur within the first 2h of the task (Deacon, 2009). Therefore, as the food burrowing task was conducted roughly 7 hours following LPS administration, mice are still heavily under the influence of the LPS-induced sickness syndrome during this period, resulting in the task being confounded. Supporting the food burrowing task being confounded, LPS administration marginally reduced locomotor activity in the spontaneous alternation task. Taken together with the concentration of inflammatory mediators, the persistence of LPS-induced behavioural suppression appears to be distinct from the systemic pro-inflammatory response. This is not surprising, as the data from the CLAMS^{TM} study in Chapter 3 have found mice suffering from the LPS-sickness syndrome to not eat or drink and have profound sleep disturbances during this period. Consequently, while recovery in the inflammatory aspect of sickness syndrome may occur within 24h, recovery in the physical aspects is slower which according to Chapter 3 occurs within roughly 48h.

The main objective of this chapter was to determine whether LPS-induced tau dephosphorylation persisted following the resolution of the pro-inflammatory response. Both pS202 and pS396/pS404 levels continued to be lower in LPS treated hTau/mTau$^{+/-}$ mice even after the resolution of the inflammatory response at 24h. As with the 4h time-point, there were no alterations in the levels of total tau, suggesting tau dephosphorylation as the source of decreased levels of phosphorylated tau. As the later time-point would allow for any non-immediate effects of inflammation to affect tau phosphorylation, these results comprehensively indicate that low doses of LPS decrease tau phosphorylation. Furthermore, similar time-points between this study and the Bhaskar *et al.,* 2010 study suggests that the contrasting effects on tau phosphorylation between the studies is indeed the result of difference in doses. This again supports that severe inflammation is required to induce tau

pathology and is substantiated in the literature where acute low doses of LPS appear to decrease tau phosphorylation (Pearson, 2014) while septic doses induce tau phosphorylation (Bhaskar et al., 2010, Liu et al., 2016a). However, similar to the 4h time-point, PP2A activity did not significantly change with LPS administration. The amelioration of the trend for increased PP2A activation following LPS administration in Chapter 5 at the 24h time-point suggests that the pro-inflammatory response is driving potential alterations in PP2A activity leading to tau dephosphorylation. Further validation with a dedicated PP2A assays is required as well as assessment of kinase activity to determine the exact mechanism of LPS-induced tau dephosphorylation.

In addition to assessing tau phosphorylation in the current chapter, tau aggregation was assessed 24h following LPS administration. Similar to the previous chapter, only induction of tau aggregation could occur due to hTau/mTau$^{+/-}$ mice not expressing aggregated tau at the age utilised in the current study. Nevertheless, if LPS were to induce tau aggregation, it is likely to be a slower process than its effect on tau phosphorylation and could require a later time-point. However, there was no evidence of sarkosyl insoluble tau 24h following LPS administration, indicating LPS did not induce tau aggregation. Further studies are required at a later age when aggregated tau is present to determine whether the tau dephosphorylating effects of mild inflammation decrease its aggregation. However, any potential effect of acute mild inflammation on tau aggregation is likely to be an effect separate from its tau dephosphorylation effects. Instead decreases in tau aggregation would likely be the result of increased tangle clearance, with LPS well known to induce autophagy (Xu et al., 2007) – a major clearance mechanism of aggregated tau (Wang et al., 2010). This is due to any decrease in tau aggregation caused by tau dephosphorylation would be due to slowing the generation of tau aggregates through promoting its binding to microtubules and reducing the levels of free tau. This would likely require sustained tau dephosphorylation which would require a method of chronically inducing mild inflammation such as the CIA model of rheumatoid arthritis.

To determine whether a low systematic dose of LPS induced a central inflammatory response, Iba1 staining of microglia was conducted. Upon activation microglia undergo two processes easily observable by IHC – proliferation (Hovens et al., 2014) and shortening of processes while increasing in soma size (Kozlowski and Weimer, 2012). In the current chapter, there was evidence for a single non-septic dose of LPS to increase microglial soma

size, indicating microglia activation had occurred. This suggests that modulation of the neuroinflammatory environment could be driving the LPS-induced tau dephosphorylation.

A limitation of the current study was the inability to assess whether tau dephosphorylation results in decreased tau aggregation. Despite this, the novel role for systemic inflammation on tau phosphorylation has implications for the contribution of inflammation in AD, irrespective of whether it has an effect on tau aggregation. While aggregated tau species and in particular tau oligomers have been implicated as the most pathogenic species in AD (Ward et al., 2012), the hyperphosphorylation of tau and hyperphosphorylated tau species have pathogenic effects separate from simply initiating the tau aggregation process. For example, tau hyperphosphorylation results in loss of affinity for microtubules leading to decreased microtubule function, impaired organelle transport, fragmentation of the Golgi apparatus and general cellular stress (Liazoghli et al., 2005, Trinczek et al., 1999, Mandelkow et al., 2003). Furthermore, the resulting increase in free tau within the cell has been implicated in both pre- and post-synaptic dysfunction and may contribute to excitotoxicity (Mondragon-Rodriguez et al., 2012, Usardi et al., 2011). Therefore, the chronic mild inflammatory environment in AD could potentially produce beneficial through reducing tau hyperphosphorylation. Further studies will have to assess the implication of LPS-induced tau dephosphorylation both at a physiological level and its effect on cognitive function following the resolution of the physical effects of the LPS-induced sickness syndrome.

Chapter 7 General Discussion

7.1 Review of aims and hypothesis

Systemic inflammation is seen as a key mechanistic driver of early tau pathology based largely on findings from LPS administration in animal models. However, these models do not necessarily model inflammation in a physiologically relevant manner to AD, confusing as to the exact role of systemic inflammation in AD. This thesis was aimed at understanding the pathogenic and symptomatic interaction between systemic inflammation induced by a low dose of LPS and tau pathology. In order to do this, a cognition task which would not be confounded by the LPS-induced sickness syndrome, as well as a method for accurately describing the neuroinflammatory response, needed to be developed. Furthermore, a tau model which is pathologically relevant to AD was required. The hTau model is largely seen as the most AD relevant tau model due to the development of tau pathology without the need for mutations in the tau gene. However, a tau isoform imbalance exists in the model which is not representative of AD (Andorfer et al., 2003). Furthermore, in-house observations have suggested that hTau mice are prone to systemic pathologies due to the ablation of the mTau gene. To improve the tau isoform ratio and avert the systemic pathologies, an altered hTau model partially expressing mTau was validated. Finally, the thesis aimed to comprehensively evaluate the effect of systemic inflammation induced by a low dose of LPS on tau pathology through assessing its effects during the initial pro-inflammatory phase at 4h and following its resolution at 24h. We hypothesised that based on the aetiological evidence associating systemic inflammation with AD, the higher levels of endogenous LPS in AD patients and the evidence from previous LPS studies in animal models that systemic inflammation will similarly augment tau pathology under the current experimental conditions.

7.2 Summary of key findings:

A contextual fear conditioning paradigm involving a pre-exposure to the context was developed and although, contextual fear was expressed, it failed to show LPS-induced impairments. This was potentially due to issues with the protocol which likely meant mice learnt the context during the conditioning session instead of needing to rely on the pre-exposure session. The development of strong allergies to mice meant further extensive behavioural studies could not be conducted and the objective was abandoned. Furthermore, an *ex vivo* MAS NMR technique was validated which had similar resolution to *in vivo* NMR and reproduced observations in the literature that LPS increases levels of NAA. Despite

failing to achieve the resolution originally hoped for, this finding again highlights the value *ex vivo* MAS NMR as a translatable tool for understanding metabolic changes in animal models.

Potential issues have been highlighted in the hTau model such as a tau isoform ratio imbalance (Andorfer et al., 2003) and the development of systemic pathologies which have been observed in-house. Chapter 4 was aimed at validating an altered hTau model involving the partial expression of mTau. As expected, an isoform ratio more akin to those observed in AD was exhibited in hTau/mTau$^{+/-}$ mice. Necroscopies conducted suggest that the model does not develop systemic pathologies. Furthermore, hTau/mTau$^{+/-}$ mice had augmented levels of phosphorylated tau at both the pS202 and pS396/404 epitopes which outweighed any increases observed in total tau levels. Further investigations are required to determine the exact mechanisms behind increased tau phosphorylation within the model. Despite overt tau phosphorylation, neither hTau/mTau$^{+/-}$ nor hTau/mTau$^{-/-}$ mice exhibited tau aggregation by 9 months of age. Behaviourally, both hTau genotypes were similar, expressing early food burrowing impairments and decreased bodyweight. Together, these results indicate that irrespective of tau aggregation, hTau/mTau$^{+/-}$ mice are an attractive model for assessing early pathological changes in tau pathology.

Overriding evidence exists in the literature that high doses of LPS induce tau phosphorylation in tau models through the upregulation of various kinases (Kitazawa et al., 2005, Sy et al., 2011, Bhaskar et al., 2010). However, limited evidence suggests that lower doses of LPS potentially have an inverse effect through inducing tau dephosphorylation (Pearson, 2014). To determine whether severe inflammation is required to induce tau phosphorylation, the current thesis aimed to understand the effect of mild systemic inflammation on tau pathology induced by low dose LPS. However, a limitation of the current study was that LPS did not induce mild inflammation; instead the level of systemic inflammation expressed was still too strong to be compared to that observed in AD (Swardfager et al., 2010). Despite this, and contrary to our hypothesis, results from this thesis support a role for lower doses of LPS in tau dephosphorylation. A single 100, 250 or 330µg/kg dose of LPS rapidly induced tau dephosphorylation at the pS202 and pS396/404 epitopes in hTau/mTau$^{+/-}$ mice during the initial pro-inflammatory response at 4h. This appeared to be a selective effect in the genotype, with hTau/mTau$^{-/-}$ mice only seeing tau dephosphorylation with the 100 µg/kg dose exclusively at the pS202 epitope. This suggests that altering the isoform ratio resulted in increased susceptibility in hTau/mTau$^{+/-}$ mice. Moreover, the effect on pS202 appeared to be strongest at the lowest dose, which leads to

the hypothesis that low doses LPS result in tau dephosphorylation while high doses in phosphorylation. Furthermore, effects on tau phosphorylation persisted even after the resolution of the pro-inflammatory response at 24h, giving sufficient time for slower effects of inflammation on tau phosphorylation to manifest. Although not significant, PP2A activity appeared to increase 4h following administration and return toward baseline levels as the pro-inflammatory response subsided, possibly providing a potential mechanism which requires further validation. Together, these results suggest that low doses of LPS result in rapid tau dephosphorylation which could have implications for our understanding of how inflammation interacts with tau pathology in AD.

7.3 Is LPS useful for modelling systemic inflammation in AD

Systemic LPS administration has been the most commonly utilised model for understanding the effect of systemic inflammation on tau pathology. Indeed, LPS does have high pathogenic relevance to AD. Circulating concentrations of LPS are increased in AD patients (Zhang et al., 2009c) and single cell RNAseq technology suggests acute, systemic, LPS administration results in a similar microglial phenotype compared to those which are found in AD (Friedman et al., 2018). However, there are limitations associated with utilising LPS to model systemic inflammation in AD. AD is associated with persistent systemic inflammation (Swardfager et al., 2010) and as such, chronic inflammatory models are more relevant. However, inducing chronic systemic inflammation with LPS is not possible due to the development of tolerance following repeated LPS administration. While initially LPS tolerance was thought to be resulting from receptor desensitisation (Medvedev et al., 2002, Medvedev et al., 2000), studies in tolerant murine macrophages demonstrate a change in phenotype to anti-inflammatory rather than receptor desensitisation (Foster et al., 2007). Supporting this, LPS tolerance in mice results in silencing of the pro-inflammatory response while promoting an anti-inflammatory one (Wysocka et al., 2001, Fensterheim et al., 2017). The shifting of phenotype instead of receptor desensitisation means that even increasing the dose does not avert tolerance (Fensterheim et al., 2017). Elevated levels of circulating LPS in AD (Zhang et al., 2009c) would suggest that a tolerant response might be more representative of AD. However, elevated levels of circulating cytokines in AD patients (Swardfager et al., 2010) coupled with a similar or exaggerated pro-inflammatory PMBC response to LPS from AD patients suggests LPS tolerance is not a feature of the disease (Vida et al., 2017, Ciaramella et al., 2010, Magaki et al., 2007, Rosenberg et al., 2009). Because of the issue of LPS tolerance, acute administration is more informative as to the contribution of systemic inflammation in AD but fails to mimic the chronic aspects of the disease.

A limitation of LPS in respect to AD which is apparent in this thesis is the strength of the systemic inflammatory response. Despite utilising low doses of LPS, the extent of the systemic inflammatory response was much greater compared to what is observed in AD. The cytokine with the highest concentration in the current study was IL-6 which had an average concentration across all genotypes of 2389 pg/ml following the 100 µg/kg dose at the 4h time-point. This compares to a level of around 2.3 pg/ml in AD patients (Wu et al., 2015). Even AD patients with a high concentration of IL-6 only have a circulating level of 26.8 pg/ml (Mooijaart et al., 2013). Therefore, the inflammatory response in the current study is an order of magnitude higher compared to the underlying mild inflammation which is expressed in AD. This raises an important question: what level of inflammation is mild inflammation in mice. Mice are less responsive to inflammatory stimuli than humans. In one study, a 250x greater LPS dose was required in order to achieve a similar induction of IL-6 in mice compared to humans (Copeland et al., 2005). Furthermore, in humans the concentration of IL-6 in severe sepsis, septic shock and during bacterial infection was reported to be 70, 126.3 and 85 pg/ml respectively (Lvovschi et al., 2011). Despite, the concentration of IL-6 in the current thesis being much higher than these levels, it is clear sepsis was not induced due to the rapid recovery from the LPS-induced sickness syndrome. Models of sepsis are associated with an IL-6 induction of around 300-30,000 ng/ml – much greater than the current study (Sasaki et al., 2017, Beurel and Jope, 2009, Soromou et al., 2014). Therefore, while the extent of systemic inflammation expressed in the current thesis was not mild, it is difficult to make exact comparisons between humans due to differences between mice and human immune systems. Not to mention, larger animals have a slower metabolic rate meaning higher doses are often required in murine models of pharmacological intervention (Nair and Jacob, 2016).

Nevertheless, the strength of the immune response and the failure to induce chronic inflammation associated with LPS administration means systemic inflammatory models which induce chronic mild inflammation may be more relevant to AD. Models which mimic peripheral inflammatory conditions and are associated with systemic inflammation provide an interesting approach to model chronic mild systemic inflammation in mice. Three peripheral inflammatory models which could serve such purpose were highlighted in this thesis: the ovalbumin model of asthma, the high fat diet model of Type 2 diabetes and the CIA model of rheumatoid arthritis. All three models are associated with chronic systemic inflammation which is truly mild (Srivastava et al., 2010, Kim et al., 2012, Amdekar et al., 2011, Tsubaki et al., 2015). Furthermore, all three models are risk factors for AD, increasing

their relevance (McGeer and Rogers, 1992, Eriksson et al., 2008, Arvanitakis et al., 2004) However, in a similar manner to asthma, and other atopic disorders, the ovalbumin model is associated with a predominant Th2 response which is characterised by high levels of IL-4 (Srivastava et al., 2010, Barnes, 2001). While IL-4 is associated with detrimental inflammatory effects in the periphery, it is thought to be protective in the CNS through shifting microglia to a, protective anti-inflammatory phenotype (Liu et al., 2016b). Furthermore, elevated immunoglobulin levels in the model, which share a similar molecular weight as tau, make the assessment of tau phosphorylation by western immunoblotting inappropriate (Sarlus et al., 2012, Bazin et al., 1974, Petry et al., 2014). Therefore, despite inducing chronic mild systemic inflammation, the ovalbumin model is not suitable for understanding the contribution of systemic inflammation to tau pathology.

The high fat diet model of Type 2 diabetes is similarly not desirable due to aberrations in insulin signalling. The high fat diet model is associated with insulin resistance which has been shown to induce tau phosphorylation in itself (Vagelatos and Eslick, 2013, El Khoury et al., 2014). Interestingly, insulin in itself has been reported to induce tau phosphorylation (Lesort and Johnson, 2000) suggesting a balance in insulin signalling is required. Nevertheless, confusion occurs in tau models fed with a high fat diet as to whether systemic inflammation or insulin signalling abnormalities are the source of pathological alterations. Instead, the most attractive model is the CIA model of rheumatoid arthritis. A major advantage of the model is that the primary pathology is limited to the joint affected, leaving purely systemic inflammation associated with the model to affect tau pathology. The only drawback of the model is its effect on locomotor activity which has the potential to confound behavioural tasks which rely on performance related behaviours (Hartog et al., 2009).

7.4 Can systemic inflammation be a beneficial to tau pathology?

Traditionally, systemic inflammation has been thought to augment tau phosphorylation largely based on findings from LPS administration in animal models. However, the doses of LPS utilised are simply too high to be modelling the underlying inflammatory response in AD. In the current thesis, the dose of LPS was lowered to determine whether milder inflammation would have a similar effect. Although, the level of systemic inflammation expressed was not as mild, it does provide use for comparing to the initial studies which found acute LPS administration to induce tau phosphorylation at high doses. Few studies have assessed lower doses of LPS on tau pathology. Although a limited

study, Pearson *et al.*, 2014 found a 250 µg/kg dose of LPS to decrease high molecular weight pT205 in Wt mice. Conversely, a 100 µg/kg dose of LPS was found to temporarily increase tau phosphorylation in Wt mice which subsided within 4h (Roe et al., 2011). However, the immediate induction of tau phosphorylation in the study suggests that the stress of injection could be inducing tau phosphorylation rather than the inflammatory response. It is therefore imperative to include a control group when conducting these sorts of studies. The results entailed in this thesis were the first to comprehensively assess the effect of an acute low-dose of systemically administered LPS on tau pathology. This provides a model where high doses of LPS result in tau phosphorylation while low doses result in dephosphorylation. Such a model is supported by the decrease in tau dephosphorylation as the dose of LPS was increased in the current study. There are a variety of potential mechanisms for tau dephosphorylation following LPS administration including: activation of PP2A as well as inhibition of CK1, GSK3β and CDK5 (Figure 7.1) – all of which are known to affect the pS202 and pS396 sites (Martin et al., 2013). However, due to the wide reaching affects of inflammatory stimuli, the source of tau dephosphorylation could just as likely be down to an indirect mechanism.

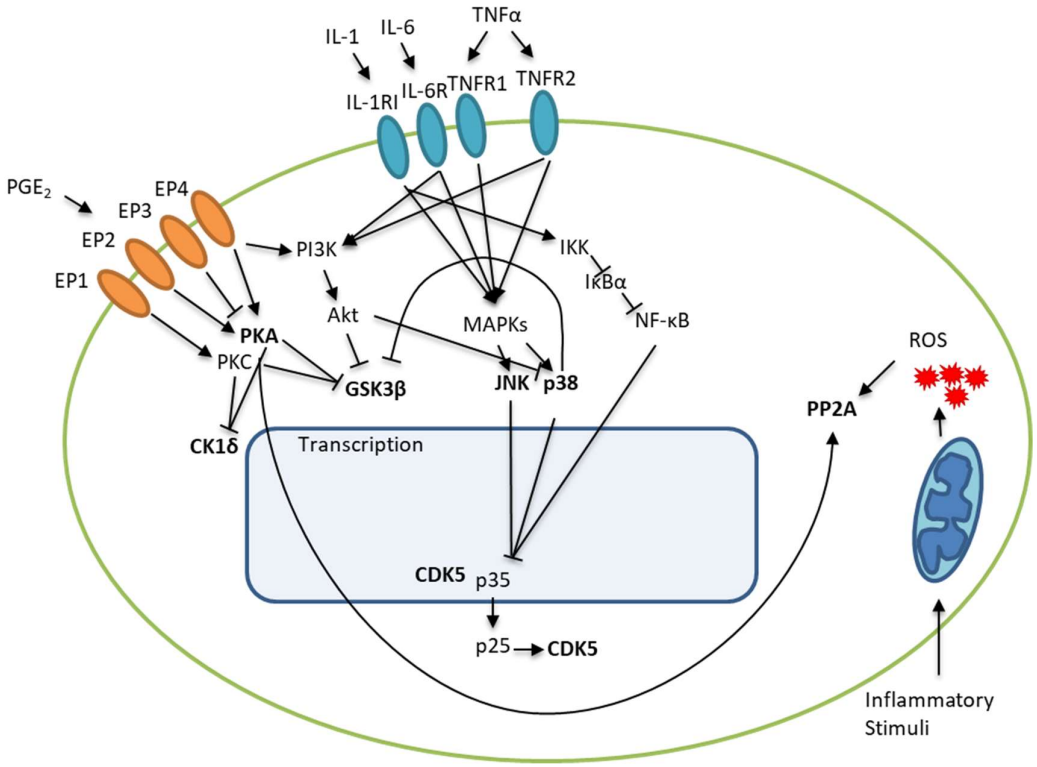

Figure 7.1: Potential mechanisms for LPS-induced tau dephosphorylation. Systemic inflammation could be reducing tau phosphorylation through inhibition of GSK3β, CK1δ and CDK5. Other potential mechanisms could be through the stimulation of PP2A activity. Figure drawn by author and adapted from Chapter 1.

The finding that tau was dephosphorylated following LPS administration demonstrates the possibility that systemic inflammation can have a beneficial effect on tau pathology. Whether this happens in AD is unclear, as the extent of inflammation was too strong to mimic the underlying mild inflammation associated with the disease. Suggesting that systemic inflammation might play a beneficial role on tau pathology in AD, chronic mild inflammation in the CIA model is associated with both a decrease in tau phosphorylation and aggregation in the P301S model (Lang et al., 2017). Furthermore, the study found an increase in microglial phagocytosis of extracellular tau, providing a potential mechanism for decreased tau pathology through inhibiting the spread of extracellular seeds. A certain degree of inflammation being beneficial in terms of tau pathology is supported by attempts to modulate TNFα activity in the 3xTg model whereby over-expression or ablation of its receptor resulted in increased tau phosphorylation (Montgomery et al., 2011, Janelsins et al., 2008).

The potential beneficial role of systemic inflammation on tau pathology could be the source of mixed findings of studies assessing the effect of anti-inflammatory treatment on tau pathology. For example, chronic treatment with the NSAIDS: ibuprofen or r-flurbiprofen both failed to affect tau phosphorylation in the 3xTg model (Carreras et al., 2013, McKee et al., 2008). In the most beneficial study, treatment with the tetracycline derivative: minocycline reduced both tau phosphorylation and aggregation in the hTau model (Noble et al., 2009b). This compares to a mixed effect of minocycline treatment in the 3xTg model with some phosphorylation epitopes increasing, decreasing or remaining unchanged (Parachikova et al., 2010). Further evidence is required to determine the exact effect of anti-inflammatory effect on tau pathology. However, the less than promising findings could support the findings from this thesis and the randomised controlled anti-inflammatory trials held in AD patients (Jaturapatporn et al., 2012).

Finally, the possibility exists that the results entailed in this thesis might not be representative of AD. For example, chronic LPS studies may be more relevant to AD. A chronic 500µg/kg (*i.p.*) LPS treatment regime was found to exacerbate tau phosphorylation and aggregation in the 3xTg model (Kitazawa et al., 2005, Sy et al., 2011). However, a recent attempt which lowered the LPS dose to 150µg/kg (*i.p.*) in the P301S model found a reduction in tau aggregation through stimulation of the autophagy-lysosome pathway (Qin et al., 2016). The most promising evidence supporting a detrimental effect of inflammation on tau pathology is centred upon studies which modulate the chemokine: fractalkine. Fractalkine possesses potent anti-inflammatory properties through its receptor on microglia cells (Jones et al., 2010). Tau phosphorylation is reported to occur when the fractalkine receptor is ablated in the hTau model (Bhaskar et al., 2010). Similarly, when overexpressed in the Tg4510 model, fractalkine reduces tau phosphorylation and aggregation (Nash et al., 2013). One issue with both studies is that they are not modelling the effect of systemic inflammation. Instead they are modulating the direct neuroinflammatory environment through the effect of fractalkine on microglia. The possible discrepancy between the fractalkine studies and the current could be due to differences between directly modulating microglial activity opposed to inducing systemic inflammation. A further possibility is that fractalkine may be acting in a manner which is independent of the neuroinflammatory environment. A major function of fractalkine is to stimulate microglial phagocytosis (Jones et al., 2010). Therefore, alterations in tau pathology could be due to phagocytosis of extracellular tau or debris which might impact on tau phosphorylation. Only through further

studies with a wide variety of inflammatory models will the effect of systemic inflammation on tau pathology be determined.

7.5 Potential consequences of tau dephosphorylation following systemic inflammation

There are multiple potential consequences associated with tau dephosphorylation following induction of systemic inflammation. Tau phosphorylation is the first stage in a cascade leading to generation of toxic tau species which include tau oligomers and to a lesser extent insoluble aggregates (Flament and Delacourte, 1989, Papasozomenos and Binder, 1987, Lasagna-Reeves et al., 2012a). Therefore, any decrease in tau phosphorylation has the potential to slow the development of toxic tau species which are thought to be involved in the neurodegenerative process. Suggesting that systemic inflammation can reduce the development of toxic tau species, the Lang *et al.* 2017 study demonstrated decreased levels of tau phosphorylation and aggregated tau following chronic systemic inflammation induced by CIA arthritis model in the P301S model. While the decrease in tau phosphorylation could be affecting its aggregation, the authors additionally reported a significant increase in microglial phagocytosis of extracellular tau which could limit the seeding of tau pathology. Future studies are required to determine whether tau dephosphorylation following systemic inflammation does affect the development of later stage tau pathology.

Irrespective of its effect on later stage tau pathology, tau dephosphorylation following induction of systemic inflammation has potential beneficial effects in itself. Firstly, a decrease in tau phosphorylation could potentially lead to increased affinity, decreased dissociation and increased microtubule stabilisation (Bancher et al., 1989). Microtubule destabilisation in tauopathies is suggested to contribute to Golgi fragmentation and impaired microtubule transport (Stamer et al., 2002, Mandelkow et al., 2003, Dixit et al., 2008, Liazoghli et al., 2005). Therefore any increase in microtubule affinity could slow down both these processes. Furthermore, while in the somatodendritic compartment, hyperphosphorylated tau results in stabilisation of the PSD-95 complex through its interaction with the Src family kinase: Fyn (Mondragon-Rodriguez et al., 2012, Usardi et al., 2011, Roberson et al., 2007, Miyamoto et al., 2017). It is this interaction which is thought to increase the susceptibility to excitotoxicity and any decrease in somatodendritic tau has the potential to protect against excitotoxic insult. Furthermore, pre- and post-synaptic hyperphosphorylated tau has been found to impair synaptic plasticity (Mondragon-

Rodriguez et al., 2012, Usardi et al., 2011). As such, any decrease in tau phosphorylation which is irrespective of later stage tau pathology has the potential to affect both excitotoxic neurodegeneration and cognitive function. Future studies should aim to understand the pathological consequences of systemic inflammation induced tau dephosphorylation through the use of behavioural studies, *in vivo* electrophysiology and IHC analysis.

7.6 Limitations of study

There are limitations when trying to understand pathological mechanisms in rodent models. For example, does the model really mimic a human disease? The answer is no. Instead, models which mimic a certain aspect of a disease are relied on, for example in the current thesis on tau pathology. This means any other pathological features of AD such as Aβ pathology, metabolic dysfunction or any of the other co-morbidities of the disease are not present (Cai et al., 2012, Taylor et al., 2001). Furthermore, the issue exists as to whether the specific aspect being modelled has validity to the disease. As discussed in Chapter 1, there are issues with tau models in respect to AD. The majority of tau models are mutational compared to AD which is associated with no known mutations in the tau gene (Kwon et al., 2000). Even the hTau model which has been suggested to be the tau model with highest validity to AD has its limitations. The biggest of which, is the fact that hTau mice express an isoform ratio imbalance: possessing a greater ratio of 3R to 4R isoforms (Andorfer et al., 2003) which is not representative of AD (Schmidt et al., 2001). Alterations in the tau isoform ratio have in themselves been associated with the generation of tau pathology and the development of specific forms of dementia such as FTDP-17 (Hutton et al., 1998, Hong et al., 1998) due to altered microtubule binding dynamics (Goode et al., 2000, Panda et al., 2003). Therefore, despite not being genetically susceptible to generate tau pathology in terms of tau mutations, the model is nonetheless genetically predisposed to develop tau pathology and may not share a pathophysiology with AD. Suggestive of an altered pathophysiology, only 3R tau isoforms is found in aggregates from hTau mice (Andorfer et al., 2003) compared to both 3R and 4R found in tau aggregates from AD patients (Espinoza et al., 2008). A further limitation with tau models in general is that they assume the pathophysiology of tau pathology in AD is due to the tau gene. In reality, AD is a complex multi-faceted disease which genetic models fail to account for (Talwar et al., 2016). Therefore, there is not a single tau model which shares an exact pathophysiology with AD at least in terms tau pathology and caution is required when interpreting findings from animal studies.

A further limitation when utilising murine models of AD is that the generation of tau pathology begins at an early age. Even in the hTau model, which is relatively mild, tau hyperphosphorylation occurs from two months of age (Andorfer et al., 2003). As systemic inflammation is thought to be an early event in AD, it is imperative to assess its effect on early tau pathology which requires assessment at a young age. The use of young mice created two important limitations of the study. The first, which has already become apparent, is that only induction of tau aggregation can be observed due to the mice not expressing aggregated tau at this time point. Therefore, the current study only modelled the effect of inflammation on pre-tangle pathology. Furthermore, failure to find aggregated tau in the tau aggregation assay indicates that even the intermediate of tau oligomers were not expressed at 3 months of age. The second limitation is that AD is an age associated disease and utilising young mice means any effect of senescence is not recapitulated. This is particularly relevant to inflammation which is well known to become dysfunctional during the ageing process (Streit, 2006). Therefore, unless utilising an inducible/suppressible tau model such as the rtg4510 (SantaCruz et al., 2005), there is a trade-off between assessing effects on early tau pathology and capturing senescence associated with the ageing process.

As previously mentioned, there were limitations in the specific method for inducing systemic inflammation in the current thesis: the extent of inflammation expressed and the failure to induce chronic inflammation. However, there is a greater limitation which is irrespective of the inflammatory model utilised: is the inflammatory system in a mouse similar to that of a human? 75 million years of evolution since the two evolutionary lines diverged would suggest there are some key differences between human and mouse immune systems (Mestas and Hughes, 2004). The fact that mice are less reactive to inflammatory stimuli such as LPS compared to humans (Copeland et al., 2005) suggests that there are underlying differences between the two. Comparison of white blood cell counts between the two species shows a marked difference. Mice have a much greater proportion of lymphocytes where humans have a greater proportion of neutrophils (Haley, 2003). An epitomising example of the differences between human and mouse immune systems is the protective effect of INFγ on experimental encephalitis (ECE) in mice (Lublin et al., 1993) opposed to its detrimental effect in the human disease for ECE: multiple sclerosis (Panitch et al., 1987). Furthermore, there are differences in the TLR superfamily in humans and mice. For example, mice have 13 TLR's compared to the 9 functional TLR's which are expressed in humans (Roach et al., 2005, Andrade et al., 2013). For two comprehensive reviews on the differences between the mouse and human immune system see (Zschaler et al., 2014,

Mestas and Hughes, 2004). Therefore, even if systemic inflammation is modelled in a manner which represents AD, it will nevertheless produce a different response to AD.

Much of the rationale for systemic inflammation driving tau pathology stems from animal models. The limitation of utilising murine tau and inflammatory models means it is difficult to determine the biological significance of these findings. A degree of validation is required in humans to provide relevance. The effect of modulating inflammation on tau pathology has been poorly investigated in humans; with only a few studies having assessed CSF tau levels following anti-inflammatory treatment. In the largest of anti-inflammatory trials for AD, NSAID treatment with celecoxib or naproxen failed to impact on CSF tau levels (Breitner J, 2013). Likewise, resveratrol, a natural phenol with anti-inflammatory properties, failed to impact on both CSF total tau and pT181 tau concentrations (Moussa et al., 2017). However, CSF levels are far from ideal in terms of deciphering alterations in tau pathology as mixed results have been achieved when trying to correlate the two (Buerger et al., 2007, Buerger et al., 2006, Engelborghs et al., 2007). Although there are issues with specificity of tau PET tracers (Saint-Aubert et al., 2017), no attempt has been made to utilise the technique to understand the effect of systemic inflammation or anti-inflammatory treatment on tau pathology in humans. Without verification in humans, it is difficult to accurately determine the effect of systemic inflammation on tau pathology.

7.7 A dichotomic effect of systemic inflammation in AD?

Despite having a potentially beneficial effect on tau pathology, systemic inflammation could nevertheless be a detrimental force in AD. For example, it is still unknown whether tau pathology is a causative factor in AD. The first tau aggregation inhibitor to make it to a Phase III clinical trial: leuco-methylthioninium (LMTM) missed all primary endpoints for mild-moderate AD (Gauthier et al., 2016). However, the authors did report a potential beneficial effect as a monotherapy, although a separate clinical study is required to verify the claim. Therefore, it is far from clear whether tau pathology is a causative factor or whether it is merely associated with AD. Even if tau pathology was found to be a causative factor in AD, systemic inflammation could be detrimental in AD irrespective of tau pathology.

Potentially the strongest rational for systemic inflammation being a negative force in AD is the finding that peripheral systemic events lead to an increased rate of cognitive decline in AD patients (Holmes et al., 2009, Ide et al., 2016, Holmes et al., 2003). However, none of the studies assessed markers of tau pathology – either through assessment of CSF

tau or through PET tracers. Therefore, the effect of systemic inflammation on cognitive decline could be simply due to symptomatic effects rather than pathological. Unfortunately, due to the development of allergies to mice, the symptomatic effect under the current experimental conditions could not be assessed. However, inflammation is well known to affect cognition. IL-1β, TNFα and LPS have been reported to depress LTP in organotypic hippocampal sections (Gonzalez et al., 2013, Cunningham et al., 1996). LPS administration in humans is associated with slower working memory performance and long term emotional memory impairments in healthy individuals (Grigoleit et al., 2011). In a similar manner, systemic inflammatory events could simply be impairing cognitive function in AD patients. However, increased cognitive decline following the resolution of the systemic inflammatory event could argue that symptomatic exacerbation is not its sole effect (Holmes et al., 2003). However, the neuroinflammatory response has the potential to persist until long after the resolution of the systemic inflammatory response in mice (Qin et al., 2007). Therefore, systemic infections in AD patients could still produce symptomatic effects until long after the resolution of the systemic inflammatory response due to persistence of neuroinflammation.

Systemic inflammation could likewise be a detrimental force through affecting pathogenic processes other than tau aggregation. One potential pathogenic effect of systemic inflammation is through the exacerbation of amyloid pathology. In animal models, systemic inflammation induced by LPS administration has been well documented to increase amyloid aggregation through the generation of the amyloid precursor protein (APP) (Lee et al., 2008, Sheng et al., 2003). However, it is unclear as to whether systemic inflammation is overall a detrimental or positive force on amyloid in AD patients due to stimulation of its clearance by microglia. Amyloid pathology is found in the extracellular compartment, with microglia phagocytosis an important clearance mechanism (D'Andrea et al., 2004). Systemic inflammation is well known to stimulate microglial phagocytosis. Both systemic and central LPS administration in mice have been found to induce microglial phagocytosis of amyloid, leading to a decrease in amyloid burden in some studies (Herber et al., 2007, Herber et al., 2004, Quinn et al., 2003). Supporting a beneficial role for systemic inflammation on amyloid pathology in AD, mild chronic inflammation in the CIA model reduced soluble and insoluble amyloid burden in the APP/PS1dE9 model (Park et al., 2011).

While it is unclear whether systemic inflammation is detrimental in terms of amyloid pathology, it has the potential to exert additional pathological effects through the sustained elevation of noxious pro-inflammatory mediators within the CNS. While some of these

mediators can exert potential beneficial effects on amyloid and tau pathology as well as clearance of pathological debris, others could be contributing to the disease. For example, elevated levels of the membrane attack complex (MAC): C5b-9 is found elevated in brains from AD patients (Webster et al., 1997). While both aggregated amyloid and tau induce formation of MAC (Shen et al., 2001), systemic inflammation induced by LPS has been reported to induce the complement cascade within the CNS of mice (Bodea et al., 2014). Formation of the MAC has the potential to be beneficial through initiating cell death of pathogenic neurons (Müller-Eberhard, 1988). However, MAC formation can affect healthy, neighbouring "bystander" cells (Park et al., 1997) and has the potential to increase the rate of neurodegeneration. A further mechanism for neuroinflammation to increase neuronal loss is through increasing susceptibility to excitotoxic neurodegeneration. IL-1β has been reported to exacerbate excitotoxic neuronal loss *in vitro* through activation of the apoptic protein p53 (Rossi et al., 2014, Takahashi et al., 2003). Furthermore, neuroinflammation has been reported to affect synapse loss. For example, C1q, C3 and the microglia complement receptor: CR3 were found to be required for Aβ induced synaptic loss in multiple amyloid models (Hong et al., 2016). Therefore, especially acting in conjunction with both classical hallmarks, systemic inflammation has the potential to exacerbate AD which is irrespective of its effect on tau pathology.

Finally, the possibility remains open that inflammation may not be a good disease modifying target in AD. Much of the rationale for systemic and neuroinflammation being involved in AD stems from epidemiological evidence. Every single randomised controlled anti-inflammatory trial in AD has failed regardless of the class of drug utilised (Jaturapatporn et al., 2012). Even when administered prodromally, the NSAIDs: naproxen and celecoxib failed to show efficacy, although the trials were halted early due to safety concerns (Breitner J, 2013). However, as diverging effects of systemic inflammation are being found on underlying AD pathology, future studies are required which selectively modulate the immune system.

7.8 Future directions

To further our understanding of how systemic inflammation interacts with tau pathology, it is important to expand on the findings from this study. Firstly, future studies should aim to clarify a mechanism for systemic inflammation to reduce tau phosphorylation. The current study saw a trend for increased PP2A activity, but further clarification is required in a dedicated PP2A activity assay. Furthermore, as the state of phosphorylation is

dependent on the balance between kinase and phosphatase activity, future studies should aim to assess kinases implicated in tau phosphorylation as well.

One of the limitations of the current study was that the magnitude of the systemic inflammatory response was too strong. Future, studies should focus on lowering the dose of LPS to determine whether the tau dephosphorylating effects still occur when more mild inflammation is occurring. Even more useful would be modelling the effect of systemic inflammation on tau pathology in a chronic mild manner. The CIA model of rheumatoid arthritis is not only a risk factor for AD (McGeer et al., 1996), but an interesting method for inducing chronic mild systemic inflammation in animal models (Amdekar et al., 2011, Tsubaki et al., 2015). Only one study to date has assessed the effect of CIA induced arthritis on tau pathology. Lang *et al.,* 2017 saw a decrease in phosphorylated tau, decrease in the number of neurons affected by NFTs and increased microglial phagocytosis of tau when CIA was induced in the P301S model. While the study provided tantalising evidence that chronic mild inflammation can have a beneficial effect on tau pathology, the study was limited. Firstly, it failed to assess multiple phosphorylation epitopes and relied on IHC observations instead of a technique more appropriate for quantification such as western blotting or enzyme-linked immunosorbent assays. Secondly, it failed to comprehensively assess a mechanism. While it did report an increase in microglial phagocytosis of extracellular tau, it does not rule out other potential mechanisms (Lang et al., 2017). Further studies are required which assess additional potential mechanisms for example kinase/phosphatase activity and other tau clearance mechanisms such as the autophagic and proteasome pathways.

The Lang *et al.* 2017 study provides the first *in vivo* evidence that microglia phagocytose extracellular tau following systemic inflammation. Future studies are needed to elucidate the physiological consequences of this interaction through utilising the tau propagation model. The tau propagation model involves the sequential spreading of tau pathology into neighbouring brain regions following injection of aggregated P301S tau in Wt mice (Clavaguera et al., 2009). By inducing systemic inflammation in the model, the effect of microglial tau phagocytosis on the propagation of tau pathology can be discerned. In addition to assessing the effect of systemic inflammation in the tau propagation models, future studies are required to determine how ageing affects the interaction between systemic inflammation and tau pathology. AD is a disease associated with ageing and age is the greatest risk factor (van der Flier and Scheltens, 2005). To this end, the use of inducible

tau models such as the rtg4510 model (SantaCruz et al., 2005) would enable assessment of early tau pathology at an old age. This is especially important in terms of inflammation which undergoes profound senescence with ageing.

Finally, future studies need to validate observations in humans. This thesis has described some of the potential issues when assessing the effect of systemic inflammation on tau pathology using murine models. However, the methods for measuring *in vivo* tau pathology in humans relies either on CSF samples or through PET imaging – both of which have potential pitfalls. While measuring CSF tau samples provides specificity in terms of assessing tau phosphorylation, there is conflicting evidence as to whether CSF tau levels correlate with tau deposition (Buerger et al., 2007, Buerger et al., 2006, Engelborghs et al., 2007). On the other hand, tau PET tracers have been found to correlate with tau pathology (Brier et al., 2016) but suffer from specificity issues (Saint-Aubert et al., 2017). Nevertheless, both techniques can provide some insight into the effect of systemic inflammation on tau pathology in humans. To date, the effect of systemic inflammation on tau pathology in humans has been poorly studied and focussed solely on CSF tau levels. Human immunodeficiency virus (HIV) infection, which is associated with the development of dementia, was found to have no effect on CSF total and pTau (Gisslén et al., 2009). Furthermore, the red wine extract: resveratrol which possess anti-inflammatory properties was found not to affect CSF total tau or pTau in individuals with mild-moderate AD (Moussa et al., 2017). Similarly, CSF total tau levels were found unaltered following treatment with the NSAIDs: naproxen and celecoxib in healthy individuals at risk of developing AD (Breitner et al., 2011). Together, these studies suggest inflammation may indeed not be a modulator of tau pathology in humans. However, further studies including the use of PET studies in a much greater variety of inflammatory conditions is required to truly understand whether systemic inflammation affects tau pathology within the disease.

7.9 Conclusions

Through understanding how systemic inflammatory stimuli affect tau pathology in animal models, the therapeutic potential of inflammation in AD can be determined. This thesis describes a potentially beneficial role for systemic inflammation on early tau pathology through inducing its dephosphorylation. The positive role of mild inflammation on tau phosphorylation suggests inflammation is not as detrimental as initially thought and is adding to growing evidence for a limited pathogenic effect of systemic inflammation on tau pathology in AD. While PP2A appears to be a likely mechanistic candidate, future studies will

have to confirm its involvement. However, these observations are based on murine models of tau pathology and systemic inflammation – both of which have fundamental limitations. Ultimately, verification is required in AD patients to provide weight behind these observations. Nevertheless, despite a potential positive role of systemic inflammation on tau pathology, there is the possibility for systemic inflammation to be a detrimental force in AD. Finally, only through validation in humans with novel techniques that non-invasively measure tau pathology will the true role of inflammation on tau pathophysiology in AD be realised.

Chapter 8 Appendix

8.1 Preliminary tau phenotype in hTau/mTau$^{+/-}$ mice

To negate the development of systemic pathologies and potentially improve the tau isoform ratio, hTau mice were bred on an mTau$^{+/-}$ background. Spare cortical tissue from 6 month old mice was assessed by western blotting to preliminary determine the tau phenotype in hTau/mTau$^{+/-}$ mice. There was an overall significant effect in total tau levels between genotypes [$F_{(5,15)}$ = 149.27, p < 0.001] with post hoc analysis revealing both hTau genotypes were significantly elevated compared to their corresponding Wt controls (p<0.001). In addition to being elevated compared to Wt controls, hTau/mTau$^{+/-}$ mice had significant higher levels of total tau compared to hTau/mTau$^{-/-}$ mice (P < 0.001, Figure 8.1B) which is likely due to the extra expression of mTau in the model. Furthermore, significant alterations in the pre-tangle associated phosphorylation epitope; pS202 and the post-tangle associated phosphorylation epitope; pS396/404 were observed among genotypes ([$F_{(5,15)}$ = 44.66, p < 0.001] and [$F_{(5,15)}$ = 33.32, p < 0.001] respectively). In both cases Tukey's post hoc analysis indicated that hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice have significantly more tau phosphorylation compared to their Wt controls (p < 0.001). Furthermore, Tukey's post hoc analysis revealed that pS202 levels were significantly increased in hTau/mTau$^{+/-}$ compared to hTau/mTau$^{-/-}$ mice (p < 0.05, Figure 8.1C-D). Suggesting the increase in phosphorylated tau was resulting from increased tau expression in hTau/mTau$^{+/-}$ mice, one way ANOVA analysis showed there were no alterations in the ratio of pS202:total tau among genotypes (Figure, 8.2A, [$F_{(5,15)}$ = 0.46, p = 0.79]). One way ANOVA analysis revealed there were significant differences in the ratio of pS396/404:total tau between genotypes [$F_{(5,15)}$ = 10.01, p < 0.001]. Tukeys post hoc indicated that while mTau$^{+/-}$ and hTau/mTau$^{+/-}$ were significantly altered (p < 0.001), hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice were similar (p > 0.05, Figure 8.2B).

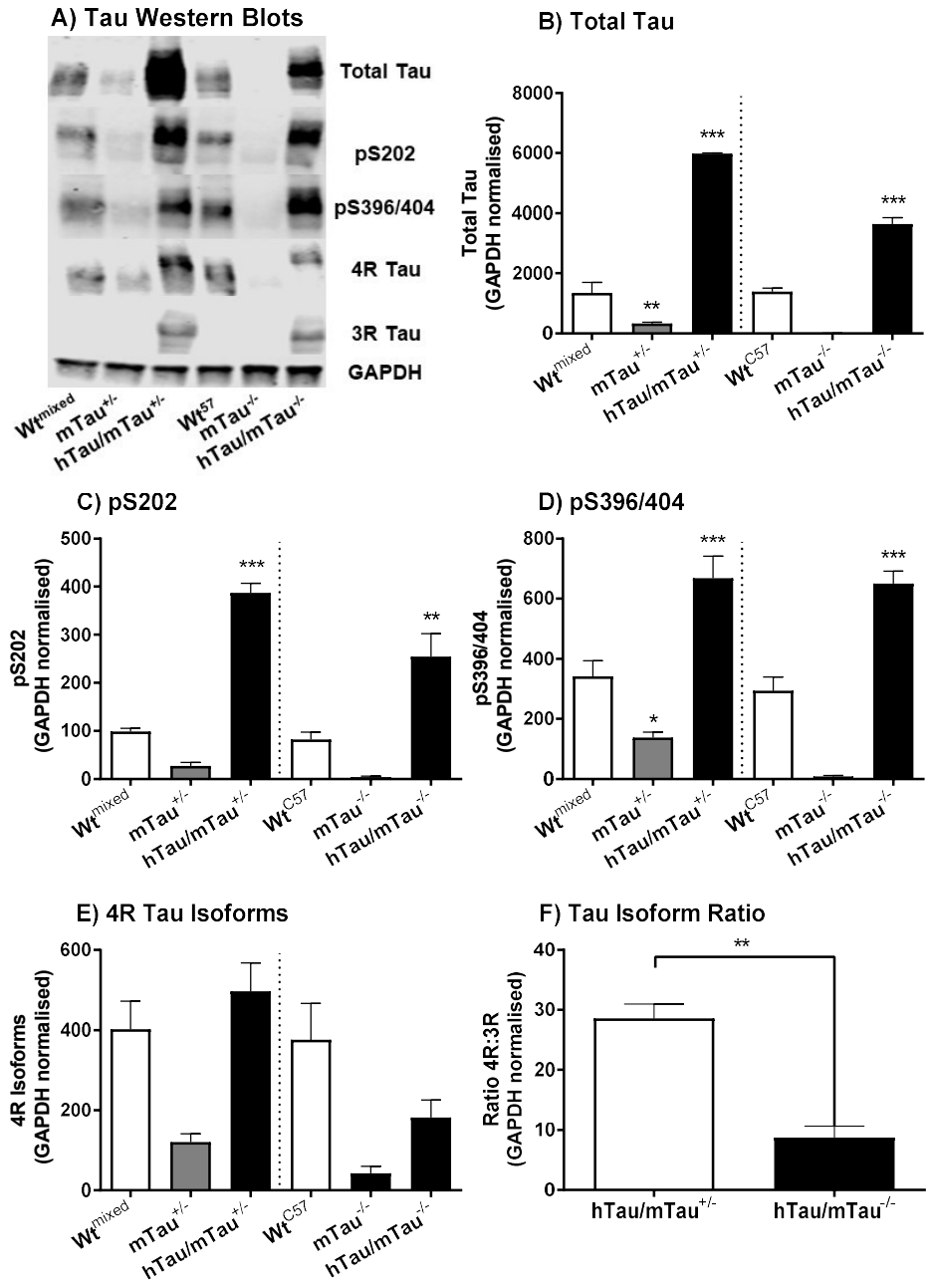

Figure 8.1: Preliminary tau phenotype in spare cortical tissue from 6 month hTau mice. Bars represent mean ± SEM (n=3-4/genotype) and dotted line separates mice on different background strains. ***A)*** *Representative western blots. Preliminary findings suggest hTau/mTau$^{+/-}$ mice have elevated* ***B)*** *total tau levels but similar* ***C)*** *pS202 and* ***D)*** *pS396/404 levels compared to hTau/mTau$^{-/-}$ mice.* ***E)*** *hTau/mTau$^{+/-}$ mice have elevated levels of 4R tau isoforms and a higher* ***F)*** *ratio of 3R:4R tau isoforms. Significance vs. corresponding Wt mice: * p < 0.05, ** p < 0.01 and *** p <0.001.*

One rational for utilising hTau/mTau$^{+/-}$ mice over their KO counterparts is the expression of mTau would hypothetically increase the 4R:3R tau isoform ratio, making the model less genetically susceptible to tau aggregation and more akin to AD. There was a

significant alteration in the levels of 4R tau isoforms among genotypes [$F_{(5,18)}$ = 9.3, p < 0.001] with post hoc analysis revealing that while neither of the hTau genotypes differed compared their Wt controls (p > 0.05), hTau/mTau$^{+/-}$ mice had increased levels of 4R tau compared to hTau/mTau$^{-/-}$ mice (p < 0.05, Figure 8.1E). Interestingly, human 4R tau isoforms appeared to be of a higher molecular weight compared to their Wt counterparts, suggesting the human tau to be hyperphosphorylated in hTau/mTau$^{+/-}$ mice (Figure 8.1A). As expected, mice expressing solely mTau did not exhibit any traces of 3R tau isoforms (Figure 8.1A). When comparing the ratio of 4R:3R tau isoforms between hTau/mTau$^{+/-}$ and hTau/mTau$^{-/-}$ mice, the former were significantly elevated [t(5) = -8.83 = p < 0.001] suggesting their isoform ratio was more akin to that observed in AD. While caution is required when interpreting this preliminary tau classification due to different background strains, hTau/mTau$^{+/-}$ mice appear to have a more favourable isoform ratio and appear to have similar extent of tau phosphorylation compared to hTau/mTau$^{-/-}$ mice, providing an interesting model to study early pathological tau alterations.

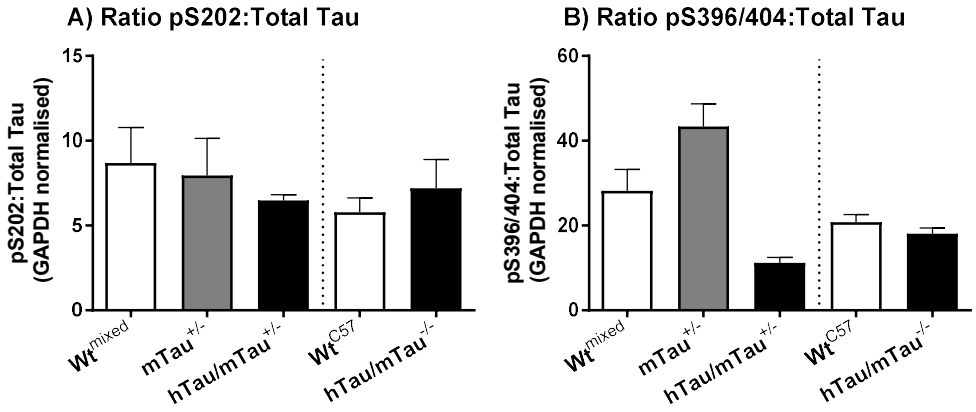

Figure 8.2: Preliminary tau phenotype in spare cortical tissue from 6 month hTau mice. Bars represent mean ± SEM (n=3-4/genotype) and dotted line separates mice on different background strains. ***A)*** *pS202:total tau ratio.* ***B)*** *pS396/404:total tau ratio.*

8.2 Microglia staining 4h following LPS administration

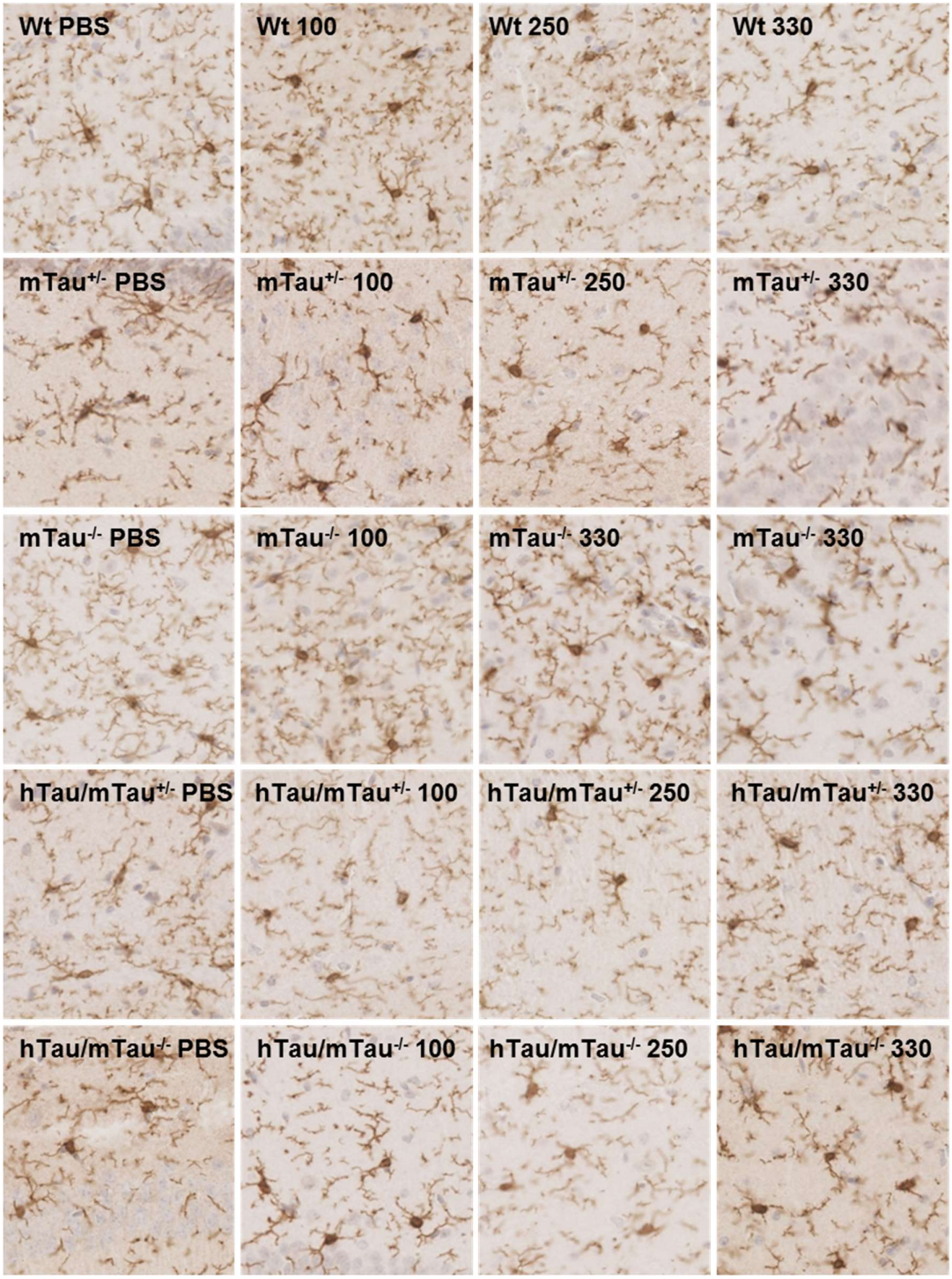

Figure 8.3 Representative section from Iba1 staining 4h following LPS administration.

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