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The Behavioural Phenotype of pThr175-Tau Expression in the Hippocampus of Female Adult Sprague Dawley Rats

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Abstract

Amyotrophic lateral sclerosis with cognitive impairment (ALSci) can be characterized by pathological inclusions of microtubule associated protein tau (tau) uniquely phosphorylated at Thr175 (pThr175-tau). The purpose of this study was to characterize the behavioural consequences of expressing a pseudophosphorylated tau mimic of pThr175-tau (Thr175Asp-tau) in rat hippocampus. Expression was hypothesized to lead to pathological tau fibril formation resulting in cognitive and behavioural deficits. Expression was accomplished in female Sprague Dawley rats through stereotactic inoculations of recombinant adeno-associated virus (rAAV9) vector with human tau gene. Pathological tau fibrillary structures were identified, but behavioural testing up to 12 months post-surgery revealed no deficits in Thr175Asp-tau group when compared with the controls. Control inoculums included: wt-human tau, phosphorylation inhibition (Thr175Ala-tau), and green fluorescent protein. There were age-related behavioural changes across testing time points. This study serves an important step towards the development of an animal model for ALS with cognitive syndromes, which is essential for understanding disease progression.

Key Words: Amyotrophic lateral sclerosis, Frontotemporal Dementia, Tau, Tauopathy, ALS-FTD, ALSci, Non-transgenic Rat Model, Behavioural Analysis, Morris Water Maze, Open Field, Acoustic Startle Response, rAAV9

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Abbreviations	Term
ALS	amyotrophic lateral sclerosis
ALSbi	ALS with behavioural impairment
ALS-bvFTD	ALS with behavioural variant FTD
ALSci	ALS with cognitive impairment
ALS/PDC	ALS/Parkinsonism-dementia complex
ALS-PNFA	ALS- progressive non-fluent aphasia
ALS-SD	ALS with semantic dementia
ASR	acoustic startle response
c9orf72	chromosome 9 open reading frame 72
CA	Cornu Ammonis (hippocampal regions)
CNS	central nervous system
DG	dentate gyrus
fALS	familial ALS
FTD	frontotemporal dementia
FTLD	frontotemporal lobar degeneration
FUS/TLS	fused in sarcoma/ translated in liposarcoma
GFP	green fluorescent protein
ISI	interstimulus interval
LTH	long-term habituation
LTM	long-term memory
MWM	Morris water maze
OFT	open field testing
PPI	prepulse inhibition
rAAV	recombinant adeno-associated viral vector
sALS	sporadic ALS
SOD1	superoxide dismutase I
SLS	superficial linear spongiosis
STH	short-term habituation
Tau	microtubule associated protein tau
TDP-43	transactive response DNA binding protein 43 kDa

PART 1: INTRODUCTION

1. Introduction

1.1 ALS: Clinical Definition and Symptoms

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease defined by progressive death of upper and lower motor neurons in the central nervous system. Motor neuronal cell loss results in progressive paralysis in patients and death generally ensues within an average of 3 to 5 years of symptom onset (Factor-Litvak et al. 2013).

Under normal physiological function, signals from the upper motor neurons in the brain are transmitted to the lower motor neurons in the spinal cord and brain stem, which then stimulate movement of voluntary muscles. In ALS, however, both upper and lower motor neurons progressively degenerate and die. This leads to diffuse loss of motor function accompanied by muscle atrophy and ultimately paralysis with death due to respiratory failure. Our conventional understanding of ALS is that in the majority of patients, the degenerative process of ALS remains restricted to the motor system, sparing modalities such as eye motor control, bowel and bladder function. ALS is one of the most common neuromuscular diseases worldwide, and all ethnicities and races are affected, with a higher prevalence in men over women in the premenopausal period.

Approximately 90% of ALS cases are sporadic (sALS) and have no clearly associated risk factors, while roughly 10% of cases are considered familial (fALS), or inherited (Ince et al. 2011). fALS is genetically heterogeneous. Greater than 50% of fALS cases can be accounted for by mutations in the genes encoding the following: superoxide dismutase I (SOD1), transactive response DNA binding protein 43 kDa (TDP-43), fused in sarcoma/ translated in liposarcoma (FUS/TLS), or chromosome 9 open reading frame 72 (c9orf72) (Fiesel and Kahle 2011; Kwiatkowski et al. 2009; Rollinson et al. 2011).

Though ALS is generally considered either sporadic or familial, this boundary is not absolute. For instance, up to 10% of clinically sporadic cases of ALS show mutations in several of the associated fALS genes, such as SOD1, FUS, and TDP-43 (Alexander et al. 2002; Chiò et al. 2011; Kirby et al. 2010). In these cases of sALS, the observed genetic mutations could be arising as new, sporadic mutations, or may be a result of insufficient family history to support a finding of fALS (Ince et al. 2011).

1.2 ALS and features of frontotemporal dysfunction

While ALS and frontotemporal lobar degeneration (FTLD) have been considered to be independent disorders, there is increasing evidence to suggest that both ALS and FTLD overlap in a significant portion of the patient population. Frontotemporal lobar degeneration (FTLD) refers to the progressive degeneration of the frontal and temporal lobes of the brain. The distributions of pathology throughout those lobes and the attendant disruption of their interconnections results in an array of syndromes of frontotemporal dysfunction, including frontotemporal dementia (FTD) (Freedman et al., 1998; Strong et al., 2009).

While ALS has classically been considered a disease of the motor neurons alone, upwards of 50% of patients with ALS are now known to develop features of frontotemporal dysfunction (Strong et al., 2009). These syndromes of frontotemporal dysfunction can manifest as a behavioural or cognitive impairment (ALSbi, ALSci), a dysexecutive syndrome, or a frontotemporal dementia (ALS-FTD) in which cases the Neary or Hodge's criteria for FTD are met (Freedman et al., 1998; Gregory, Orrell, Sahakian, & Hodges, 1997; Strong et al., 2009).

Each of these clinically defined syndromes is characterized by different criteria. ALSci patients exhibit impaired executive functions or verbal fluency, while ALSbi patients show behavioural features associated with frontotemporal dysfunction and meet some of the Neary or Hodge's criteria for FTD (Strong et al., 2009). The term ALS-FTD refers to a clinical spectrum that includes ALS with behavioural variant FTD (ALS-bvFTD), progressive non-fluent aphasia (ALS-PNFA), as well as semantic dementia (ALS-SD) (Strong et al. 2009). These ALS-FTD subtypes are defined as follows: bvFTD shows impaired executive function determined through neuropsychological assessment, PNFA is characterized by effortful speech production, errors in grammar and difficulties in retrieving words, whereas SD exhibits naming and word comprehension impairment while speech remains fluent and grammatical (Neary et al. 1998). Aside from the utilization of the Neary and Hodge's criteria, further extensive neuropsychological testing is, of course, conducted when diagnosing such disorders. These features serve as an effective screening process for ALS patients with frontotemporal dysfunctions.

1.3 ALS and Frontotemporal Degeneration: impact and relevance

ALS is amongst the most devastating of adult-onset neurodegenerative diseases. The concomitant presence of frontotemporal dysfunction further worsens the condition. The presence of these frontotemporal syndromes in ALS is associated with a shorter survival rate when compared to ALS alone (Murphy et al. 2006). In addition, caregivers of ALS patients with neurobehavioural symptoms have been shown to have a lower quality of life, increased rates of depression, and undergo a significantly higher burden (Chiò et al. 2011). Given the peak age of onset of ALS is in the 6th decade of life, higher prevalence of ALS are anticipated as the baby boomers age. From an economic standpoint this will

result in greater numbers of patients and a greater burden to the health care system in the near future.

1.4 ALS and Frontotemporal Degeneration: common ties

The frequent clinical overlap of the syndromes of frontotemporal dysfunctions and ALS has led to the notion that these disorders may be a continuum (Strong et al. 2009). The development of frontotemporal dysfunction can precede, coincide, or follow the motor neuron dysfunction of ALS, and there is frequent co-existence between these two conditions (Ringholz et al. 2005; Robberecht and Philips 2013). For each new gene mutation discovered in fALS there has been a parallel discovery that those genes contribute to the pathobiology of FTD. These associated mutations have become important in the neuropathological differentiation amongst distinct subtypes of frontotemporal syndromes of ALS (Geser et al. 2013; Ince et al. 2011). For example, FTD's are further classified based on whether TDP-43, FUS/TLS, or hexanucleotide repeats in c9orf72 are the predominant pathologies (Rohrer et al. 2011). Altogether, this has led some to postulate that ALS and FTD are but two states on a single disease continuum, based on the genetic linkages provided by these discovered mutations (Ince et al. 2011).

1.5 The function and dysfunction of microtubule associated protein tau

Conversely, all forms of FTD are divided into those presenting with alterations in the microtubule associated protein tau (tau) – known as "tauopathy" – and those that do not. Forms of FTD without a tauopathy are those mutations mentioned above (TDP-43, FUS/TLS, c9orf72). Approximately 40% of remaining FTD's have an underlying tauopathy. There is abundant evidence of alterations of tau metabolism in FTD's. These

tauopathies include; Pick's disease, progressive supranuclear palsy (PSP), and corticobasal degeneration (Mackenzie and Rademakers 2007). Recently, similar evidence of a tauopathy has been observed, in ALS with frontotemporal dysfunctions. This section will address the normal function of tau protein, and the abnormalities observed in ALS.

Tau protein stabilizes the cytoskeleton of a cell (Weingarten et al. 1975). Within nerve cells, microtubules run along the axon from the soma to the synaptic terminal and act as tracks to facilitate axonal transport (Brown 2003). Axonal transport allows for the movement of synaptic vesicles, proteins, lipids, and organelles throughout the neuron and is an essential process for proper neuronal functioning (Brown 2003). Tau protein binds to, and supports, the microtubules in the axonal processes to prevent microtubule breakdown. This binding stabilizes the cytoskeleton of the cell (Weingarten et al. 1975).

Human tau proteins are translated from chromosome 17q21 (Buee et al. 2000). A total of six isoforms of tau exist in the human adult brain as a result of alternative splicing of N-terminus domains (N1, or N2) and a C-terminus multiphosphorylation repeat (R2, Figure 1, Buee et al., 2000). N1, N2, and R2 are exon 2, 3, and 10 encoded respectively. Each can either be included, or not, through alternative splicing (Buee et al. 2000). There are three other multiphosphorylation repeats (R1, R3, R4) always present (Buee et al. 2000). Thus, there are the 3R or 4R isoforms, containing 0, 1, or 2 N-terminus inserts for a total of six isoforms of tau (in order of increasing size): 0N3R, 0N4R, 1N3R, 1N4R, 2N3R, 2N4R. The smallest isoform, 0N3R, has a molecular weight of 36.7 kDa, whereas the largest, 2N4R, has a molecular weight of 45.9 kDa (Mandelkow and Mandelkow 2012). The isoforms with more repeats, like 2N4R, tend to bind more strongly to microtubules than shorter isoforms, such as 0N3R (Mandelkow and Mandelkow 2012).

A tauopathy is a diseased state where tau protein becomes hyperphosphorylated, loses its affinity for the microtubule, forms aggregates, and relocates then from the axon to the cell body (Kowall and Kosik 1987). Tau protein aggregation is a pathological hallmark for a number of neurodegenerative diseases such as Pick's disease, PSP, and Alzheimer's disease (Ferrer et al. 2005). The prominence of tau pathology in these neurodegenerative diseases has been taken to suggest that tau protein abnormalities are linked to mechanisms of brain degeneration.

The normal function of tau is physiologically regulated by phosphorylation. Normal physiological changes in the phosphorylation state of tau protein can be observed during development. Specifically, the phosphorylation state of tau is high in fetal stages, and decreases with age as a result of activation of phosphatases (Mawal-dewan et al. 1994; Vanmechelen et al. 1995). In the fetal state, tau is hyperphosphorylated, containing approximately four phosphates per tau molecule, whereas adult tau contains roughly two phosphates per tau molecule (Mandelkow and Mandelkow 2012). In either case, tau is extensively phosphorylated. As such, hyperphosphorylation of tau protein is not a total indicator of a disease state (Mandelkow and Mandelkow 2012).

There is a complex balance between phosphorylation and dephosphorylation. This complex balance results from the activity of specific kinases and phosphatases towards the phosphorylation sites within or outside the microtubule-binding domain (Hasegawas et al. 1992; Paudel and Li 1999; Roder et al. 1997; Takahashi et al. 1995; Vanmechelen et al. 1995). Ultimately, under normal physiological conditions there is a complex balance of phosphorylation and dephosphorylation of tau protein, and it is not simply phosphorylation of tau that leads to a diseased state. Rather, it may be the abnormal

phosphorylation of tau, and consequent loss of affinity for the microtubule, that leads to a disease state.

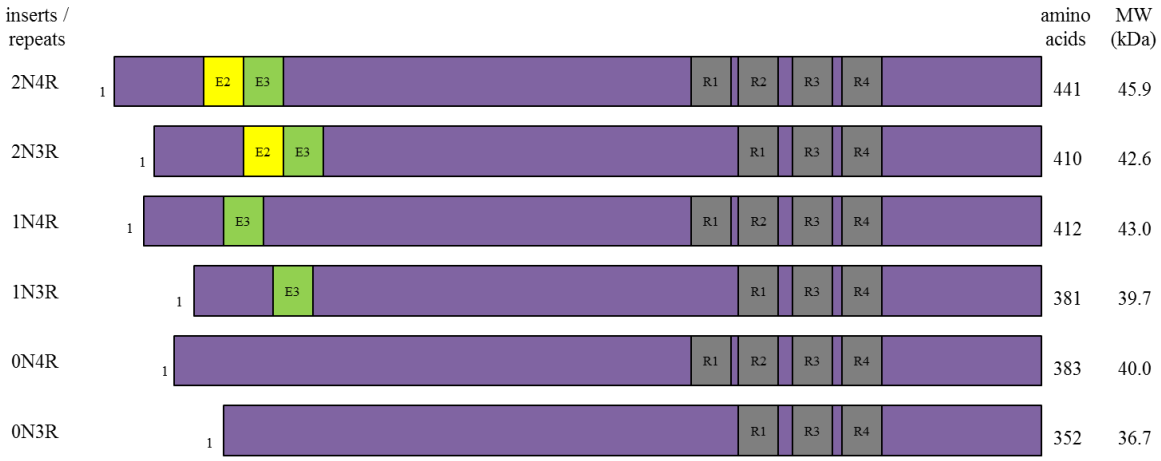

Figure 1: Schematic of human tau protein isoforms

The sizes of the tau isoforms range from 352 to 441 amino acids. Their molecular weights range from 36.7 to 45.9 kDa, with the tau variants differing from each other by the presence of either three (R1, R3, R4) or four repeat regions (R1, R2, R3, R4) in the C-terminal part of the molecule, as well as the presence or absence of one or two inserts (E2, E3) in the N-terminal part (Mandelkow and Mandelkow 2012).

1.7 Propagation of tau pathology

As previously stated, pathologically phosphorylated tau is a prominent component of the intracellular inclusions of many neurodegenerative diseases. In ALSci, it has been proposed that neuronal and glial tau inclusions first appear in the entorhinal cortex, and that this then forms the apparent epicenter of pathology (Yang & Strong, 2012). It has been further proposed that from the entorhinal cortex the tau pathology spreads to regions such as the superior frontal and anterior cingulate cortex, regions of the hippocampus, the substantia nigra, and the basal ganglia (Nakano, Nakaso, and Nakashima 2004; Yang and Strong 2012).

There is evidence of this described propagation of tau pathology from animal model studies. A neuropsin-tTA-tau transgenic mouse model with inducible expression of pathological human tau in the entorhinal cortex was established. This model showed changes in the distribution of tau pathology at different time points (Liu et al. 2012). Relatively young (10-11 months old) transgenic mice showed tau pathology mostly limited to the entorhinal cortex, whereas aged transgenic mice (>22 months old) demonstrated evidence of trans-synaptic propagations of tau accompanied by mature tangle formation along anatomically connected neurons (Liu et al. 2012).

1.8 ALS with cognitive impairment (ALSci)

The most commonly impaired functions in ALSci patients are verbal fluency, visual memory, immediate verbal memory and executive dysfunction (Raaphorst et al. 2010). Although tau pathology is only rarely observed in motor neurons in ALS, there is

abundant evidence of disturbances in tau metabolism in cortical and subcortical neurons in ALSci.

At a tissue level, ALSci is characterized by a spectrum of neuropathological abnormalities including gross frontal atrophy, and superficial linear spongiosis (SLS) in the frontal cortex (Wilson et al. 2001). There are also neuronal and glial tau protein inclusions located most prominently in the entorhinal cortex, hippocampus, and anterior cingulate gyrus (Yang et al. 2003). Tau protein aggregation in the frontal cortex is a disease-specific process as opposed to a normal function of aging (Yang, Ang, and Strong 2005). Further, tau isolated from the cortical and subcortical white matter of cognitively impaired ALS patients is highly insoluble and tends to form pathological fibrils *ex vivo* (Strong et al. 2006). These tau fibrillary inclusions in ALSci are aberrantly phosphorylated at Threonine residue 175 (pThr175) and associated with an upregulation of GSK3β activity (Strong et al. 2006; Yang, Leystra-Lantz, and Strong 2008; Yang and Strong 2012). The extent of fibril formation, as well as aberrant phosphorylation, is significantly greater in ALSci-derived tau than in controls, with ALS-derived tau falling intermediately between these (Strong et al. 2006). These observations suggest that aberrant phosphorylation of tau may be a common occurrence in ALS (Behrouzi et al. 2016; Yang and Strong 2012).

1.9 *In vitro* effects of pseudophosphorylated (Thr175Asp-tau) tau

To investigate the above-mentioned tau pathology, full-length tau constructs were subjected to site-directed mutagenesis to replace Thr175 with either aspartic acid (Asp) or alanine (Ala). The expression of Asp at Thr175 (Thr175Asp-tau) mimics Thr175

phosphorylation, whereas expression of Ala at Thr175 (Thr175Ala-tau) causes inhibition of Thr175 phosphorylation (Gohar et al. 2009). This model of pseudophosphorylated tau (Thr175Asp-tau) has been shown to induce tau aggregate formation in human embryonic cells (HEK293T) and Neuro2A cells at 24, 48, and 72 hours post-plating (Gohar et al. 2009). Additionally, it was found that the 2N4R tau isoform shows the greatest tendency for fibril formation (Gohar et al. 2009).With pseudophosphorylation of tau, there is an enhanced rate of caspase-mediated cell death 96 hours post-plating (Gohar et al. 2009). Using the construct that inhibits phosphorylation at Thr175 (Thr175Ala-tau), has conversely been shown to significantly inhibit aggregate formation (Gohar et al. 2009). These *in vitro* findings suggest that phosphorylation of Thr175 on tau is pathologically relevant to ALSci (Moszczynski et al. 2015).

1.10 A potential mechanism of tau pathology in ALSci

The normal or abnormal function of tau is regulated by the phosphorylation of specific sites along the protein (Forman, Trojanowski, and Lee 2004). For example, phosphorylation of tau at Threonine residue 231 (Thr231) by the well-established tau kinase glycogen synthase kinase-3β (GSK3β) plays a major role in regulating the binding of tau to microtubules (Cho and Johnson 2004). GSK3β activation has been linked to tau pathology, and its subsequent phosphorylation of Thr231 is known to cause a conformational change in tau decreasing its ability to bind microtubules (Cho and Johnson 2004; Lin et al. 2007). It has been shown *in vitro* that Thr175Asp-tau induces GSK3β phosphorylation and activation, which in turn induces Thr231 phosphorylation and the formation of pathological intracellular tau fibrils (Moszczynski et al. 2015). Of direct relevance to ALSci, inhibition of GSK3β activity reduced both fibril formation and

cell death in Thr175Asp-tau transfected cells indicating a role of GSK3β in fibril formation (Moszczynski et al. 2015). Based on these *in vitro* results, a potential mechanism is proposed where pThr175-tau induces GSK3β activation, which leads to Thr231 phosphorylation and subsequent fibril formation (Moszczynski et al. 2015).

1.11 Animal models of neurodegeneration

Animal models play an important role in our understanding of the pathogenesis of neurodegenerative diseases such as tauopathies and ALS. They also provide the basis for developing and testing potential therapeutic agents for such diseases.

Transgenic mice and rat models of pathological tau deposition have been developed to examine the role of tau in human neurodegenerative diseases. Tau transgenic mice expressing the smallest human tau isoform under the PrP promotor develop an age-dependent tauopathy pathology (Ishihara et al. 1999). Ishihara et al. found that the tauopathy included tau-immunoreactive intraneuronal inclusions in cortical, brainstem and spinal cord motor neurons in association with progressive motor neuron degeneration (1999). These latter authors subsequently demonstrated progression of the tau pathology with increasing age (Ishihara et al. 2001). Similar expression of the smallest tau isoform in transgenic mice, while not inducing neurofibrillary tangles even at 19 months of age, did induce ‘pretangle’ tau deposition with immunoreactivity to several pathological tau phosphorylation sites observed in human neurofibrillary tangles (Brion and Tremp 1999). Taken as a whole, these studies reveal that transgenic models are effective for the investigation of tauopathies and neurodegenerative disease, and that a significant amount of time may be required to lead to measurable neurodegeneration.

There is also abundant evidence in rat models demonstrating the development of behavioural and neuropathological features consistent with a tauopathy in a wide range of paradigms. For example, transgenic rats expressing truncated human tau protein showed a progressive behavioural impairment, and widespread hyperphosphorylated tau deposits (Zilka et al. 2006, 2010). In another study, transgenic rats expressing Alzheimer's disease-associated tauP301L displayed prominent dendritic pathology (Korhonen et al. 2011). In summary, there have been a number of studies that were successful in developing transgenic mice and rat models that mimic pathologies observed in humans. However it is important to note that such models also present with the drawbacks of late onset, and lack of expression control.

As an alternative to transgenic models, somatic gene transfer by viral vectors is a method used extensively in animal studies. This method leads to efficient expression of a protein of interest in defined neuronal populations (Klein, Wang, and King 2009; McCown 2005). The recombinant adeno-associated viral vector serotype 9 (rAAV9) has been shown to produce the greatest expression in the neurons of the CNS with a reporter gene such as green fluorescent protein (GFP) and with neurodegenerative disease-related tau protein in comparison to other AAV serotypes (Klein et al. 2006, 2008). This allows for the ability to induce expression of the protein of interest at specific ages, which is especially beneficial to produce age-related models of neurodegenerative diseases (Klein et al. 2010). Ultimately, the somatic gene transfer technique, using rAAV viral vector, offers the benefits of being able to control the expression of the protein of interest in region-specific and age-specific ways.

1.12 Expression of tau constructs in the hippocampus

The cognitive and behavioural studies, for which this thesis reflects, involve of the use of somatic gene transfer using the rAAV9 viral vector to express a range of human tau constructs in the adult Sprague Dawley rat hippocampus (Strong Lab, unpublished data). The hippocampus was the targeted brain region because of the range of substantiated behavioural paradigms that serve to indicate abnormalities in hippocampal function of animal models. There were four inoculums used in the study including: an aberrantly phosphorylate tau mimic (Thr175Asp-tau); Thr175Ala-tau in which phosphorylation at Thr175 is inhibited; wt-tau; and EGFP controls. Each of these tau constructs was tagged with EGFP.

EGFP expression was confirmed in the hippocampus of all four treatment groups (wt-tau, Th175Asp-tau, Thr175Ala-tau, EGFP) for the entirety of the study (12 months post-surgery, Figure 2, Figure 3, Strong Lab, unpublished data). This indicated that the rAAV9 viral vector was effective for the expression of the human tau protein constructs in the hippocampus of the rat host. Expression was specific to the hippocampus (the structure of injection) as there was no EGFP expression in the cerebellum or thalamus (Figure 2, Strong Lab, unpublished data). However, there was variability in expression of the viral constructs throughout the hippocampal structure, as observed in Figure 5 (Strong Lab, unpublished data).

Additionally, tau fibrillary structures were identified at 3 months post-surgery in the experimental (Thr175Asp-tau) group (Figure 4, Strong Lab, unpublished data). The extent of the pathology and frequency within groups is not yet known.

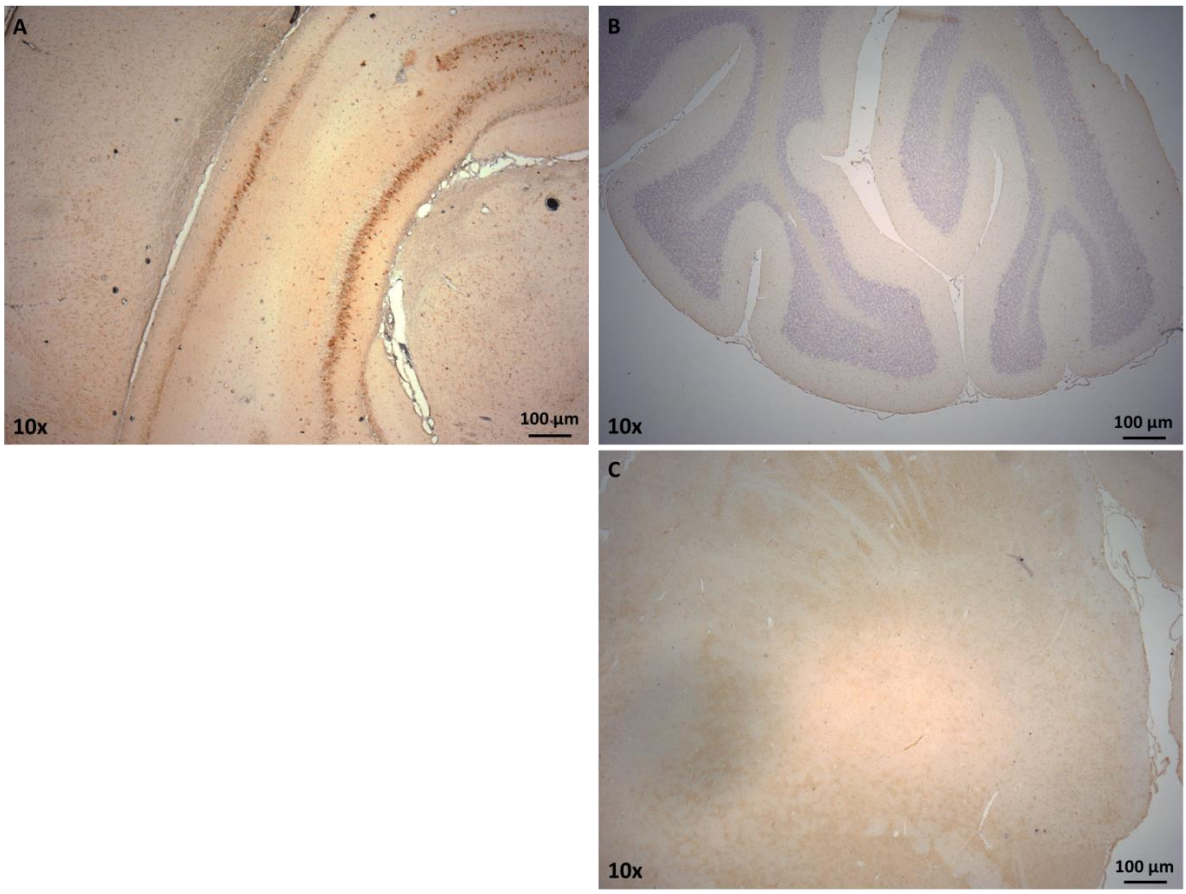

Figure 2: Hippocampal expression of EGFP-tagged rAAV9 Thr175Ala-tau at 1 month post-surgery

Rabbit anti-GFP 1:200 DAB stain. (A) Section from a Thr175Ala-tau injected rat brain at 1 month post-surgery confirms expression of the viral vector in neurons of the hippocampus. Section from a Thr175Asp-tau injected rat brain at 3 months post-surgery shows (B) no expression of EGFP in the cerebellum, and (C) no expression of EGFP in the thalamus.

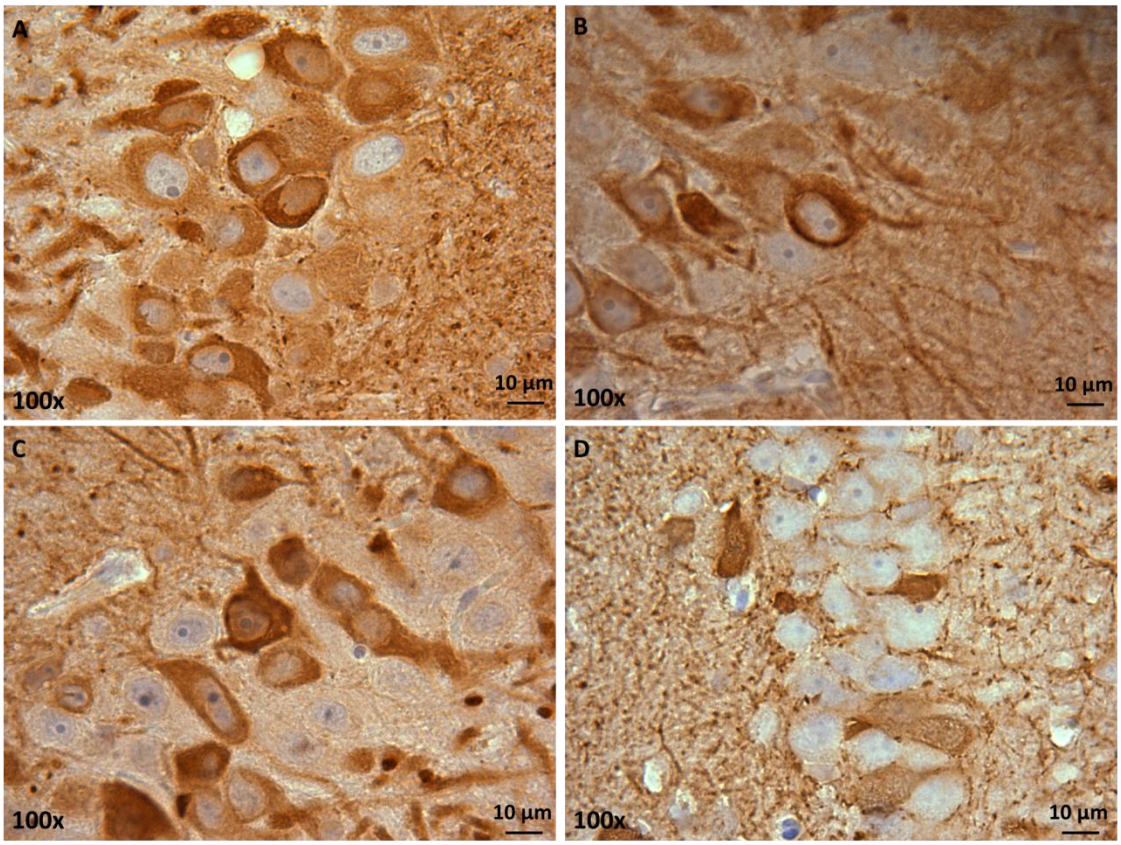

Figure 3: Expression of EGFP in all four treatment groups at 12 months post-surgery

Rabbit anti-GFP 1:200 DAB stains. Sections from (A) wt-tau injected rat hippocampus, (B) Thr175Asp-tau injected rat hippocampus, (C) Thr175Ala-tau injected rat hippocampus, and (D) an EGFP injected rat hippocampus. Each brain was collected 12 months post-surgery. These images confirm expression of the viral vector in the hippocampus.

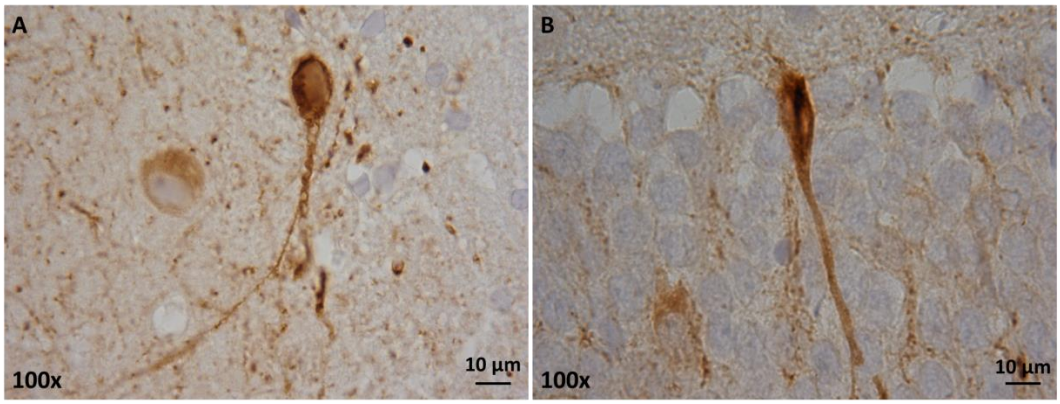

Figure 4: Expression of tau fibrillary structures in Thr175Asp-tau expressing rats

Rabbit anti-GFP 1:200 DAB stain. Section from a Thr175Asp-tau injected rat brain at 3 months post-surgery. (A) Tau fibril with corkscrew pathology, (B) tau fibrillary structure.

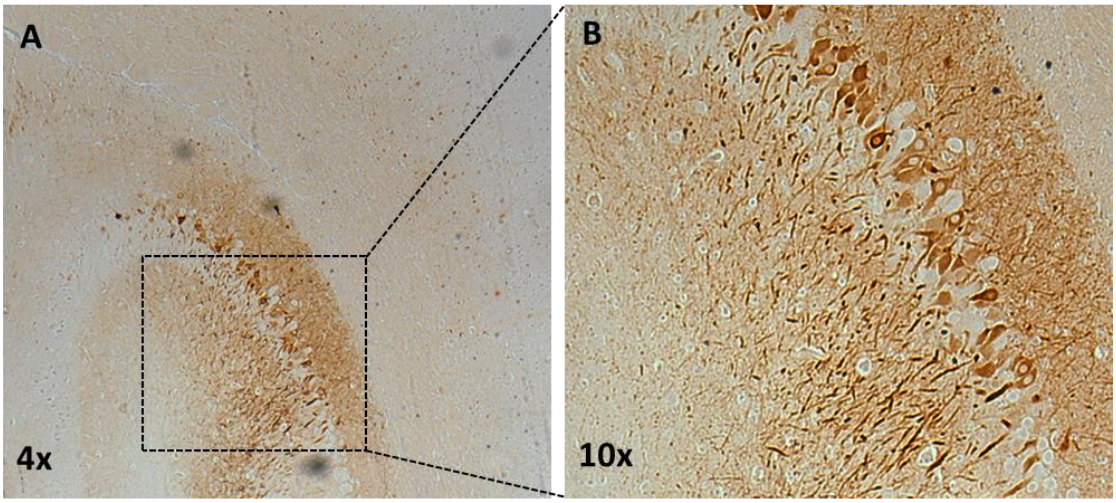

Figure 5: Variable expression of EGFP-tagged rAAV9 Thr175Asp-tau

Rabbit anti-GFP 1:200 DAB stain. Section from a Thr175Asp-tau injected rat brain at 3 months post-surgery. Expression is observed in the dentate gyrus (DG), and in CA3-CA2 regions, but not in the CA1 region.

1.13 Rationale

It has been established that significant proportions of ALS patients either present with, or develop frontotemporal dysfunction, whether it be ALSbi, ALSci or ALS-FTD. These neuropsychological deficits are also associated with shorter patient survival and an increased caregiver burden and depression. Previous observations reveal neuronal and extraneuronal tau depositions in ALSci with a unique tau phosphorylation site at Threonine residue 175 (pThr175-tau), which has led to the postulation that ALSci can be associated with alterations in tau metabolism. It is also known that pseudophosphorylated tau (Thr175Asp-tau) induces tau aggregate formation and increased cell death *in vitro* (Gohar et al. 2009). The effects of pThr175-tau have not been investigated *in vivo*. Therefore, the purpose of this study is to investigate the effects of expressing pseudophosphorylated tau (Thr175Asp-tau) in a rat model and characterize its pathological and behavioural consequences.

1.14 Hypothesis and Aims

Hypothesis:

Expression of aberrantly phosphorylated tau protein at pThr175 in the hippocampus leads to cognitive and behavioural deficits.

Aim:

Characterize the behavioural effects of expressing aberrantly phosphorylated (human 2N4R) tau at Threonine residue 175 (Thr175Asp-tau) in rat hippocampus by evaluating the effects on different hippocampus-related cognitive tasks, such as spatial learning and memory, exploratory and anxiety-like behaviour, as well as sensory filtering, and sensorimotor gating at 1, 3, 6, 9, and 12 month post-surgery time points.

PART 2: METHODOLOGY

2. Methodology

2.1 Stereotactic Injections and tau protein constructs

Somatic gene transfer

A somatic gene transfer technique was used to express recombinant adeno-associated virus (rAAV9) vectors: rAAV9 tau (wt-human tau), rAAV9 Thr175Alanine-tau (phosphorylation inhibition), rAAV9 Thr175Aspartic-tau (phosphorylation mimic), rAAV9 EGFP (control) in the hippocampus of wild-type female Sprague Dawley rats (Figure 6).

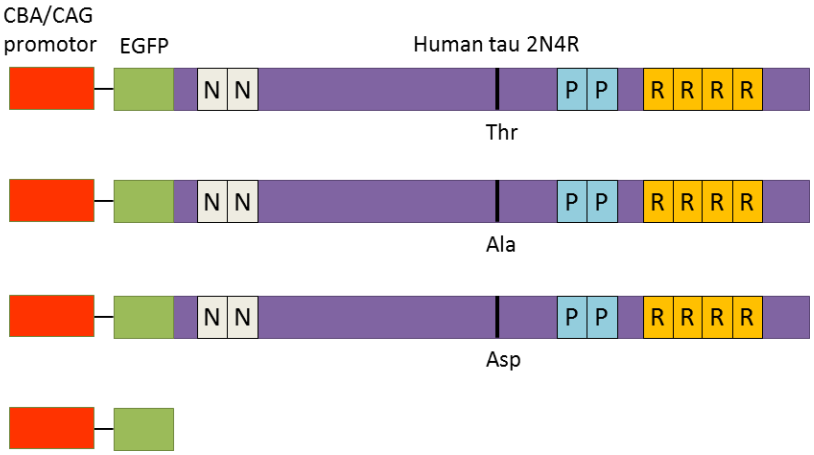

Figure 6: rAAV9 constructs injected as different treatment groups

Recombinant adeno-associated virus (rAAV9) vectors incorporating green fluorescent protein (EGFP) and 2N4R human tau. From top to bottom the constructs are as follows: rAAV9 tau (wt-human tau), rAAV9 Thr175Alanine-tau (phosphorylation inhibition), rAAV9 Thr175Aspartic-tau (phosphorylation mimic), rAAV9 EGFP (control).

Stereotactic injections procedure

All procedures followed the guidelines of Canadian Council on Animal Care and Western University Animal Use Subcommittee. Two-month old wild type female Sprague Dawley rats (Charles River Canada) were housed in pairs prior to surgery and randomly assigned to an experimental or control group. Food and water were provided *ad libitum*. Ten rats were used per inoculum, thus a total of 40 rats underwent surgery. Each rat weighed approximately 250 g at the time of surgery. All rats in this study were female because of a planned follow-up study which will utilize a double transgenic rat line that exhibits X-chromosome-linked transmission of the transgene of interest (further detail in 'Future Studies' section of Discussion).

Rats were weighed, anesthetized under an induction dose of 5% isofluorane and 2 L/min oxygen, and shaved at the top of their heads. With a stereotaxic apparatus, their heads were immobilized using ear bars as well as a mouth-piece. A plastic mask covered the nose and delivered a maintenance dose of 2.5% isoflurane anesthetic with 1% oxygen. Eye lube was applied to the eyes to avoid them from drying out during the procedure, and the bald area of the head was disinfected using a three-stage preparation: rub with soap, then ethanol, and finally iodine. Body temperature was maintained by placing a 37˚C isothermal pad beneath the rat for the duration of the procedure. A rostrocaudal incision was made along the midline of the scalp, and the subcutaneous tissue and periosteum were elevated to expose the underlying bone. Four small holes (1mm) were bored into the skull using an electric drill. Injections of the rAAV9 vectors were conducted using stereotactic coordinates, with Bregma as the reference point. Four inoculations per animal were conducted at two sites per side (3 µL per site), all within the hippocampus at the

following coordinates: A/P: -5.5 mm, M/L: ±4.6 mm with D/V: -3.2 mm, and M/L: ±6.0 with D/V: -6.0 mm (Figure 7). Using Hamilton syringes, a total volume of 12 µL (3 µL per site) injected over the course of 20 minutes (5 minutes per site) were injected for a total of 1.32×10^{10} vector genomes. After the injection, the syringe tip remained in place for 2 minutes, before being slowly withdrawn. The coordinates and vector volume were adopted from Mustroph et al. (2012).

Rats were then sutured and administered 0.1 mL/100 g Baytril, and 0.1 mL/100 g Meloxicam subcutaneously. After surgery, rats were placed in a recovery cage under a heat lamp until fully recovered from the anesthesia. Rats were then singly housed for 1 week to allow for the incision to close, and then were paired with another rat.

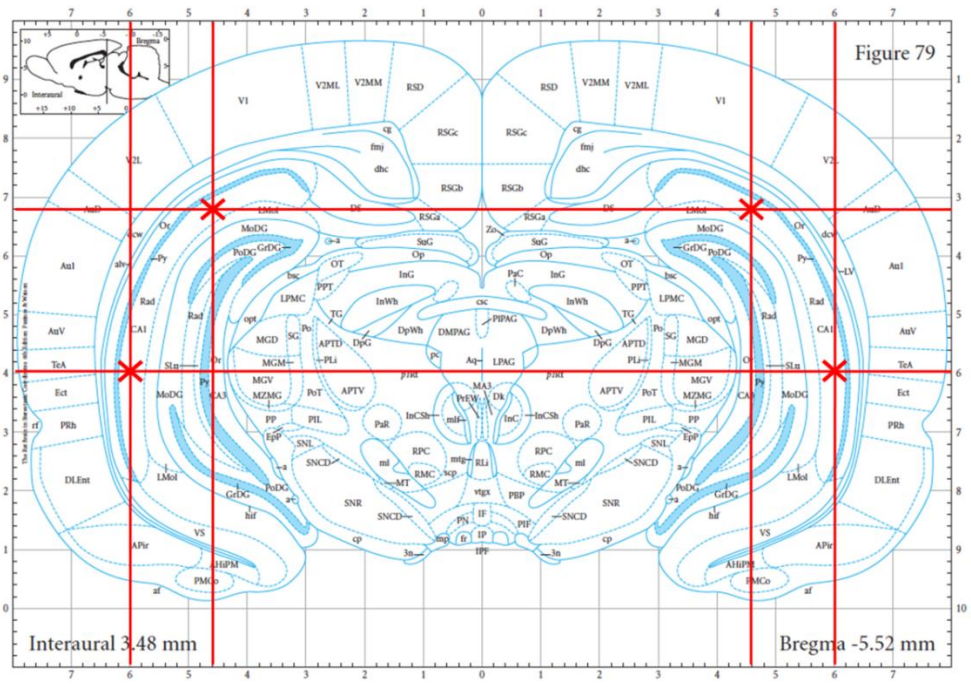

Figure 7: Stereotactic coordinates represented on cross-section of rat brain

Injection sites in the rat hippocampus; 4 injection sites with 3 µL injected into each site (total of 12 µL per rat). The injection coordinates from Bregma are as follows: left lateral injection; A/P: -5.5 mm, M/L: +6.0 mm, D/V: -6.0 mm, left medial injection; A/P: -5.5 mm, M/L: +4.6 mm with D/V: -3.2 mm, right lateral injection; A/P: -5.5 mm, M/L: -6.0 mm, D/V: -6.0 mm, right medial injection; A/P: -5.5 mm, M/L: -4.6 mm with D/V: -3.2 mm.

2.2 Behavioural Testing

Open Field Testing

The open field test (OFT) was used to determine exploratory behaviour and to provide a measure of potential anxiety-like behaviour. Animals were placed in the testing room at least 1 hour before testing. The open field is a square enclosure 45.7 cm x 45.7 cm with surrounding walls 40.6 cm high that prevent escape (Figure 8). The open field boxes were divided into two zones: the center is defined by a conceptual square of approximately 27.5 x 27.5 cm, and the periphery by the perimeter of the box (Figure 8). The open field boxes were cleaned with 95% ethanol before testing, and between each test session to remove any scent of urine and feces. Rats were placed in the open field box for 30 minutes and monitored by an overhead camera. The analysis of activity was performed using ANY-MAZE software version 4.82. The total time spent moving, the distance traveled, as well as the time spent in each zone was analyzed.

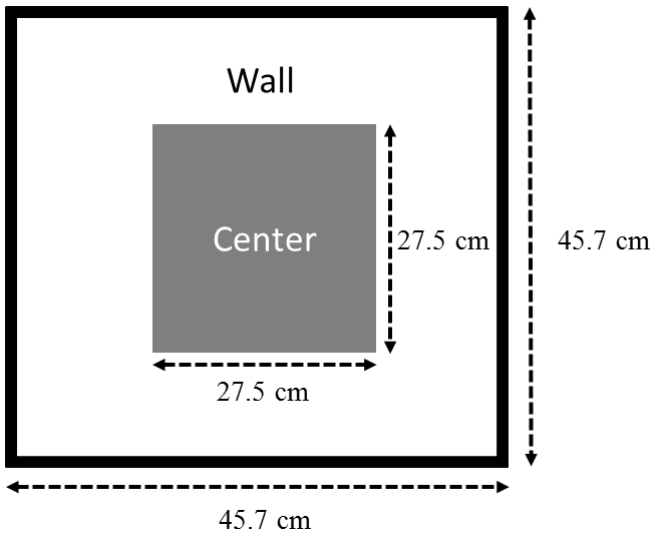

Figure 8: Open field test (OFT) box schematic

Overhead schematic of the open field test (OFT) box. The open field is a square enclosure 45.7 cm x 45.7 cm with surrounding walls 40.6 cm high that prevent escape. The center is defined by a conceptual square of approximately 27.5 x 27.5 cm.

Acoustic Startle Response

The acoustic startle response (ASR) was measured to analyze sensory filtering, and sensorimotor gating. To measure the ASR, Med Associate startle boxes were used (Figure 9). These sound-proof boxes contain a force plate on which the rats are placed, and the whole-body startle response results in the vertical dislocation of the platform. This vertical dislocation of the platform is transduced into a voltage and translated to digital startle amplitude readout on a computer for data analysis. Before placement on the platform, rats were placed in a clear cylindrical plastic tube to prevent escape from the platform. The tubes contain bored holes to allow the sounds from the speakers (startle stimuli) within the boxes to be heard without being muffled by the clear cylinder.

An acclimation phase was used for the rat to adapt to the animal holder and startle box with background noise. During a 5 minute acclimation period, the rat was placed in the cylindrical tube, placed in the startle box, and presented with constant background noise of 65 dB white noise with no startle stimuli. This lasted a total of 5 minutes.

Next, an input/output (i/o) function was established for each individual rat tested. This started with a 5 minute acclimation period, followed by 12 startle stimuli of 20 ms white noise and increasing intensity from 65 dB to 120 dB in 5 dB steps, with an interstimulus interval of 15 seconds. The readout of the startle response amplitude gives the experimenter an idea of each animal's startle intensity. With this information, the experimenter can increase or decrease the sensitivity of the platform if the animal has low or high startle intensity, respectively. This i/o function protocol lasts a total of 9 minutes, with the white background noise continuing through the entirety of the test.

For assessing sensory filtering and sensorimotor gating, short-term habituation (STH) and prepulse inhibition (PPI) were measured respectively, within the following startle testing protocol. It started with a 5 minute acclimation period, followed by Block I: Habituation, and Block II: PPI. Block I contained 50 startle stimuli that consisted of a white noise of 20 ms duration and 110 dB intensity, with an interstimulus interval of 20 sec. Block II consisted of pseudorandomized trials of startle stimuli with and without a prepulse. The prepulse was a white noise of 4 ms duration with either 75 or 85 dB intensity, preceding the startle stimulus by either 30 or 100 ms. The pseudorandomized trials are as follows:

- 10 startle stimuli with no prepulse
- 10 startle stimuli with prepulse at 75 dB and ISI of 30 ms
- 10 startle stimuli with prepulse at 75 dB and ISI of 100 ms
- 10 startle stimuli with prepulse at 85 dB and ISI of 30 ms
- 10 startle stimuli with prepulse at 85 dB and ISI of 100 ms

This adds to a total of 50 trials in Block II and lasted 31 minutes, with the white 65 dB background noise continuing through the entire test. The startle testing lasted over 3 days in order to acclimate the animal to the startle boxes, to establish an input/output (i/o) function, and to actually test the animals. The protocol outline was as follows:

- Day 1: Acclimation → 2 hour break (minimum)→ Acclimation
- Day 2: Acclimation → 2 hour break (minimum) → i/o function
    - The i/o function establishes the Gain for each rat to be used on Startle testing
- Day 3: Startle (STH and PPI)

Figure 9: Med Associates acoustic startle box

Photograph of a Med Associates startle box. Inside at the back of the box is a speaker, with the platform in front of it. The rat is placed in a clear, plastic tube before being placed on the platform. To the right of the platform are the knobs for calibrating the box and setting the Gain for each rat. The two swivel doors on the front are closed during testing.

Morris Water Maze (MWM)

The MWM task was used to assess spatial learning and memory in the four groups of rats in this study. The water maze consisted of a circular pool with a depth of 58 cm, and 145 cm in diameter, which was conceptually divided into four quadrants (Q1, Q2, Q3, Q4) using cardinal coordinates North, East, South and West (not the true magnetic coordinates, Figure 10). The pool was filled with water (20-23˚C to a depth of 33 cm) and turned opaque using black dye. In one of the quadrants a hidden, movable, transparent circular acrylic platform of 11.4 cm diameter was placed in the pool 1-2 cm below the water surface. The quadrant containing the platform was referred to as the target quadrant. Distinctive, distal visual cues were placed on the walls of the room at each cardinal coordinate for facilitating the spatial orientation of the animal. Animals were placed in the testing room at least 1 hour before testing on each day of testing.

To test spatial learning, the platform location remained constant in one quadrant. The rats were trained in 4 trials per day for 4 consecutive days. For each trial, the rat was placed in a different start location around the pool and allowed 60 seconds to find the hidden platform. If the rat did not find the platform in the allotted 60 seconds, it was gently guided to the platform. The rat was allowed 15 seconds on the platform for each trial before being picked up. Each trial was recorded with a camera, and the analysis of activity was performed using ANY-MAZE software version 4.82. The average of the time taken to find the platform during the 4 trials was calculated for each of the 4 days to compare performance over the 4 days of learning. The performance of the spatial learning task was expressed in terms of latency and distance traveled to find the platform.

To test reference memory, a single probe trial was conducted on the 5th day of testing (24 hours after the 4th spatial learning trial) where the hidden platform was removed from the pool. The rat was placed in the pool at a novel start location and allowed to swim for 30 seconds. The distance traveled and the time spent within the target quadrant, as well as an area surrounding where the platform was during the learning trials, were measured during the 30 seconds of swimming. An area larger than the area of the actual platform (twice the diameter of the platform) was used in the analysis to account for the fact that the computer tracking system considers the rat as a single point, as opposed to observing the rat's whole body. Note that the time spent in the area where the platform was during the learning trials is a more specific index of spatial location compared to the time spent within the target quadrant.

The protocol for MWM testing was altered at different time points (adding to the original protocol used at month 1 post-surgery), using tests with increasing sensitivity. Specific changes to the protocol were as follows: a probe trial 7 days after the first probe trial was added to test long-term memory for months 6 and 9 post-surgery, and reversal learning was added to the month 12 post-surgery time point. The long-term memory test was not conducted at month 12 because the reversal learning protocol interfered with the long-term protocol, and the previous long-term results had been inconclusive. The test for LTM may have been compromised by the fact that other behavioural tasks were conducted during the 7 days between the probe trials. The different MWM protocols for all time points are summarized briefly below:

Time point	MWM Protocol outline
Month 1	• 4 days learning • Probe day 5
Month 3	• 4 days learning • Probe day 5
Month 6	• 4 days learning • Probe day 5 • Probe day 12
Month 9	• 4 days learning • Probe day 5 • Probe day 12
Month 12	• 4 days learning • Probe day 5a • Reinforcement day 5b • Reversal learning day 6a • Reversal learning day 6b • Reversal probe day 7

Each time point had a different platform location than the previous time point. The experimentation was performed and the data was collected and analyzed by an individual blind to the experiment. All experimentation was carried out during the light cycle.

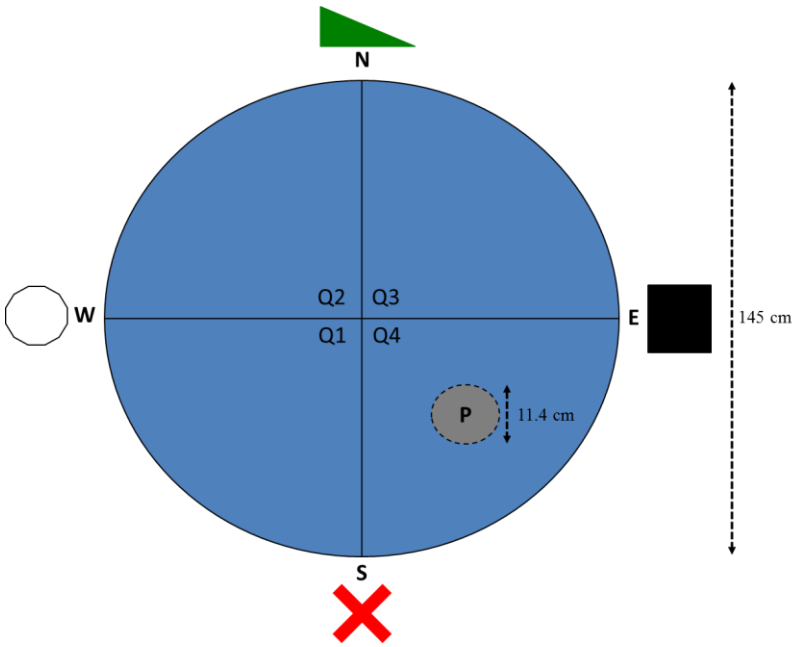

Figure 10: Morris Water Maze schematic

Overhead schematic of the Morris water maze (MWM). The MWM consists of a circular pool of 145 cm in diameter; conceptually divided into 4 quadrants (Q1, Q2, Q3, and Q4) using cardinal coordinates North, East, South and West (not the true magnetic coordinates). This pool is filled with water (20-23˚C to a depth of 33 cm) and turned opaque using black dye. In one of the quadrants a hidden, movable, transparent circular acrylic platform (P) of 11.4 cm in diameter is placed in the pool 1-2 cm below the water surface. The quadrant containing the platform is referred to as the target quadrant (Q4 in the schematic). Distinctive, distal visual cues were placed on the walls of the room at each cardinal coordinate for facilitating the spatial orientation of the animal (represented by the different shapes of varying colours in the schematic).

2.3 Statistical Analysis

IBM SPSS Statistics 20 was used for statistical analysis. Data are expressed as group means (groups are defined by the four treatments) ± the standard error of the mean (SEM). Student's t-tests, one-way, and two-way analysis of variance (ANOVA) testing were used where appropriate. Repeated measures ANOVA were also used to compare groups. These tests were followed by post hoc tests and Bonferroni corrections. The symbol " * " indicates statistical significance between group means ($p<0.05$).

PART 3: RESULTS

3. Results

3.1 Changes in n-values across testing time points as a result of aging

Rats were subjected to behavioural testing at 1, 3, 6, 9, and 12 month post-surgery time points. There was an age-related decline in the health of the rats in this study. This lead to the death of many rats in the different treatment groups at various time points (Table 1). Some lethal health issues included respiratory disease, as well as tumours in a range of anatomical locations. Further details of health-related issues are outlined in the 'Study Limitations' section of the Discussion.

Table 1: animal treatment groups and their respective n-values at the given testing time points.

	Behaviour testing time point				
	Month 1	**Month 3**	**Month 6**	**Month 9**	**Month 12**
Wt-tau	n=10	n=10	n=10	n=7	n=6
Thr175Asp-tau	n=10	n=10	n=9	n=8	n=7
Thr175Ala-tau	n=9	n=9	n=9	n=9	n=8
EGFP	n=10	n=9	n=9	n=9	n=9

Months refer to the number of months post-surgery. The sample size (n value) of each treatment group changed across testing time points. This occurred due to the death of rats typically from health related issues (refer to 'Study Limitations' section in Discussion for more details).

3.2 Open Field Testing Results

3.2.1 Normal habituation of exploratory behaviour in all groups at all time points

Rats were subjected to open field testing (OFT) at 1, 3, 6, 9, and 12 month post-surgery time points to examine exploratory and anxiety-like behaviour. The rats were tracked over a time period of 30 minutes in the open field box, and data was separated into 5 minute blocks. One rat had to be excluded from analysis since the video tracking system did not track it properly. Repeated measures ANOVA showed an effect of block numbers ($F(5,25)=91.257$, $p<0.01$), but not of treatment ($F(3,25)=0.094$, $p=0.963$), or interaction of the two factors ($F(15,25)=0.847$, $p=0.624$), indicating that all groups habituated without significant differences in locomotion between groups. This was the case at all behavioural testing time points (representative graphs at Month 12 testing shown, Figure 11).

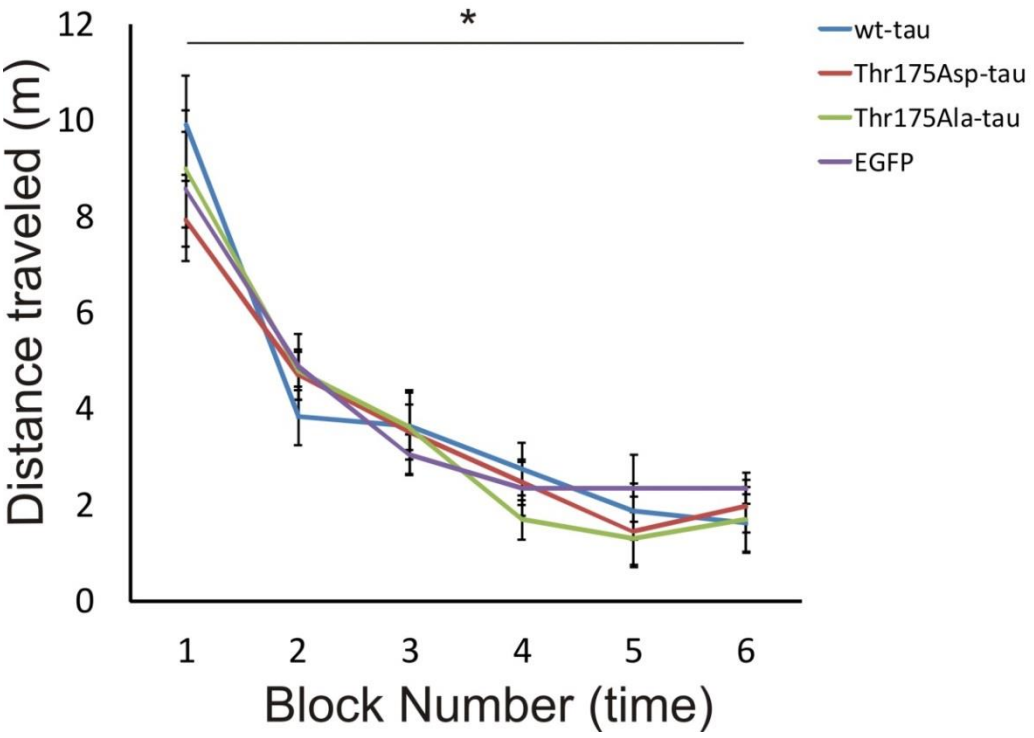

Figure 11: Ambulatory distance traveled over 30 minutes in OF box at 12 months post-surgery

Rats expressing either wt-tau (n=6), Thr175Asp-tau (n=7), Thr175Ala-tau (n=8), EGFP (n=9). Each Block represents 5 minutes (total of 30 minutes inside the OF box). The figure shows a trend of decreased activity as time progressed for each group. Data are expressed as means ± SEM.

3.2.2 Total distance traveled by each treatment group across all time points

The total distances traveled at each time point was analyzed, however a different testing location with different open field boxes were used for the first two versus the latter three time points. As such, the distance traveled for months 1 and 3 cannot be compared with subsequent time points. When comparing total distance traveled there was a significant decrease in the total distance traveled between months 6-12, with no significant difference between the treatment groups (Figure 12). Two-way ANOVA revealed a significant effect of time point on total distance ($F(3,87)=19.560$, $p<0.01$), with no significant differences between treatment groups across time points ($F(6,87)=0.729$, $p=0.628$) for the 6-12 month time periods.

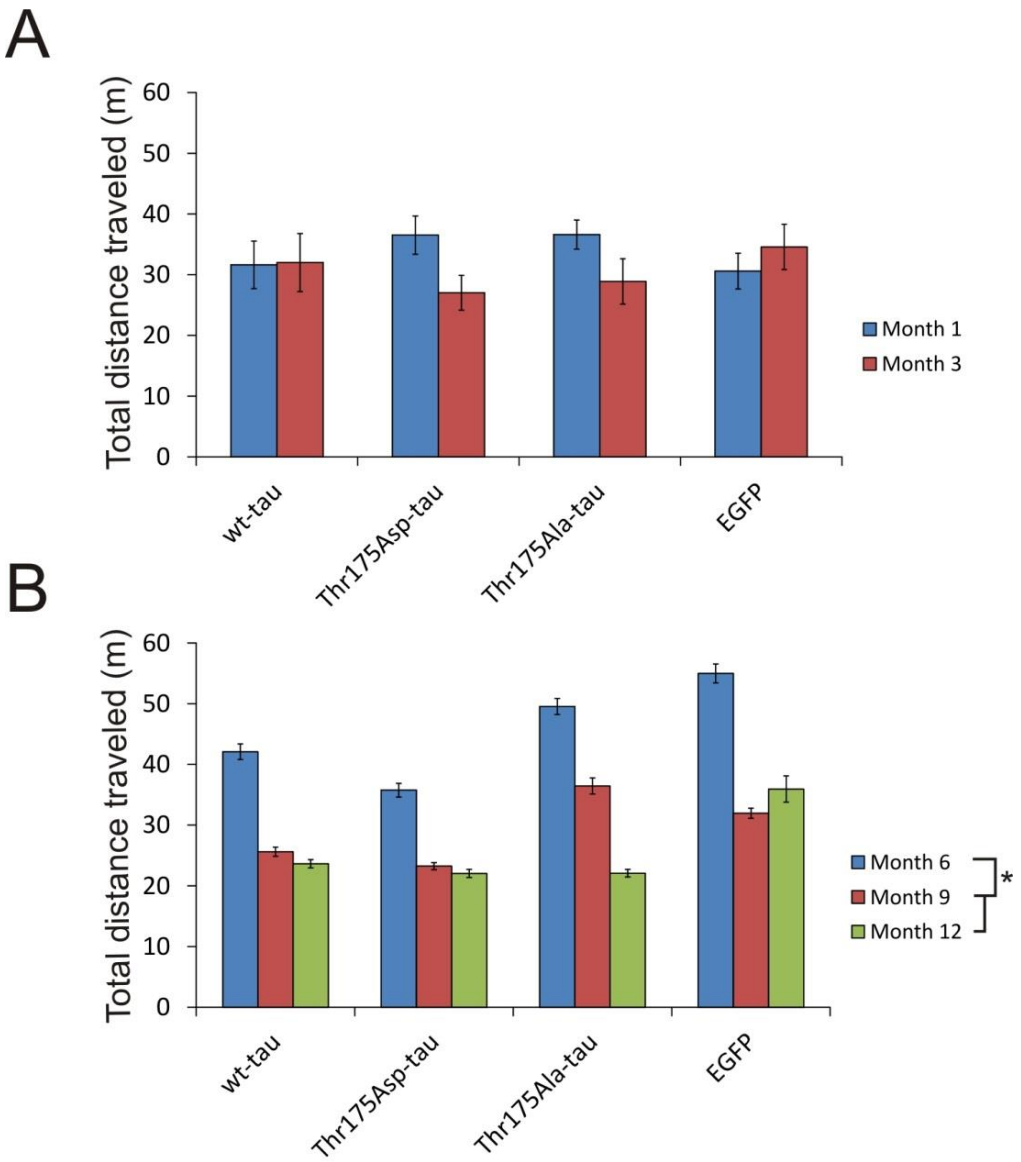

Figure 12: Total distance traveled in open field test at all time points

Rats expressing either wt-tau, Thr175Asp-tau, Thr175Ala-tau, EGFP (refer to Table 1 for n values). This graph shows the total distance traveled by each treatment group at (A) months 1 and 3, and (B) months 6, 9, and 12 of testing in the OFT. (B) There was a significant effect of total distance in terms of time points, with no significant differences between treatment groups across time points. Data are expressed as means ± SEM. * indicates statistical significance, $p < 0.05$.

3.2.3 No anxiety-like behaviour indicated between treatment groups at any time point

The ratio of distance traveled in the center of the box versus wall of the box is an indication for anxiety-like behaviour, since anxious animals tend to stay close to the walls. ANOVA revealed no significant difference between treatment groups at any of the time points ($F(3,26)=0.177$, $p=0.911$), indicating no differences in anxiety-like behaviour (representative graphs at Month 12 testing shown, Figure 13).

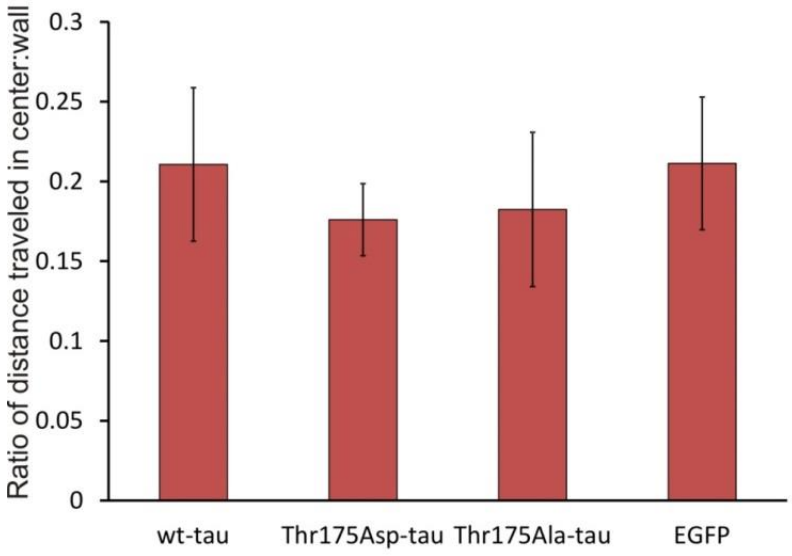

Figure 13: Center to wall ratio as an Anxiety measure at month 12

Rats expressing either wt-tau (n=6), Thr175Asp-tau (n=7), Thr175Ala-tau (n=8), EGFP (n=9). Open field test data at 12 months post-surgery. All four treatments showed a strong preference for the periphery of the open field box as opposed to the center of the box, with no statistically significant differences in the ratio of center to wall distance traveled between the four treatment groups. Data are expressed as means ± SEM.

3.3 Startle Reflex Results

3.3.1 Habituation of the acoustic startle reflex (ASR)

The amplitude of startle in response to an acoustic stimulus at months 3, 6, 9 and 12 post-surgery was measured to assess short-term habituation and prepulse inhibition as a measure for sensory filtering and sensorimotor gating, respectively. Habituation of the startle response was analyzed over 50 trials. Figure 14 depicts absolute startle amplitudes over 50 trials at the 3-12 month time points. All groups show habituation to the acoustic startle stimuli over the repeated trials at every time point (Figure 14). Repeated measures ANOVA revealed habituation to the 50 acoustic stimuli, without significant interactions of trial and treatment groups, and without significant differences between the four treatment groups at all time points. At month 3, treatment groups showed habituation ($F(49, 1666)=2.674$, $p<0.01$), no significant interaction of trials and treatment groups ($F(147, 1666)=0.793$, $p=0.965$), and no significant differences between the treatment groups ($F(3, 34)=0.118$,$p=0.949$). At month 6, treatment groups showed habituation ($F(49, 1617)=2.349$, $p<0.01$), no significant interaction of trials and treatment groups ($F(147, 1617)=1.084$, $p=0.241$), and no significant differences between the treatment groups ($F(3, 33)=0.389$,$p=0.761$). At month 9, treatment groups showed habituation ($F(49, 1421)=2.268$, $p<0.01$), no significant interaction of trials and treatment groups ($F(147, 1421)=0.959$, $p=0.621$), and no significant differences between the treatment groups ($F(3, 39)=0.251$,$p=0.885$). At month 12, treatment groups showed habituation ($F(49, 1274)=2.624$, $p<0.01$), no significant interaction of trials and treatment groups ($F(147, 1274)=0.912$, $p=0.759$), and no significant differences between the treatment groups ($F(3, 26)=1.391$,$p=0.268$).

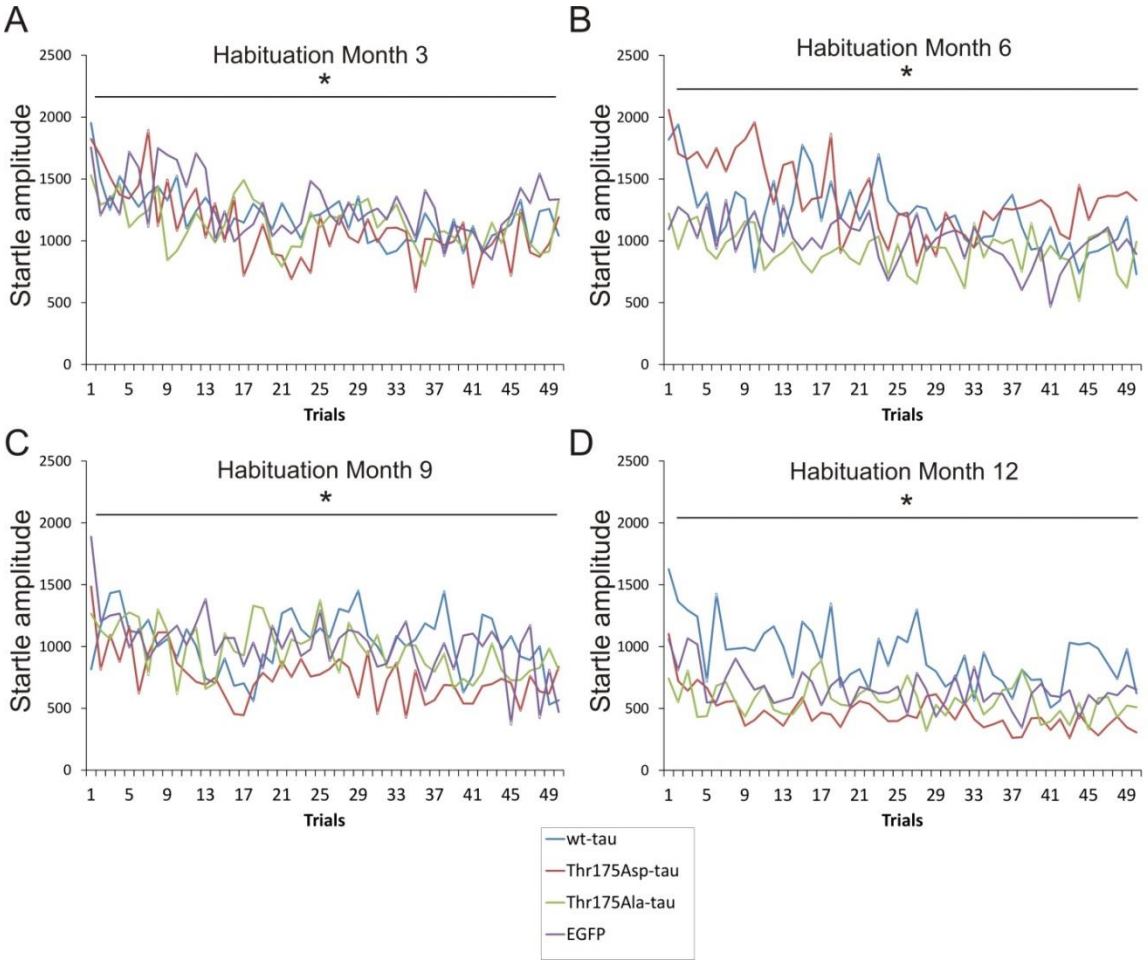

Figure 14: Short-term habituation at months 3, 6, 9, and 12 post-surgery

Rats expressing either wt-tau, Thr175Asp-tau, Thr175Ala-tau, or EGFP (refer to Table 1 for n values). Short-term habituation data at the (A) 3 month, (B) 6 month, (C) 9 month, and (D) 12 month post-surgery time points. All four treatment groups showed habituation to the startle stimuli when presented with repeated trials. There was no significant interaction of trials and treatment groups, and there were no significant differences between the four treatment groups at any time point. Data are expressed as means.

3.3.2 Prepulse inhibition (PPI)

Rats were tested for PPI with prepulse intensities of 85 and 75 dB. A prepulse intensity of 85 dB produces a more robust and maximum PPI, whereas PPI with a 75 dB prepulse is more subtle. Both of these prepulse intensities were tested at two interstimulus intervals (ISI's) of 30 and 100 ms. Different receptor subtypes have been suggested to mediate PPI at these different ISI's (Yeomans et al. 2010).

For the 85 dB prepulse, ANOVA followed by post hoc test with Bonferroni corrections revealed a significant effect of ISI at month 3 ($F(1,34)=8.048$, $p<0.01$), without a significant interaction of ISI and treatment ($F(3,34)=1.788$, $p=0.168$), and no significant differences between the four treatment groups ($F(3, 34)=1.774$, $p=0.171$) (Figure 15). At the subsequent time points there were no significant effects of ISI, treatment, or an interaction of ISI and treatment. At month 6 there was no significant effect of ISI ($F(1,33)=0.242$, $p=0.626$), no significant interaction of ISI and treatment ($F(3,33)=1.207$, $p=0.322$), and no significant differences between the four treatment groups ($F(3, 33)=0.513$, $p=0.676$). At month 9 there was no significant effect of ISI ($F(1,29)=1.350$, $p=0.255$), no significant interaction of ISI and treatment ($F(3,29)=0.449$, $p=0.720$), and no significant differences between the four treatment groups ($F(3, 29)=0.278$, $p=0.841$). At month 12 there was no significant effect of ISI ($F(1,26)=0.270$, $p=0.608$), no significant interaction of ISI and treatment ($F(3,26)=1.459$, $p=0.249$), and no significant differences between the four treatment groups ($F(3, 26)=0.153$, $p=0.927$).

For the 75 dB prepulse, ANOVA followed by post hoc test with Bonferroni corrections revealed a significant effect of ISI, without a significant interaction of ISI and treatment, and no significant differences between the four treatment groups at all time points (Figure

16). At month 3, there was a significant effect of ISI ($F(1,34)=0.411$, $p<0.01$), no significant interaction of ISI and treatment ($F(3,34)=0.122$, $p=0.947$), and no significant differences between the four treatment groups ($F(3, 34)=0.507$, $p=0.680$). At month 6, there was a significant effect of ISI ($F(1,33)=9.048$, $p<0.01$), no significant interaction of ISI and treatment ($F(3,33)=0.559$, $p=0.646$), and no significant differences between the four treatment groups ($F(3, 33)=0.248$, $p=0.241$). At month 9, there was a significant effect of ISI ($F(1,29)=11.994$, $p<0.01$), no significant interaction of ISI and treatment ($F(3,29)=0.653$, $p=0.588$), and no significant differences between the four treatment groups ($F(3, 29)=0.274$, $p=0.844$). At month 12, there was a significant effect of ISI ($F(1,26)=30.301$, $p<0.01$), no significant interaction of ISI and treatment ($F(3,26)=1.149$, $p=0.348$), and there no significant differences between the four treatment groups ($F(3, 26)=0.422$, $p=0.739$).

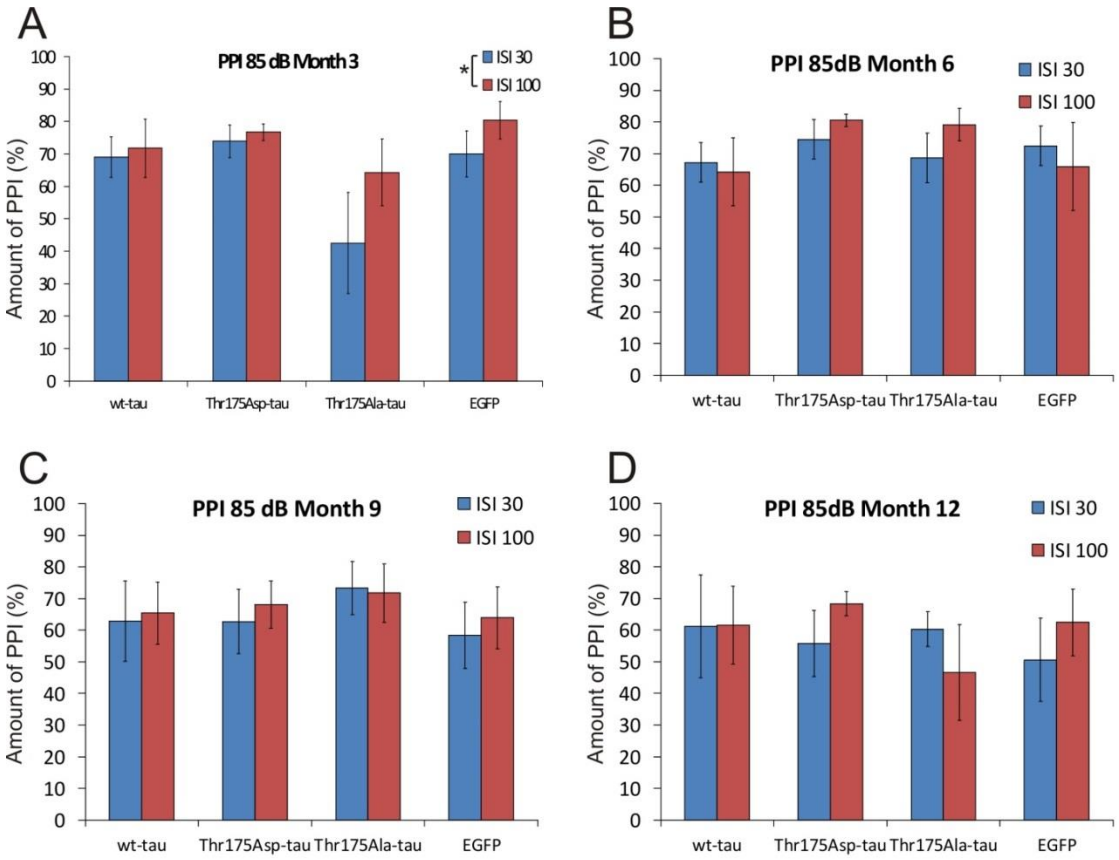

Figure 15: PPI at 85 dB with ISI 30 and 100 ms at 3, 6, 9, and 12 months post-surgery

Rats expressing either wt-tau, Thr175Asp-tau, Thr175Ala-tau, or EGFP (refer to Table 1 for n values). Prepulse inhibition with a prepulse of 85 dB and interstimulus intervals (ISI) of 30 and 100 ms. (A) At month 3 post-surgery there was a significant difference between startle ISI 30 and 100 at 85 dB. There was no significant interaction of ISI and treatment, and there were no significant differences between the four treatments. There were no significant differences at the (B) 6 month, (C) 9 month, or (D) 12 month time points in terms of either ISIs or treatment groups. Data are expressed as means ± SEM. * indicates statistical significance, $p<0.05$.

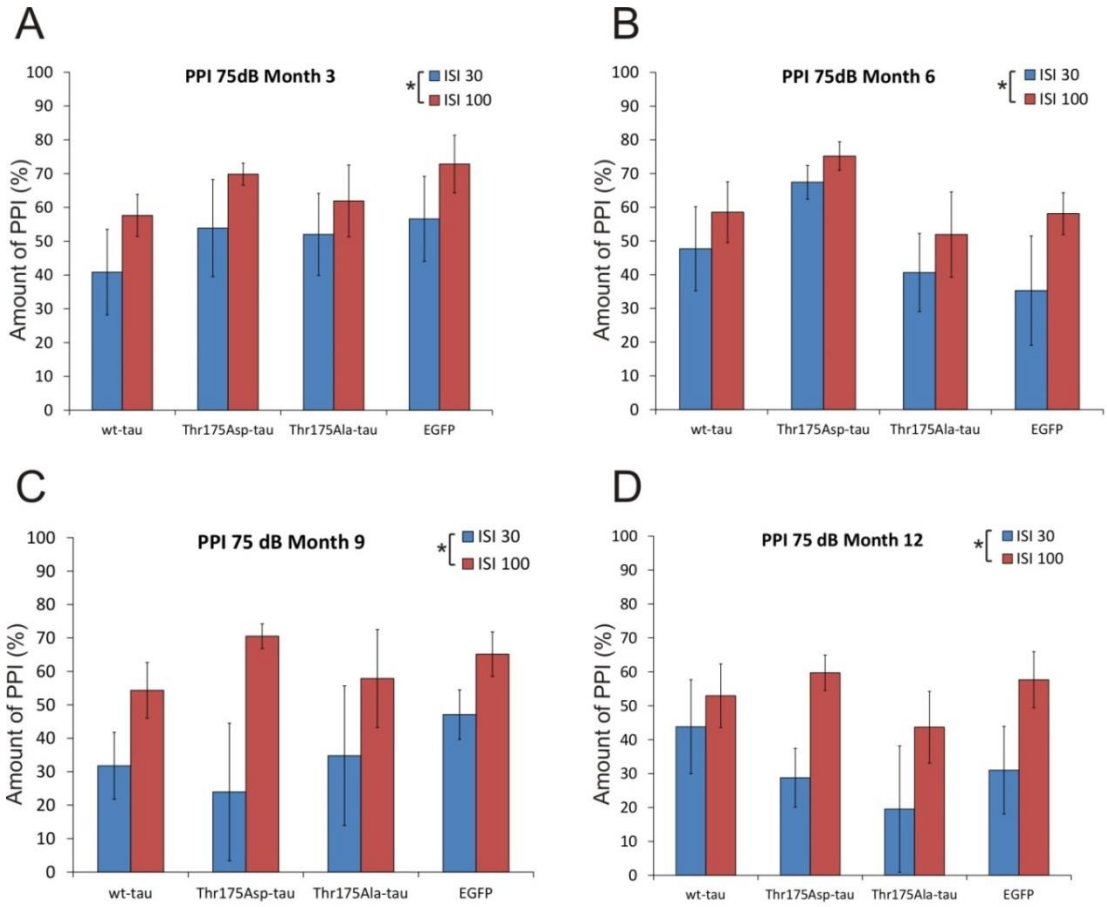

Figure 16: PPI at 75 dB with ISI 30 and 100 ms at 3 months post-surgery

Rats expressing either wt-tau, Thr175Asp-tau, Thr175Ala-tau, or EGFP (refer to Table 1 for n values). Prepulse inhibition (PPI) with a prepulse of 75 dB and interstimulus intervals (ISI's) of 30 and 100 ms. At the (A) 3 month, (B) 6 month, (C) 9 month, and (D) 12 month post-surgery time points there was a significant difference between startle ISI 30 and 100 at 75 dB. There were no significant interactions of ISI and treatment, and no significant differences between the four treatment groups at any given time point. Data are expressed as means ± SEM. * indicates statistical significance, $p<0.05$.

3.3.3 Longitudinal prepulse inhibition (PPI)

Prepulse inhibition data was also analyzed across testing periods (months 3, 6, 9, and 12 post-surgery) to assess for potential age effects. No effects of testing months were observed at either ISI 30 or ISI 100 for the 85 or 75 dB prepulses. For the 85 dB prepulse, ANOVA showed no significant effect of testing time point on ISI 30 ($F(3,134)=1.465$, $p=0.227$) or ISI 100 ($F(3,134=1.812$, $p=0.148$). For the 75 dB prepulse, ANOVA showed no significant effect of testing time point on ISI 30 ($F(3,134)=2.096$, $p=0.104$) or ISI 100 ($F(3,134)=1.318$, $p=0.271$).

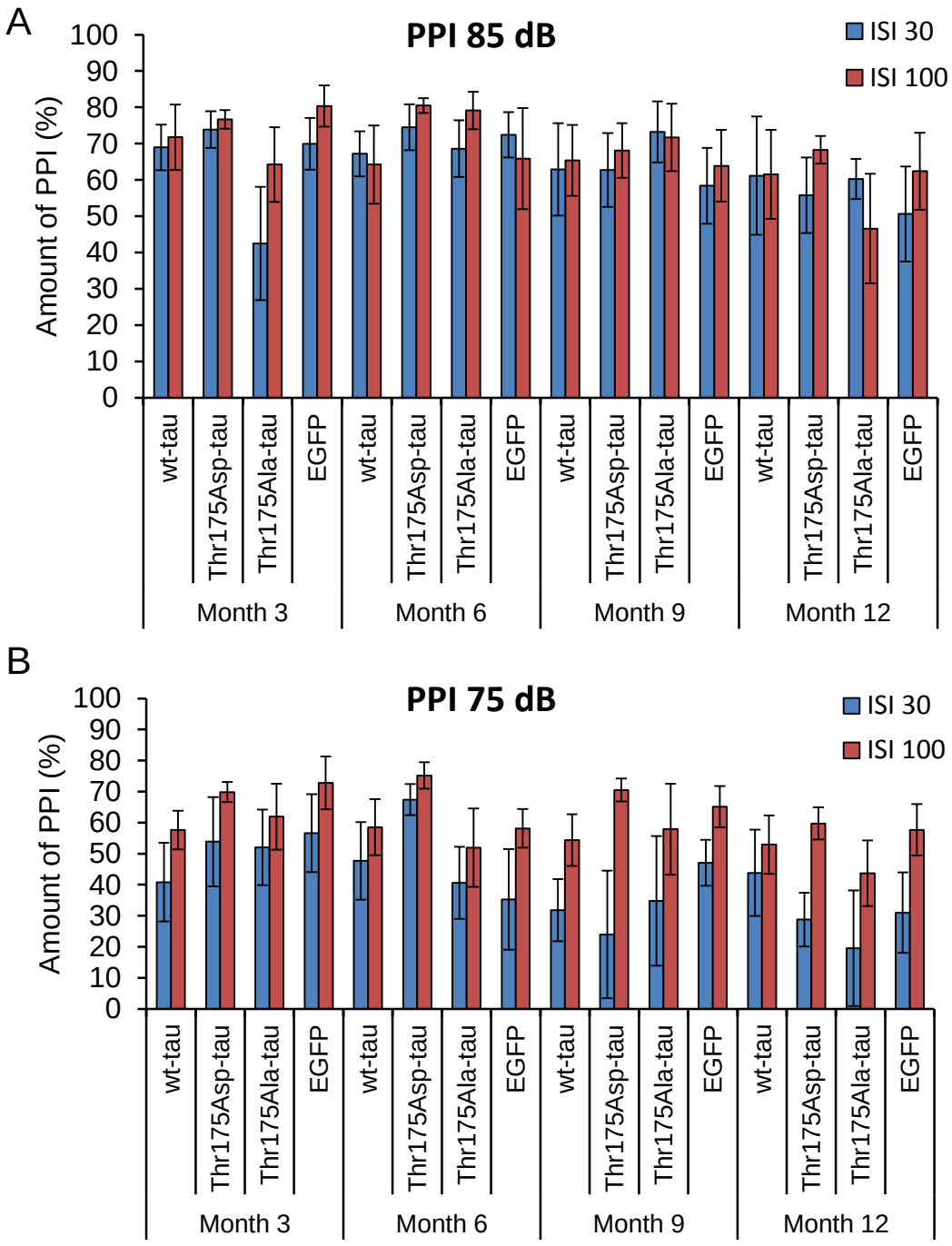

Figure 17: Longitudinal PPI at 85 dB and 75 dB

Rats expressing either wt-tau, Thr175Asp-tau, Thr175Ala-tau, or EGFP (refer Table 1 for n values). Prepulse inhibition (PPI) with a prepulse of 75 dB and interstimulus intervals (ISI's) of 30 and 100 ms at the 3, 6, 9 and 12 month time points. (A) PPI 85 dB. (B) PPI 75 dB. There was not a significant effect of time on PPI with ISI 30 or 100 at either 85 or 75 dB prepulse intensities. Data are expressed as means ± SEM.

3.4 Morris Water Maze Results

3.4.1 Spatial learning in the Morris water maze

To test for spatial learning and memory in the rats, the Morris water maze (MWM) was performed on months 1, 3, 6, 9, and 12 post-surgery. The protocol involved 4 spatial learning trials per day for 4 days. ANOVA revealed that all treatment groups were successful in learning the task after the 4 days of spatial learning as the latency to reach the platform decreased over the 4 testing days at each time point (representative data at month 12, Figure 19, $F(3,78)= 13.337$, $p<0.01$). There was not a significant difference in the time taken to find the platform over the 4 testing days between the treatment groups (representative graphs at month 12 testing shown Figure 19, $F(3,26)=3.104$, $p=0.044$). Additionally, multivariate ANOVA revealed a decrease in latency between testing months ($F(16,628)=6.876$, $p<0.01$), with no effect of treatment groups across the testing months ($F(48,628)=1.101$, $p=0.301$) (Figure 18).

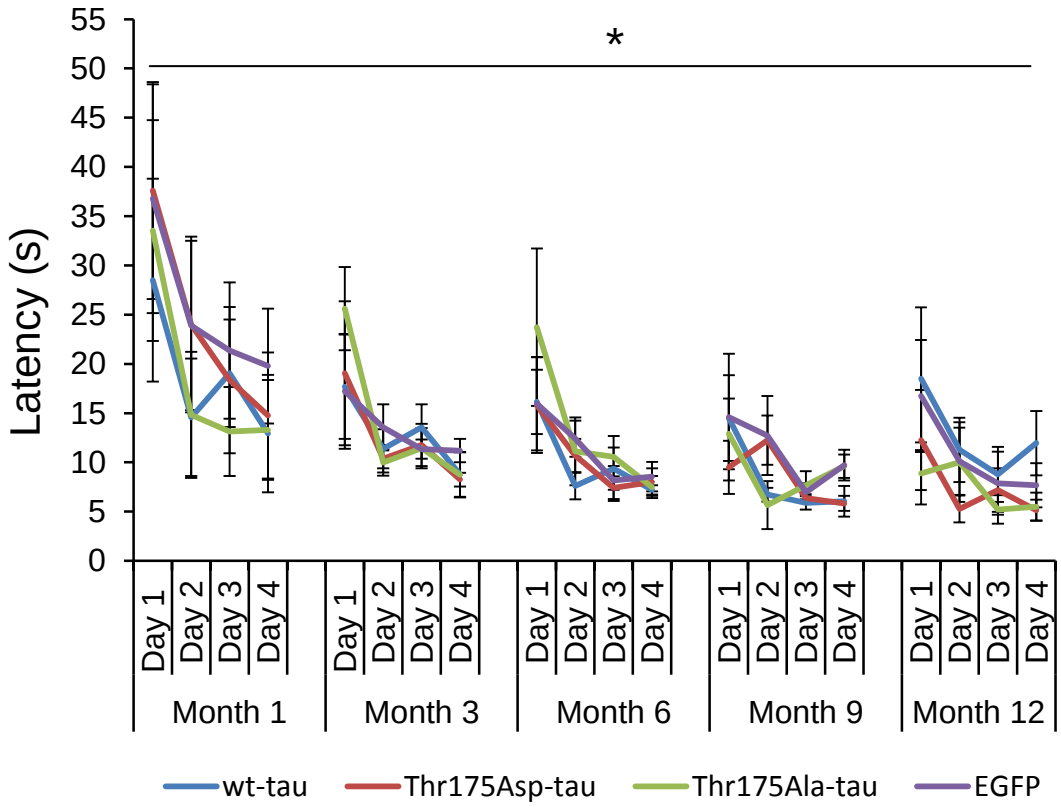

Figure 18: Mean latency to find platform across 4 learning days during 1, 3, 6, 9, and 12 month post-surgery time points

Rats expressing either wt-tau, Thr175Asp-tau, Thr175Ala-tau, or EGFP (refer to Table 1 for n values). Latency (s) to find platform over 4 learning days across testing months 1, 3, 6, 9 and 12 post-surgery. The latency depicted on each day is an average of 4 trials on that day. There was a significant decrease in latency between testing months, with no significant effect of treatment groups across the testing months. Data are expressed as means ± SEM.

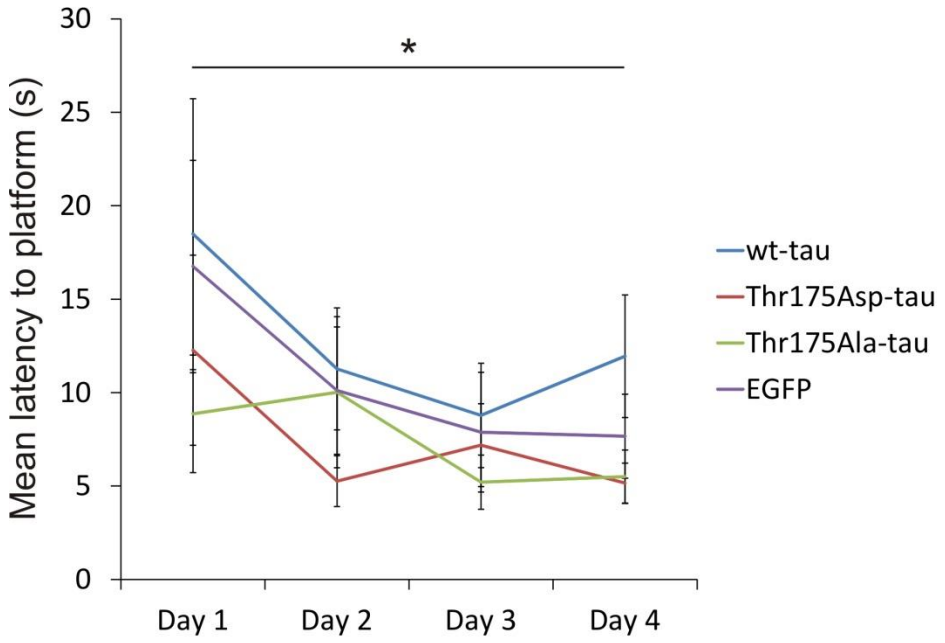

Figure 19: Mean latency (s) to reach the platform during 4 learning days at month 12 post-surgery

Rats expressing either wt-tau (n=6), Thr175Asp-tau (n=7), Thr175Ala-tau (n=8), EGFP (n=9). Mean time to find the hidden platform for each group over the 4 consecutive learning days. Rats received 4 training trials per day. All animals learned the MWM task as performance improved across days of testing, but there was no significant effect of treatment. Data are expressed in means ± SEM.

3.4.2 Test of spatial memory using probe trials – Day 5

A probe trial was conducted 24 hours after the 4 learning days in all cases. This involved the removal of the hidden platform from the water. Rats are given a total of 30 seconds in the Probe trial. Normal performance is expected to show a preference for the Quadrant that contained the platform during the learning trials. In terms of time spent in each quadrant, repeated measures ANOVA followed by post hoc paired t-test with Bonferroni corrections revealed that the four treatment groups did not explore all quadrants equally, they showed a preference for the target quadrant, and there were no significant differences between the groups at 1, 3, 6, and 9 month time points (Figure 20). At month 1, subjects did not explore all quadrants equally ($F(3,105)=26.058$, $p<0.01$), with a preference for the target quadrant (Q1) over all others (Q2, Q3, Q4, $t38=7.941$, $p<0.01$, $t38=4.446$, $p<0.01$, $t38=4.310$, $p<0.01$, respectively) across all groups, and there was not a significant difference between the treatment groups ($F(3,35)=0.209$, $p=0.890$). At month 3, subjects did not explore all quadrants equally ($F(3,90)=34.35$, $p<0.01$), with a preference for the target quadrant (Q4) over all others (Q3, Q2, Q1, $t37=4.4$, $p<0.01$, $t37=5.9$, $p<0.01$, $t73=6.7$, $p<0.01$, respectively) across all groups, and there was not a significant difference between the treatment groups ($F(3,30)=0.87$, $p=0.463$). At month 6, subjects did not explore all quadrants equally ($F(3, 99)=11.172$, $p<0.01$), with a preference for the target quadrant (Q4) over all others (Q3, Q2, Q1, $t36=5.877$, $p<0.01$, $t36=2.630$, $p<0.05$, $t36=4.720$, $p<0.01$, respectively) across all groups, and there was not a significant difference between the treatment groups ($F(3,33)=1.314$, $p=0.286$). At month 9, subjects did not explore all quadrants equally ($F(3,87)=35.568$, $p<0.01$), with a preference for the target quadrant (Q3) over all others (Q4, Q2, Q1, $t32=8.126$, $p<0.01$,

t32=6.192, p<0.01, t32=7.272, p<0.01, respectively) across all groups, and no statistical differences between the treatment groups (F(3,29)=1.286, p=0.298). At the 12 month time point subjects did not explore all quadrants equally (F(3,78)=16.325, p<0.01), however there was a significant difference between the preferences of the four treatment groups (F(3,26)=3.427, p<0.05), and a significant interaction between treatment and quadrant (F(9,78)=2.831, p<0.05, Figure 20, Figure 21). There was a preference for Q4 over all others (Q1, Q2, Q3, t29=5.8534, p<0.01, t29=3.079,p<0.01, t29=6.822, p<0.01, respectively) across all groups. Further, there were no significant differences between the groups in the time spent in the platform zone (F(3,29)=0.626,p=0.605) or area around the platform zone (F(3,29)=1.958,p=0.145, Figure 22C, E), nor was there a significant difference between the treatment groups in terms of the latency to find those zones (F(3,29)=1.949, p=0.147, F(3,29)=0.319, p=0.811,Figure 22D, F). The lack of differences between treatment groups in terms of time in the platform zone, time in the area around the platform zone, and latency to those zones held true at all testing time points.

Note: “platform zone” refers to an area encompassed by the actual circumference of the platform, whereas the “area around the platform zone” refers to an area encompassed by a circle with a diameter twice the size of the platform zone, with the platform zone at the center. The area around the platform zone is used to account for the fact that the ANYMAZE tracking system uses only a single point on the rat to track its movement, and this point may sometimes be out of the platform zone, when part of the rat is in fact in the zone.

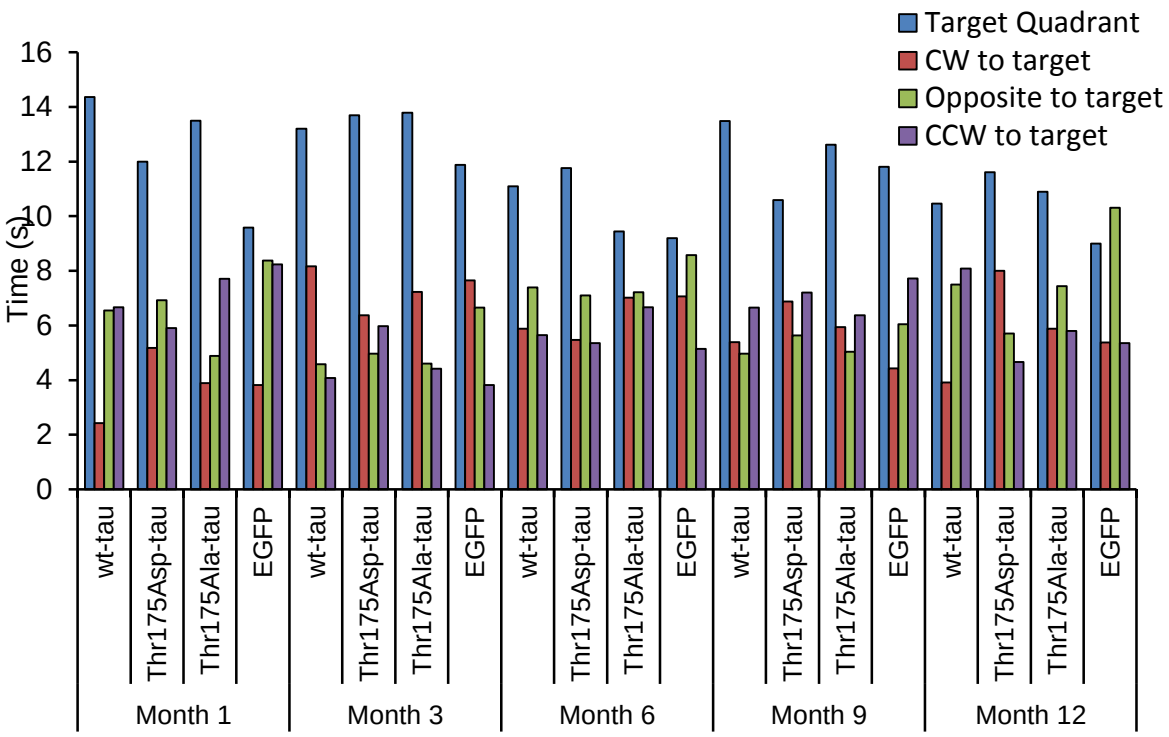

Figure 20: Time spent in each quadrant on probe trial day 5 at months 1, 3, 6, 9, and 12 of behavioural testing

Rats expressing either wt-tau, Thr175Asp-tau, Thr175Ala-tau, or EGFP (refer to Table 1 for n values).This figure is included to depict the day 5 probe trials at the 1, 3, 6, 9 and 12 month behavioural time points. Statistics were not performed on this longitudinal graph.

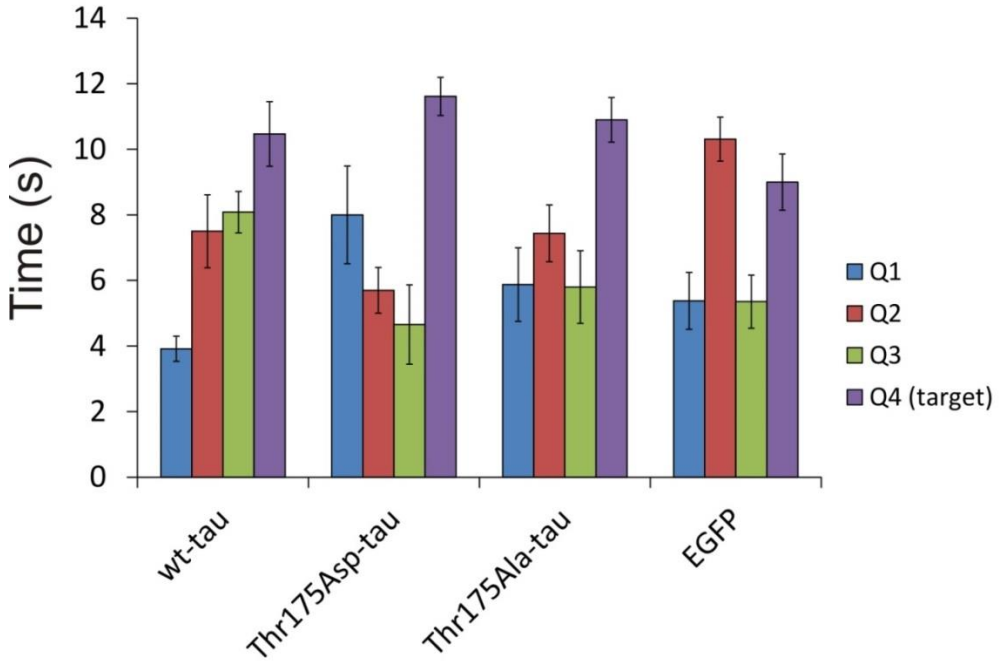

Figure 21: Time spent in each quadrant during probe trial day 5 at 12 months post-surgery

Rats expressing either wt-tau (n=6), Thr175Asp-tau (n=7), Thr175Ala-tau (n=8), or EGFP (n=9). Total time spent in each quadrant during the Probe trial (24 hours after the last learning day), where the hidden platform is removed from the MWM. Quadrant 4 (Q4) is the quadrant where the platform used to be. Rats are given a total of 30 seconds in the probe trial. Normal performance is expected to show a preference for the Quadrant that contained the platform during the learning trials. Subjects did not explore all quadrants equally. There is a statistical difference between the preferences of the four treatment groups, and a significant interaction between treatment and quadrant.

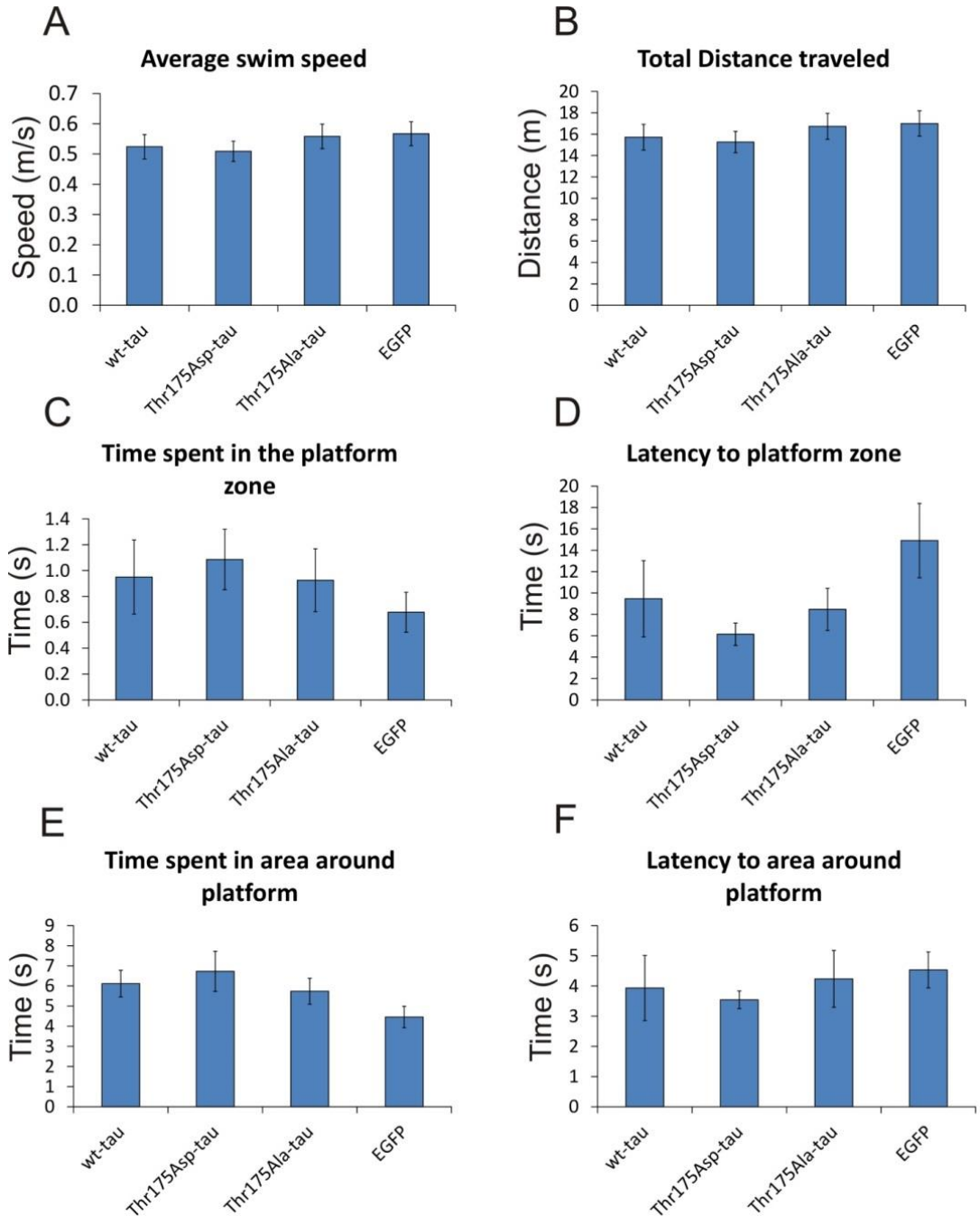

Figure 22: No significant differences in performance between treatment groups during probe trial day 5

Rats expressing either wt-tau (n=6), Thr175Asp-tau (n=7), Thr175Ala-tau (n=8), or EGFP (n=9). There was no significant effect of treatment on (A) average swim speed, (B) total distance traveled, (C) time spent in the platform zone, (D) latency to reach platform zone, (E) time spent in the area around the platform, or (F) latency to the area around the platform during probe trial day 5 at month 12 post-surgery. Data are expressed as means ± SEM.

3.4.3 Cognitive flexibility in the reversal trials

At the 12 month time point, reversal learning trials were added to the MWM protocol to control for repeated testing, and to potentially observe any differences in treatment groups that were not apparent through other protocols. The protocol involved the normal 4 days of learning, with a probe trial on the 5^{th} day, followed by a reinforcement trial (where the platform was placed back in its original location, Day 5b), and then the reversal trials, where the platform was placed in the opposite quadrant (Days 6a and 6b). This was to test the rats' cognitive flexibility. Each treatment group showed a similar increase in time to find the platform when the platform location was changed, and ANOVA revealed a steep decrease in time to find the new platform location over 4 initial trials ($F(3,75) = 7.323$, $p<0.01$, Figure 23). There was no difference in learning the new platform location between the treatment groups ($F(3,25)=1.691$, $p=0.195$).

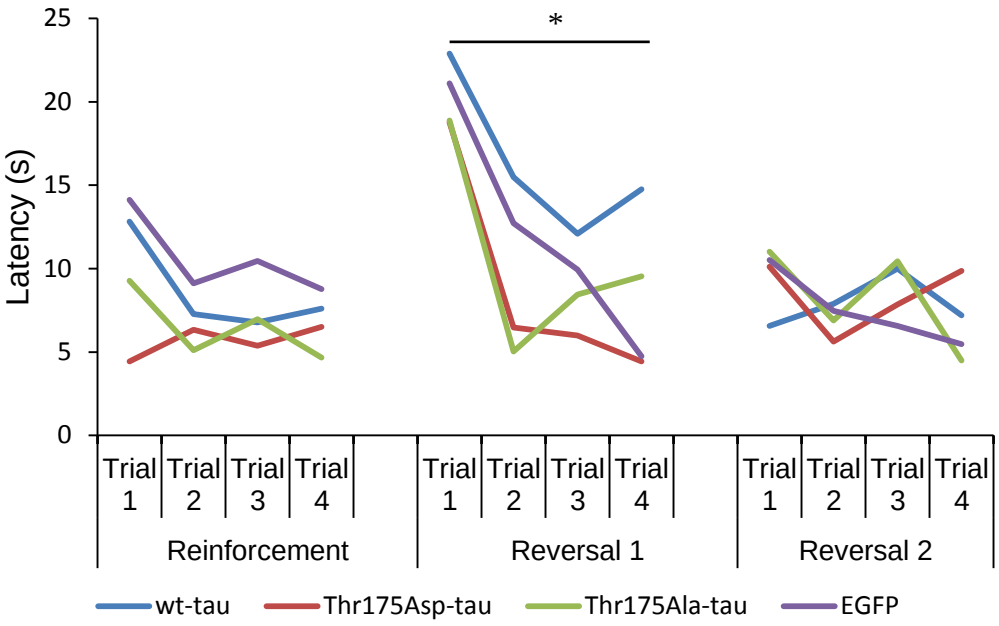

Figure 23: Latency during reversal trials

Rats expressing either wt-tau (n=6), Thr175Asp-tau (n=7), Thr175Ala-tau (n=8), EGFP (n=9). This graph shows the latency to find the new platform location during the reinforcement trials (Day 5b), the first day of reversal learning (Day 6a), and the second set of reversal trials (Day 6b). All treatment groups successfully learned the location of the new platform, as performance improved across trials on the first set of reversal trials. There was no significant difference in learning between the four treatments at month 12 post-injections. Data are expressed in means.

3.4.4 Mean speed in the Morris water maze

The mean swim speed of each rat was also assessed in the water maze. Mean speed acts as an indicator for the rats' ability to swim since it may influence the latency to reach the platform on any given testing day, and is an important control to ensure for no motor deficiencies. ANOVA revealed no significant differences between treatment groups ($F(3,25)=0.318$, $p=0.812$), and no significant change in mean speed over the 4 learning days ($F(3,75) = 2.064$, $p=0.112$ (representative graph for Month 12 incorporated in Figure 24).

Interestingly, there was an increase in mean speed over the testing days during month 12 post-surgery ($F(6,150) = 5.062$, $p<0.01$), with no significant difference between the treatment groups ($F(3,25)=0.335$, $p=0.800$, Figure 24). It is important to note that the 12 month time point was the first and only time that the rats had been tested in the MWM for 7 consecutive days as previous protocols did not require so many days.

Further, with repeated testing there was no significant effect of testing time points on mean swim speed ($F(4,157)=0.482$, $p=0.749$, Figure 25), and no significant effect of treatment groups ($F(12,157)=0.869$, $p=0.579$) in terms of the 4 learning days over months 1, 3, 6, 9 and 12.

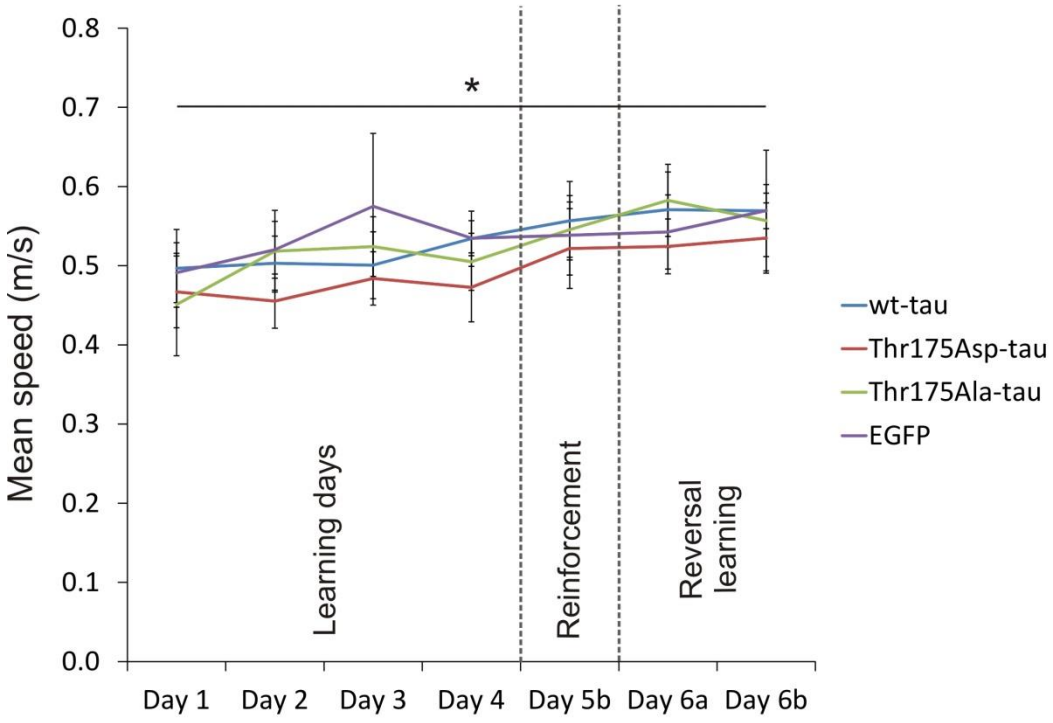

Figure 24: Mean speed over all testing days excluding probe trials at month 12 post-surgery

Rats expressing either wt-tau (n=6), Thr175Asp-tau (n=7), Thr175Ala-tau (n=8), EGFP (n=9). Mean speed during 4 days learning, reinforcement day (after probe 1), and 2 reversal days (before probe 2). Rats received 4 trials per day. There was a significant effect of day on mean speed, and from the figure it can be seen that mean speed increased over testing days. There was no significant difference in mean speed between treatment groups at 12 month post-injection. In terms of the 4 learning days, there was no significant effect of day on mean speed, and no significant difference between treatment groups. Data are expressed in means ± SEM.

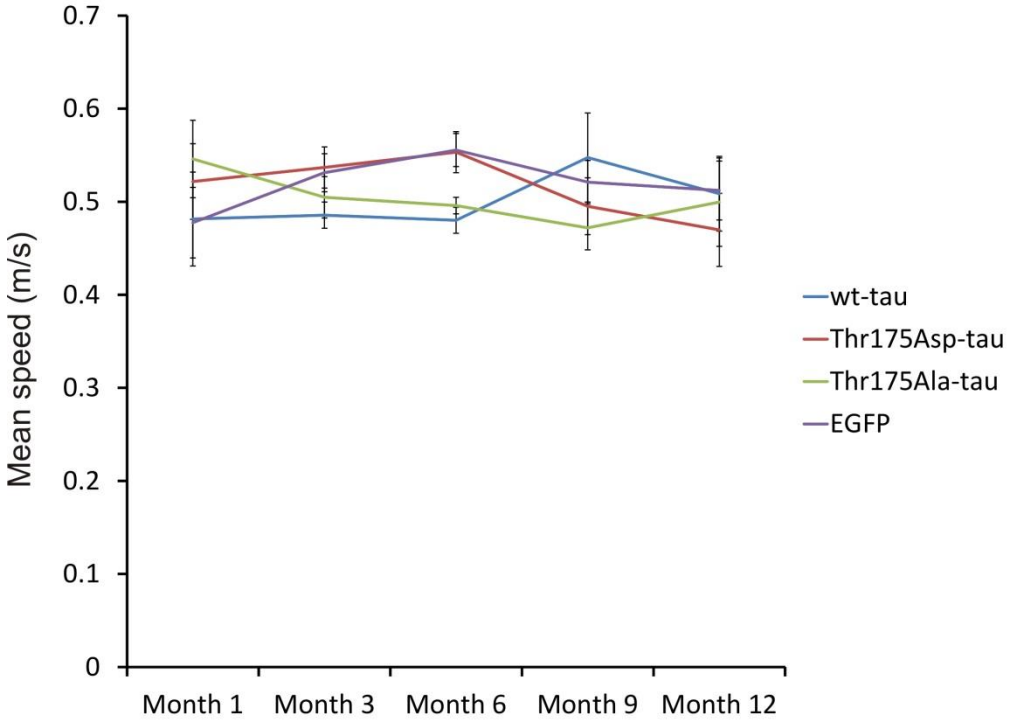

Figure 25: Mean swim speed of 4 learning days over 1, 3, 6, 9, and 12 month post-surgery time points

Rats expressing either wt-tau, Thr175Asp-tau, Thr175Ala-tau, or EGFP (refer to Table 1 for n values). Average speed of the 4 learning days in the MWM at 1, 3, 6, 9 and 12 months post-surgery. Each month consisted of 4 learning days, with 4 learning trials per day. Thus, these averages are calculated by taking the average speed over the 4 trials for each of the 4 learning days, then averaging the 4 learning days for the month. There was no significant difference in mean speed at the different testing time points, and no significant difference between treatment groups across testing time points. Data are expressed in means ± SEM.

PART 4: DISCUSSION

4. Discussion

Microtubule associated tau protein (tau) pathology is evident in ALS with frontotemporal dysfunction. Previous studies have shown that ALSci is associated with atrophy of frontotemporal lobes, neuronal and extraneuronal tau deposition, and a unique tau phosphorylation site at Threonine residue 175 (pThr175-tau) (Strong et al. 2006; Wilson et al. 2001; Yang et al. 2003, 2005). The aberrant phosphorylation of tau protein at Thr175 is hypothesized to play a role in the pathogenesis leading to the cognitive impairment in ALS (Gohar et al. 2009; Strong et al. 2006). Here, an *in vivo* rat model has been established to test the behavioural consequences of expression of an aberrantly phosphorylated tau mimic (Thr175Asp-tau) in the hippocampus of Sprague Dawley rats. It has been demonstrated in this study that injection of Thr175Asp-tau construct, as well as controls (wt-tau, Thr175Ala-tau, EGFP), into the hippocampus using the rAAV9 gene transfer technique allows for expression in the hippocampus that is maintained for the 12 month testing period (Figure 2, Figure 3). Initial histology shows some presence of tau fibrils in the Thr175Asp-tau group at 3 months post-injections (Figure 4). Further detailed analysis of tau expression will be conducted, but is not part of this thesis.

In this study, in spite of the induction of tau pathology, we have failed to observe a behavioural phenotype when comparing the experimental Thr175Asp-tau–expressing rat group to controls (wt-tau, Thr175Ala-tau, EGFP) at any given time point (1, 3, 6, 9, or 12 months post-surgery). Open-field testing (OFT) revealed normal exploratory behaviour that habituated over time (Figure 11 - Figure 13), while startle testing showed short-term habituation (STH) to an acoustic stimulus (Figure 14), and normal levels of prepulse inhibition (PPI, Figure 15, Figure 16) in all four groups. Lastly, the Morris water maze

(MWM) did not reveal any deficits in spatial learning or memory in the Thr175Asp-tau-expressing rat group when compared to controls (Figure 18 - Figure 22).

4.1 Viral vector expression in the rat hippocampus

In our study, the bilateral stereotactic hippocampal injections of the rAAV9 constructs exhibited expression of human tau protein in the hippocampus of the treatment groups (Figure 2, Figure 3). This was visualized through immunohistological staining for green fluorescent protein (GFP). These findings substantiate previous studies demonstrating that intraparenchymal injections of AAV9 to the central nervous system (CNS) shows efficient expression of the protein of interest, and serves as a highly efficient vector for neurons (Klein et al. 2009; McCown 2005). Further, it has been previously established that this method is an operational mechanism for neuropathological expression of tau protein in specific brain regions (Klein et al. 2009). With this, the viral load size used in this experiment seems effective in leading to widespread expression in the hippocampus that is sustained throughout the entire 12 month study (Figure 3). Both the amount of virus, and method of injection into the hippocampus have been verified in the literature using both mutated tau, and through lentiviral gene transfer models (Herman et al. 2011; Mustroph et al. 2012).

4.2 Normal exploratory behaviour and lack of anxiety-like behaviour

In our OFT analysis, the Thr175Asp-tau rats showed normal levels of exploratory behaviour, and habituation of exploration over 30 minutes in the open field box at all time points (1, 3, 6, 9, and 12 months post-surgery) with no significant difference from

controls (representative figure at month 12, Figure 11). This indicates that there is no difference in motor activity between treatment groups (Seibenhener and Wooten 2015).

Further, in this study, the four treatment groups did not display differences in anxiety-like behaviour, measured as the ratio of the time spent in the center of the box versus the periphery (Figure 13, Seibenhener and Wooten 2015). These results are important to also validate subsequent behavioural testing for cognitive function, which can easily be confounded by differences in motor ability or anxiety (Seibenhener and Wooten 2015).

Unfortunately, the testing location and the open field boxes themselves were changed at one point during testing. A move of the animal facility into a new building took place after the testing points of 1 and 3 months post-surgery, and before the 6 month time point. However, when comparing the 6, 9, and 12 month time points, each treatment group traveled a significantly smaller distance with repeated testing in the same boxes (Figure 12). This indicates that the animals showed long-term habituation of the locomotor activity upon repeated testing in the same boxes. Alternatively, the longer distance traveled at month 6 may have occurred because of the move to a new animal facility as well as a change in testing apparatus, indicating a sensitivity of the OFT to changing emotions in rats. In any case, there were not any significant differences between the treatment groups at any given time point. This similarity in total distance traveled shows similar locomotor activity between treatment groups and, again, eliminates confounding factors when analyzing other behavioural results (Seibenhener and Wooten 2015).

4.3 Acoustic startle response (ASR), prepulse inhibition (PPI) and short-term habituation (STH)

The acoustics startle response (ASR) is a protective reflex stimulated by a sudden acoustic stimulus. In rodents, the startle response involves a whole body flinch. Testing the ASR is a behavioural paradigm used to assess sensory filtering and sensorimotor gating, and can aid in indicating neurodegenerative disease or cognitive impairments through investigation of habituation and prepulse inhibition (PPI) (Van den Buuse 2010; Davis and Wagner 1969; Fenton, Stover, and Insel 2003; Geyer 2006; Valsamis and Schmid 2011).

Habituation is a form of non-associative learning that involves a decreased response to a repeated stimulus, and is a form of sensory filtering. Short-term habituation (STH) is habituation within a testing session (Davis and Wagner 1969). In our study, the four treatment groups exhibited STH to a repeatedly presented acoustic stimulus with no significant differences between groups (Figure 14). This was indicative of intact sensory filtering, with no indications of disruptions in habituation. Disruptions in habituation could be suggestive of mental or neurodegenerative disease (Davis and Wagner 1969; Valsamis and Schmid 2011). If significant neurodegeneration were present in any particular treatment group in this study, slower habituation, or no habituation at all would likely be observed (Braff, Geyer, and Swerdlow 2001; Kumari et al. 1999).

PPI is the inhibition of a startle response to a startle stimulus, when the startle stimulus is preceded by a weak prestimulus, or prepulse (Hoffman and Ison 1980; Koch 1999; Swerdlow, Geyer, and Braff 2001). Prepulse inhibition is a form of sensorimotor gating,

meaning it is assumed that the prepulse elicits an orienting response while avoidance responses such as startle are inhibited (Fendt, Li, and Yeomans 2001). PPI has been shown to be disrupted in many mental disorders and neurodegenerative diseases (Van den Buuse 2010; Fenton et al. 2003; Geyer 2006; Swerdlow and Geyer 1998). Although PPI is mediated by midbrain circuits, it is modulated by the hippocampus. Therefore examination of the startle response was important to assess the function of the hippocampus (Caine, Geyer, and Swerdlow 1992; Fendt et al. 2001; Jones and Shannon 2000; Li et al. 1998; Mawr 1985; Swerdlow et al. 1993; Yeomans, Hempel, and Chapman 1993). There is evidence of regional heterogeneity throughout the hippocampal formation as to the degree to which they modulate PPI and sensory filtering (Caine et al. 1992). Specifically, the CA1, dentate gyrus (DG), and ventral subiculum have been implicated in differing abilities to modulate PPI and the ASR when presented with cholinergic agonists and antagonists (Caine et al. 1992). Considering this, it will be interesting to draw potential correlations between tau expression patterns in single rats and the amount of PPI in the respective animals upon further detailed histological analysis.

Here, by assessing average group performance, we observed no indications of deficits in PPI at either 85 or 75 dB prepulse intensities in any treatment groups at any given testing time points (Figure 15, Figure 16). With an 85 dB prepulse, there was a significant difference between the interstimulus intervals (ISI) 30 and 100 ms at the 3 month time point, but not at the subsequent time points (6, 9, 12 months, Figure 15). On the other hand, there was a significant difference between the ISI's of 30 and 100 ms for all testing points (3, 6, 9, and 12 months) for PPI at 75 dB, with the 30 ms ISI exhibiting

significantly lower PPI than 100 ms (Figure 16). Therefore, a prepulse intensity of 85 dB allows for maximal PPI, whereas 75 dB does not. This is not unexpected as PPI is enhanced with increasing intensities, and 85 dB is a higher intensity than 75 dB (Hoffman and L 1970; Hoffman and Searle 1968).

Furthermore, PPI at different ISI ranges act through differing mechanisms. Ionotropic receptors mediate the fast (ISI's of 10-100 msec) effects of PPI, where metabotropic receptors mediate the long-lasting (ISI's of 100-1000 msec) effects of PPI (Jones and Shannon 2000; Yeomans et al. 2010). The purpose of measuring two different ISI's in this study was to gain an understanding of what type of transmitter system was affected in case there was a deficit in the experimental group. Ultimately, our results presented here as group averages are not indicative of any abnormalities in sensory filtering, or cognitive impairment caused by expression of the different tau constructs. Further analysis correlating the expression patterns of the constructs with PPI performance in single animals, however, might be more sensitive to subtle changes (Van den Buuse 2010; Fenton et al. 2003; Geyer 2006; Swerdlow and Geyer 1998).

When analyzing PPI across all testing time points (3, 6, 9, and 12 months post-surgery), there seems to be a trend towards decreasing PPI at 75 dB prepulse intensity across time that is not as apparent at 85 dB prepulses (Figure 17). Though this trend is not statistically significant, this observation may imply the onset of hearing loss in the aged rats, and thus variability in their ability to hear the lower intensity 75 dB prepulse.

4.4 Spatial learning, reference and working memory

From our MWM data, it is important to firstly note that there were no significant differences in mean speed between treatment groups, and the mean speed did not significantly change with repeated testing (Figure 24, Figure 25). From this, it can be inferred that swim speed did not play a role on latency measures. Interestingly, at the 12 month time point the rats were tested for 7 consecutive days, and there was a significant increase in mean speed for all treatment groups over those 7 days (Figure 25). This was the only time the rats had been tested for such a high number of consecutive days, and is an indication that the rats were still motivated in finding the hidden platform.

We had hypothesized that the expression of Thr175Asp-tau would cause neurodegeneration and thereby significant damage to the hippocampus. This would result in deficits in reference memory because it involves consolidation of memories, a function of the hippocampus (Giap 2000; Vorhees and Williams 2006). In our MWM task, we found that the Thr175Asp-tau group, along with the control groups showed intact spatial learning through a trend of decreasing time to find the platform on learning days (Figure 19), and displayed intact reference memory through a low latency to the platform zone on probe trials (Figure 22). This occurred at all time points (1, 3, 6, 9, and 12 month post-surgery).

One abnormality to note is that on probe trial day 5 at the 12 month post-surgery time point, the EGFP treated group showed preference for the quadrant opposite to the target quadrant, where the other groups showed a preference for the target quadrant (Figure 21). Upon further analysis, a similar trend can be observed in the EGFP group at the month 1

and 6 time points post-surgery (Figure 20). It is unlikely that this is indicative of a behavioural phenotype, as there was no significant difference in the latency to find the platform zone between the treatment groups on probe day 5 (Figure 22). A likely explanation is that rats in the EGFP group abandoned looking for the platform in the original location once it was realized that the platform was not present in that location.

Interestingly, with repeated testing (at the 1, 3, 6, 9, and 12 month post-surgery time points), the rats seemed to improve on the MWM task, as indicated by decreasing latencies to find the platform over time points (Figure 18). To control for repeated testing, the MWM protocol was extended to include reversal learning at the 12 month time point to test working memory and behavioural flexibility. All treatment groups showed a rapid adaptation to the new platform location within the first day of testing without significant differences between groups (Figure 23). Reversal learning was used to enhance the detection of spatial impairments as it requires the animal to extinguish the existing knowledge of the original platform location and adapt to the new location by using information from the immediate past (Bradley and Hitch 1974; Vorhees and Williams 2006). This form of cognitive flexibility has shown to be a more sensitive test than the classic form of spatial learning in the water maze (Al-onaizi et al. 2016; Vorhees and Williams 2006). If deficits in working memory were present, the rats would likely also not adapt to the new platform location as rapidly as observed here (Figure 23).

The above results may be an indication of a lack of pathology in the hippocampus, or pathology that is not present in the regions of the hippocampus necessary for spatial learning and memory. It has been shown that both the extent and region of damage within the hippocampal formation are factors that influence behavioural outcomes in tests such

as the MWM (Morris et al. 1982; Moser, Moser, and Andersen 1993). Specifically, the dorsal hippocampus plays a greater role in spatial navigation than the ventral hippocampus (Moser et al. 1993). It has been previously shown that a widespread tau pathology is required to lead to spatial memory deficits in rats (Mustroph et al. 2012). Mustroph et al. showed that deficits in the MWM correlated with robust expression in the hippocampus proper via rAAV9 viral vector, the presence of neurofibrillary tangles (NFT's) throughout the hippocampus, extensive hyperphosphorylated tau, and profound neuronal loss detected in tau-expressing cells (2012). Altogether, this indicates that pathology in the dorsal hippocampus may be of greater interest to correlate with MWM behavioural data than in the ventral hippocampus, and that pathology needs to be widespread and severe in order to result in a behavioural phenotype.

4.5 Possible explanations for lack of behavioural deficits

It must be emphasized that tissue staining of all rat brains in this study is still underway and has not yet been completed. A more advanced analysis of behavioural results is contingent upon the completion of this histology and subsequent correlation of tau expression patterns and behavioural phenotype. The results obtained here may have occurred because of the following reasons: (1) the construct expression within the hippocampus may not have been robust enough to lead to pathological tau fibril formation throughout the entirety of the hippocampus, (2) there may not have been enough time for the expression of the tau construct to lead to pathological fibril formation, subsequent neurodegeneration and a behaviourally measurable pathology, (3) the presented hypothesis of pThr175-tau playing a role in the frontotemporal syndromes

in ALS may be incorrect, or (4) there may be a need for tandem pathology of both TDP-43 and tau. Each of these reasons will be explained here.

First, expression of tau constructs in the hippocampus have been demonstrated (Figure 2, Figure 3), and tau fibrillary structures have been identified in an experimental group (Thr175Asp-tau) at month 3 post-surgery (Figure 4). However, tau construct expression was not consistently widespread throughout the hippocampus (Figure 5). The extent of these observed fibrils is not yet known and it must be verified that this is an observation limited to the experimental group alone. It is also unknown if these fibrils are correlated with activation of proteins related to tau pathology such as increased expression of activated GSK3β, or increased caspase-3 activity (Moszczynski et al. 2015). Overall, the extent of construct expression may not be widespread throughout the hippocampal structure, and so the degree of resulting tau fibrillary pathology may not be sufficient to result in a behavioural phenotype. As has been explained in detail in earlier parts of this discussion, the regions of the hippocampus where pathology is taking place, and the extent of that pathology play vital roles in interpreting behavioural data.

The second reason posited was that there may not have been enough time for the expression of the tau construct to lead to pathological fibril formation. With the above in mind, it is also important to consider the fact that this study ended at the 12 months post-surgery time point. If pathology is not present, there is the possibility that 12 months does not provide sufficient time to lead to a behavioural phenotype with the given tau construct. As outlined in the introduction section of this paper, a potential pathway was described where pThr175-tau induces GSK3β activation, which leads to Thr231 phosphorylation and subsequent fibril formation (Moszczynski et al. 2015). Since it is

likely that pThr175-tau plays an indirect, or an initiating, role in the mechanism leading to the observed frontotemporal dysfunction in ALS, there is potential for this mechanism of pathology to take longer than 12 months to lead to a behaviourally measurable phenotype in the rat model.

The third potential reason is that there is the possibility that the original hypothesis of pThr175-tau playing a role in the neurodegeneration and resulting cognitive impairments in ALS is, in fact, incorrect. To assess if there were any true differences between the four treatment groups, the brains of these rats will have to be analyzed for potential pathology, degeneration, and upregulation of specific proteins. Again, this will be further investigated with staining, and histological analysis.

Lastly, there may be a need for tandem pathology of TDP-43 as well as tau in order to observe a behavioural phenotype. Though there is abundant evidence of pathological alterations in tau metabolism in ALSci, TDP-43 pathological processing is also a significant component of the pathology of ALS (Geser et al. 2013; Ince et al. 2011). Thus it is unclear whether the observed tau pathology in the frontotemporal syndromes of ALS is a primary or secondary phenomenon, and if secondary, whether it is driven by the altered TDP-43 metabolism observed in ALS.

4.6 Study Limitations

There are a number of limitations to this study, such as a rapid decrease in the health of our rats after the 6 month post-surgery time point, the lack of histological staining, and potential variability in expression patterns.

A major limitation faced in this study was a rapid decline in health in many of the rats over time. There were sporadic health issues in the initial phases of the experiment, such as the death of a rat as early as 1 month after surgery from respiratory disease, and another rat suffered a non-operable tumour and was sacrificed approximately 3 months after surgery. One additional rat died of respiratory issues 7 months post-surgery. The most damaging issue in terms of the health of the rats was the development of tumours in a large portion of the rats after the 6 month time period. Specifically, 5 rats developed tumours approximately 9 months post-surgery, and an additional 6 rats developed tumours at approximately 12 months post-surgery. These tumours were inconsistent in both size and anatomical locations. The tumour locations included brain, oral, mammary, and genital sites. There was no evident correlation between treatment groups and development of tumours. The distribution of treatment groups to number of rats that eventually developed tumours are as follows: wt-tau; 4 rats, Thr175Asp-tau; 3 rats, Thr175Ala-tau; 2 rats, EGFP; 3 rats. Tissue analysis of the tumours confirmed that there was no viral expression in the tumours, and thus did not result from the rAAV9 injections.

Ultimately, this decline in health, which escalated after the 6 month testing period, prevented the study from being carried out past the 12 month time point. It also likely contributed to increased variability in the behavioural data in more than one way. Firstly, some rats were unhealthier than others, and this can lead to differing performances in the sensitive behavioural tasks conducted. Secondly, poor health lead to the early death of some rats, resulting in a decreased sample size in each group, which, again, increases variability in the data. To correct for this, we had performed a second round of 8

surgeries (2 rats per treatment group), to undergo behavioural testing at the 1, 3, 6, 9, and 12 month post-surgery time points and the data is to be merged from these two cohorts.

In addition to the poor health of the animals in this study, another major limitation is the lack of detailed histological analysis at this time point, which inevitably limits the behavioural analysis. With only the behavioural data we are limited in our analysis to assessing averages within treatment groups. Once the histological analysis has been completed, the behavioural data can be revisited using regression analysis to correlate behaviour with expression levels on an individual rat basis.

Another possible limitation to this study is variable expression patterns in the hippocampus. The staining that has been conducted, though minimal, typically shows widespread expression of GFP throughout the given slice of hippocampal formation (Figure 2). However, some brain slices reveal expression primarily in the DG and CA3-CA2 regions, with minimal expression in the remaining hippocampal structures, such as CA1 (Figure 5). With this, it is crucial to speculate that there may be variable expression from one injection to the next, and possibly variability in rostral-caudal expression since all injections were administered in one plain (A/P 5.5 mm caudal from bregma). These possibilities will be investigated once extensive staining of all rat brain slices is conducted.

4.7 Future Studies

In this study we have demonstrated a lack of behavioural differences in the experimental Thr175Asp-tau group compared to controls (wt-tau, Thr175Ala-tau, EGFP) after 12 months of Thr175Asp-tau expression in the hippocampus. The immediate future studies

include the use of a transgenic ALS-like rat model, and one that examines methods of inhibiting tau pathology that may result from pThr175-tau.

Currently, a double-transgenic Sprague Dawley rat line is being established to further investigate the role of pThr175-tau in a rat model with an ALS-like phenotype. The double transgenic (chat-tTA/TRE-TDP-43 M337V) rats will express human mutant TDP-43 in a suppressible manner under doxycycline control. As mentioned in the introduction, the pathological processing of TDP-43 is a significant component of the pathology of ALS (Geser et al. 2013; Ince et al. 2011). The conditional expression of ALS-associated mutant TDP-43 (M337V) in rats results in motor neuron phenotype, without evidence of brain involvement (Huang et al. 2012). The same bilateral hippocampal injections of rAAV9 with tau constructs will be administered to these rats. The idea is to investigate whether pThr175-tau is the primary pathogenic insult in the frontotemporal syndromes of ALS, or whether it is a consequence of the altered TDP-43 metabolism observed in ALS. Ultimately, this will serve to determine whether the associated disease process is modified by the expression of mutant human TDP-43.

Further, if there is a pathological effect of Thr175Asp-tau, it will be important to test methods of inhibiting the resulting pathology. It has already been established in cell culture that Thr175Asp-tau leads to upregulation of active GSK3β, which leads to subsequent tau phosphorylation and pathology (Moszczynski et al. 2015). If this mechanism holds true in our rat model, it will be important to investigate the effects of inhibitors of GSK3β *in vivo*. Eventually, the development of pharmacotherapies that target GSK3β may be critical to the resolution of pathological tau aggregates in ALS.

PART 5: SUMMARY AND CONCLUSIONS

5. Summary and Conclusions

5.1 Summary of findings

- rAAV9 viral vector injections lead to robust expression of GFP, and thus expression of the wt-tau, Thr175Asp-tau, Thr175Ala-tau, and EGFP constructs within the hippocampus of the Sprague Dawley rats (Strong Lab, unpublished data)
- Expression was maintained for the entirety of the study (12 months post-surgery) in all treatment groups (Strong Lab, unpublished data)
- Tau fibrillary structures have been observed in the Thr175Asp-tau group 3 months post-surgery
- Thr175Asp-tau group showed normal habituation of exploratory behaviour, no differences in total distance traveled, and no differences in anxiety-like behaviour at the 1, 3, 6, 9, and 12 month post-surgery time points when compared to controls (wt-tau, Thr175Ala-tau, EGFP) in the OFT
- Thr175Asp-tau group showed normal short-term habituation at the 3, 6, 9, and 12 month post-surgery time points when compared to controls (wt-tau, Thr175Ala-tau, EGFP), and no deficits in PPI were observed between the groups using the acoustic startle response
- Thr175Asp-tau group showed normal spatial learning and memory through learning trials, probe trials, and reversal trials in the MWM when compared to controls (wt-tau, Thr175Ala-tau, EGFP)

5.2 Conclusions

This study demonstrated that Thr175Asp-tau expression throughout the hippocampus of non-transgenic Sprague Dawley rats does not lead to significant behavioural changes when compared to controls (wt-tau, Thr175Ala-tau, EGFP) up to 12 months post-surgery. There was expression throughout the hippocampus, and expression was maintained for the entirety of the experiment. It is therefore assumed that the gene transfer technique using rAAV9 viral vector was successful. However, the Thr175Asp-tau experimental group exhibited normal habituation of exploratory behaviour, and a lack of an anxiety-like phenotype in the OFT. The experimental group also showed normal short-term habituation and lack of PPI deficits through ASR testing. Finally, the Thr175Asp-tau group showed normal spatial learning and memory in the MWM task, as well as normal cognitive flexibility. Ultimately, there were no measurable hippocampal or behavioural deficits observed in this study.

Our findings also showed tau fibrillary structures in the experimental group at the 3 month post-surgery time point. The extent of this pathology and whether or not it is limited to the experimental group will be further investigated with the histological analysis. This study proposes a possible mechanism for the frontotemporal dysfunction in ALS where tau protein pathology plays a critical role. Aberrantly phosphorylated tau at Thr175 is observed in this disease process, and is a potential mediator in the observed pathology. It is crucial to understand potential influence of pThr175-tau on the syndromes of frontotemporal dysfunctions in ALS *in vivo*. These results, and the results from the studies to immediately follow, will help to further our understanding of the

mechanism behind this complex disease process, and provide insight for novel pharmacotherapy targets.

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CONFERENCES/POSTER PRESENTATIONS

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"Phosphorylated tau at Threonine residue 175 and its role in ALS with frontotemporal degeneration"
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