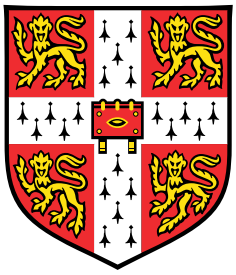
THE PROSTGLANDIN E2 RECEPTOR SUBTYPE 3E AND ITS INVOLVEMENT IN TAUOPATHIES



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I would like to dedicate this thesis to my family. For their love and unwavering support.

Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee.

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June 2021

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Abstract

Neuroinflammation is becoming increasingly recognised as key to the pathogenesis of Alzheimer's disease and tauopthies. Epidemiological studies report a delay in the onset of Alzheimer's in subjects using nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit enzymes in the cyclooxygenase-2 (COX-2) pathway which play a key role in synthesising prostaglandin E2 (PGE2) from arachidonic acid. PGE2 has been implicated in preclinical stages of Alzheimer's disease, where elevated levels of PGE2 in the cerebrospinal fluid can be found, as well as aberrant amyloid processing in experimental models of disease. PGE2 signals via 4 E-prostanoid (EP) receptors, EP1-EP4, all G-protein coupled receptors (GPCR). The EP3 receptor, the most abundant PGE2 receptor in the brain, is unique in that it is alternatively spliced giving rise to species specific isoforms. One of the EP3 receptor isoforms, EP3Re, is human specific and an incidental finding within a project to investigate its distribution in brain, suggested that it could be associated with tau tangles. The aim of this project was to further investigate the unknown distribution of EP3Re in human brain, to determine its signalling mechanism and explore whether any meaningful interaction between EP3Re, tau and its pathology exists. We use immunohistochemistry, proximity ligation assays and electron microscopy to map out the distribution of EP3Re in the human brain and explore the interaction between EP3Re and tau. We also use gene reporter and second messenger assays to characterise EP3Re signalling and what role if any this may be playing in tauopathies. We show that EP3Re is expressed throughout the

brain, with strong expression in brain stem nuclei, and signals predominantly through a Gi coupling pathway. Moreover, using a combination of human tissue, primary cell lines and neurons derived from induced pluripotent stem cells, we show that EP3Re appears to be associated with tau neurofibrillary tangles in disease. We also show, using the EP3 agonist sulprostone, that signalling through the receptor increases tau phosphorylation in our cellular systems. Further work will be required to fully clarify the specificity of the interaction and understand the mechanism behind this and if targeting inflammatory EP3Re signalling has the potential to affect tau pathology in disease.

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Chapter 1

INTRODUCTION

1.1 Neurodegeneration

Neurodegenerative disorders account for a significant proportion of neurological disease and represent a significant cause of death and disability worldwide. They are a group of heterogeneous disorders marked by progressive neuronal loss in specific neuronal populations. These include diseases such as motor neuron disease, Parkinson's, progressive supranuclear palsy, Huntington's, multiple system atrophy and the dementias, such as Alzheimer's disease and frontotemporal dementia. Among the list of neurodegenerative disorders, the dementias have the highest burden of disease with Alzheimer's disease accounting for 60-70% of all dementias (Gaugler et al., 2016). It is estimated that the global incidence of dementia in 2016 was 43.8 million, an increase by over 20 million since 1990 (Nichols et al., 2019) and it will exceed globally 100 million by 2050 (Nichols et al., 2019). With no cure at present, this creates a significant public health problem.

Neurodegenerative disorders are characterised pathologically by the accumulation of a misfolded or abnormal protein aggregates in the affected disease area (Gan et al., 2018). Examples of this include α-synuclein inclusions in Parkinson's, amyloid plaques and tau aggregates in Alzheimer's and huntingtin inclusions in Huntington's (Dehay et al., 2015; Landles and Bates, 2004). Biochemical characterisation of these inclusions has contributed significantly to our understanding of the underlying mechanism of disease with the pathological role of the protein forming the aggregates being supported by molecular and genetic studies. Indeed, the presence of aggregates in distinct brain regions is associated with neurodegeneration with mutations in genes encoding these proteins leading to familial forms of the disease (Dehay et al., 2015; Goedert, 2003; Murrell et al., 2000; Wszolek et al., 2006). Examples of this include mutations in *SNCA*, the gene encoding α-synuclein, that forms the characteristic Lewy bodies

associated with Parkinson's disease (Appel-Cresswell et al., 2013; Pasanen et al., 2014; Polymeropoulos et al., 1997; Proukakis et al., 2013; Singleton et al., 2003). The *APP* gene which encodes amyloid precursor protein (APP) leading to plaque formation and early onset Alzheimer's (Murrell et al., 1991) and the trinucleotide repeat in the *HTT* gene which encodes huntingtin leading to Huntington's (MacDonald et al., 1993).

Disorders typified by the presence of aggregated tau inclusions are referred to as tauopathies. These are a group of degenerative conditions that consist of dementias and movement disorders (Götz et al., 2019). Despite the variable phenotype, they are thought to share similar disease mechanisms due to the presence of filamentous tau inclusions(Gendron and Petrucelli, 2009). The pathological role of these inclusions is supported by genetic studies that show mutations in *MAPT*, the gene encoding tau, to be sufficient to cause disease (Strang et al., 2019).

1.2 Tau

1.2.1 Tau protein

Tau is a microtubule-associated protein encoded by the *MAPT* (microtubule-associated protein tau) gene. Under normal physiological conditions, tau is abundantly expressed in neurons and contributes to stabilising neuronal microtubules and promote their assembly, which is key to regulating transport along the axon and promoting axonal outgrowth (Evans et al., 2004; Goedert et al., 1989; Mietelska-Porowska et al., 2014). Beyond microtubule assembly and stability, several other physiological roles of tau have been (Ittner et al., 2010) described. Tau deficient mice show impaired hippocampal long term potentiation (LTP) and long term depression (LTD) consistent with a physiological role of tau in synaptic plasticity and memory (Biundo et al., 2018; Kimura

et al., 2014). Tau has also been identified under normal physiological conditions at pre- and post- synaptic terminals (Tai et al., 2012) and has been shown to mediate post-synaptic targeting of the Src kinase Fyn to NMDA receptors (Ittner et al., 2010) further supporting a role for tau at synapses. Tau's interaction with Fyn has also been shown to be an important component of oligodendroglial process growth (Klein et al., 2002). Klein et al show that disrupting Fyn-Tau binding by culturing tau deficient oligodendrocytes results in a reduction in the length and number of processes. Tau has also been shown to inhibit histone deacetylase-6 (HDAC6), the main tubulin deacetylase (Perez et al., 2009). This highlights a potential mechanism through which tau could regulate microtubule function independent of microtubule binding.

The *MAPT* gene is situated on chromosome 17q21.31 (Hutton et al., 1998) and in normal adult brain, 6 tau isoforms are expressed by *MAPT* pre-mRNA alternative splicing (Goedert et al., 1989) (Fig. 1.1). The isoforms differ by the presence or absence of one or two 29 amino acid inserts in the N-terminal domain (inclusion or exclusion of exons 2 and 3) producing 0N, 1N or 2N tau isoforms, and the presence or absence of three or four microtubule binding repeats at the C-terminus (inclusion or exclusion of exon 10) giving rise to either 3R of 4R tau (Goedert and Jakes, 1990; Goedert et al., 1989; Jakes et al., 1991). The microtubule binding domain at the C-terminal, as the name suggests, is involved in microtubule binding regulating the rate of microtubule polymerisation (Friedhoff et al., 2000; Mietelska-Porowska et al., 2014). The N-terminal region is known as the projection domain as it projects from the microtubule surface interacting with the plasma membrane, mitochondria, and, other elements of the neuronal cytoskeleton (Derisbourg et al., 2015).

The expression of the various isoforms are developmentally regulated (Goedert and

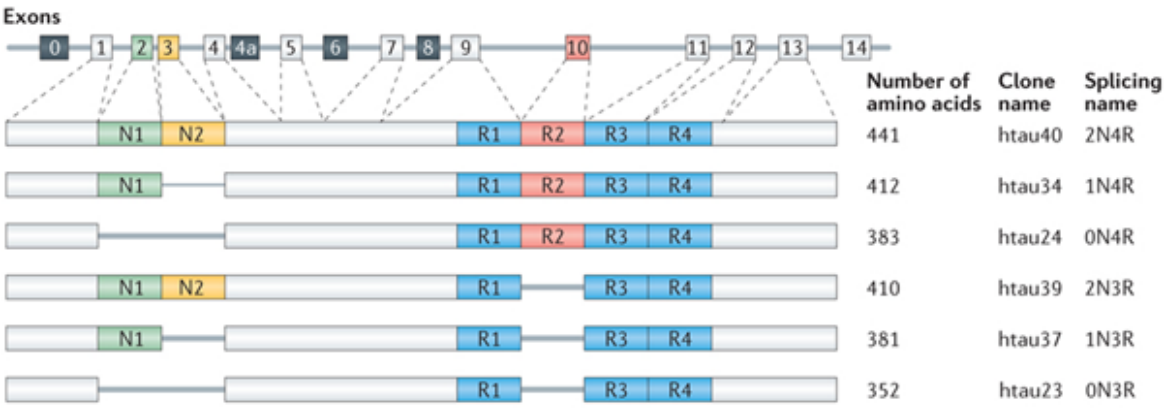


Fig. 1.1 Adapted from Wang and Mandelkow (2015). The 6 tau isoforms are formed by MAPT pre-mRNA alternative splicing of exon 2, 3 or 10

Jakes, 1990; Goedert et al., 1989). In the human foetal brain only 3R tau is expressed, whilst in the normal adult brain, both 3R and 4R tau are expressed in equal amounts (Goedert and Jakes, 1990; Goedert et al., 1989; Richey et al., 1995). Four repeat tau shows an approximate 3 fold increase in its binding affinity for microtubules compared to 3R tau and is thus more effective at promoting microtubule assembly, which has been observed using video microscopy where 4R tau greatly suppresses the rate and extent of microtubule disassembly (Panda et al., 2003). This developmental change in isoform expression suggests that tau plays a different role in development from postnatal time to adulthood possibly reflecting the different functional requirements of microtubules in foetal and adult neurons. Moreover, tau undergoes several post-translational modifications, including phosphorylation which regulates its biological function, indeed tau is hyperphosphorylated during development (Martin et al., 2011). When tau is phosphorylated it undergoes a conformational change reducing tau's affinity to microtubules and triggering its detachment from the microtubule (Andorfer et al., 2003; Goedert et al., 1992; Mietelska-Porowska et al., 2014). Its de-phosphorylation by tau phosphatases has the opposite effect restoring microtubule binding (Billingsley and Kincaid, 1997; Goedert et al., 1992). This cycle between kinase mediated phosphorylation of tau and de-phosphorylation by phosphatases is essential for axonal transport

and maintaining a balance between the two is important. When tau is bound is to the microtubule it has been proposed to impede binding of motor proteins, slowing axonal transport (Ebneth et al., 1998). Other studies propose a different view showing direct involvement of tau in axonal transport through interaction with cytoskeletal motor proteins dynein and kinesin (Cuchillo-Ibanez et al., 2008; Magnani et al., 2007; Rodríguez-Martín et al., 2013). *In vitro* binding assays have shown tau binds to the p150 domain of the dynein cofactor dynactin, whilst Hanger et al show not only does tau bind to kinesin but tau's ability to interact with kinesin is modulated by tau phosphorylation. When tau phosphorylation by GSK3β is inhibited, this impairs tau's ability to interact with kinesin and slows axonal transport of tau (Cuchillo-Ibanez et al., 2008). Other post-translational modifications include glycosylation, methylation, glycation, ubiquitination, acetylation and truncation (Martin et al., 2011).

1.2.2 Tauopathies

Structurally tau is a highly soluble natively unfolded protein, however, under pathological conditions, it becomes hyperphosphorylated forming neurofibrillary tangles comprised of insoluble tau aggregates (Fig. 1.2) (Friedhoff et al., 2000; Goedert et al., 1992). The formation of neurofibrillary tangles leads to neuronal dysfunction and eventually cell death. The presence of tau aggregates characterise a group of neurodegenerative diseases collectively known as tauopathies. These include Alzheimer's disease (AD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17T), and Pick disease (PiD) (Spillantini and Goedert, 2013).

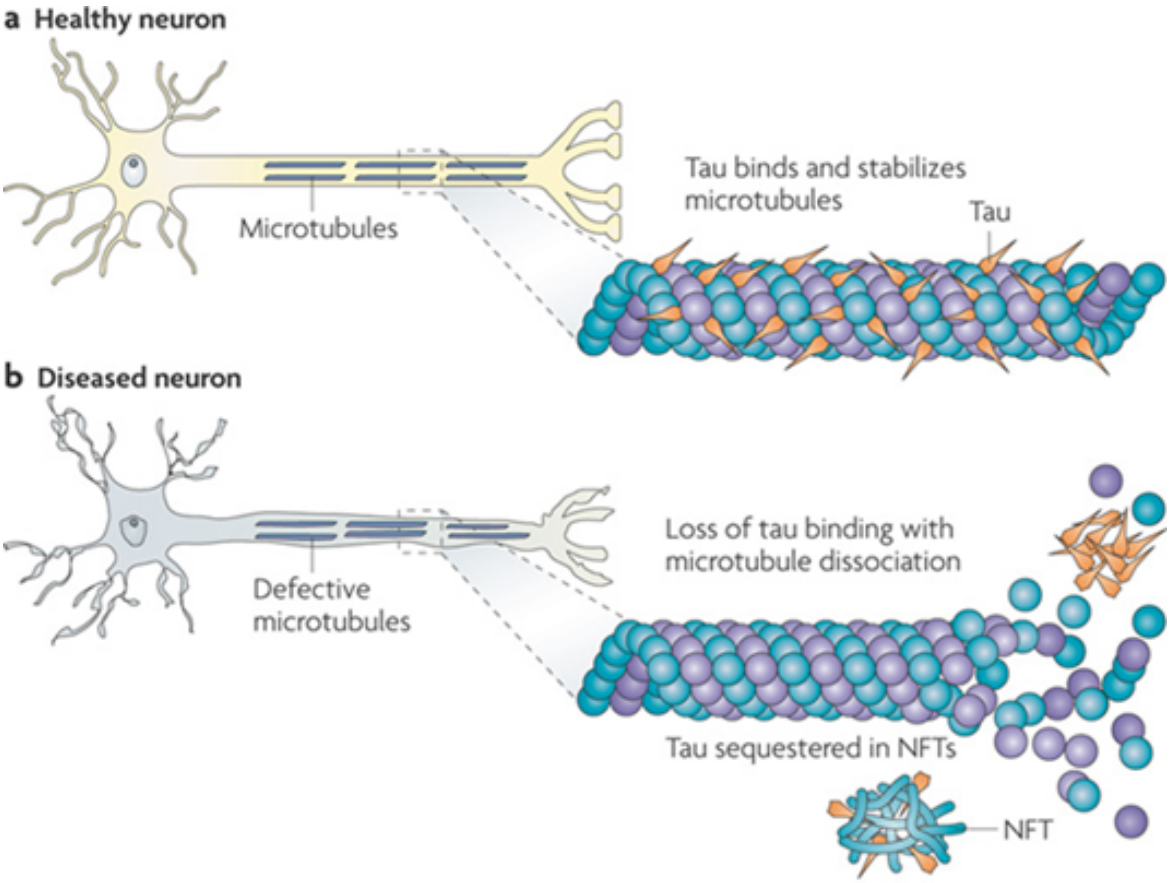


Fig. 1.2 From Brunden et al. (2009). Image depicting a healthy neuron and a diseased neuron. When tau becomes hyperphosphorylated it detaches from the microtubule and becomes sequestered into filaments forming NFTs. There is loss of stability of the microtubule which dissociates leading to impaired neuronal function and eventually neuronal death.

1.2.2.1 Alzheimer's Dementia

Alzheimer's disease is characterised by two distinct pathologies, intracellular neurofibrillary tangles and extracellular amyloid plaques. Neurofibrillary tangles consist of tau arranged as paired helical filaments or straight filaments. Electron microscopy reveals both PHFs and SFs to consist of a C-shaped double helical β stacked core with disordered N- and C- termini projecting away from the core forming the 'fuzzy coat' (Fitzpatrick et al., 2017). Disease filaments are comprised of all 6 tau isoforms (Fitzpatrick et al., 2017; Goedert et al., 1992).

Braak and Braak show a characteristic pattern of tau pathology distribution in Alzheimer's disease that correlates with the clinical presentation of dementia (Bancher et al., 1993; Braak and Braak, 1991). They identify 6 distinct neuropathological stages. In early stages (I and II) tau pathology is identified in the transentorhinal cortex with only mild involvement of the CA1 subfield in the hippocampus. This then progresses to involve the entorhinal and transentorhinal regions (stage III) with moderate involvement of the hippocampal formation by stage IV. By stage V and VI there is severe involvement of the entire hippocampal formation with pathology extending to the neocortex, involving the striate cortex in the occipital lobe by stage VI (Fig 1.3) (Braak and Braak, 1991).

Alzheimer's disease is the most common tauopathy presenting as a gradual decline in memory and cognitive functions (Cudaback et al., 2015). The onset is insidious with most patients presenting from age 65. There is a prodromal transitional stage between ageing and Alzheimer's referred to as mild cognitive impairment (MCI) characterised by some memory impairment with preservation of other higher cortical functions (Fan et al., 2008; Morris et al., 2001; Vuoksimaa et al., 2020). As the degree of neuronal

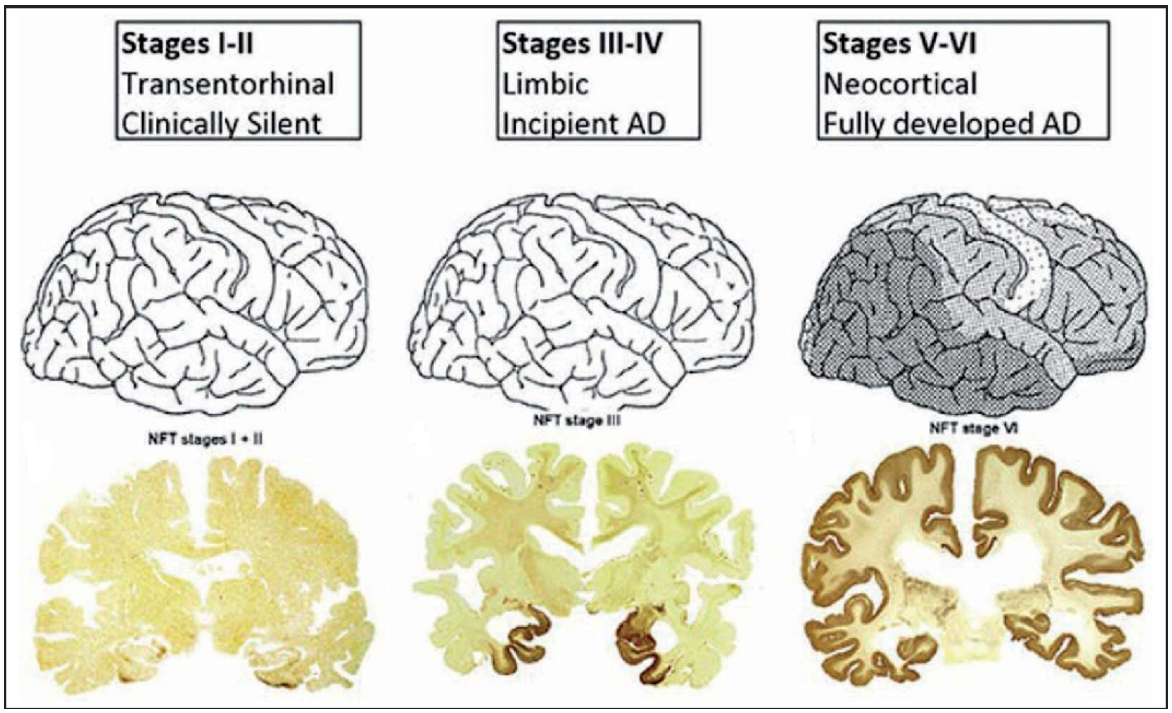


Fig. 1.3 Adapted from Vies 2016. Braak and Braak describe 6 distinct neuropathological stages of tau pathology in Alzheimer's disease. In stage I-II tau pathology is identified in a single layer of the transentorhinal cortex. In stage III-IV pathology has now spread to involve the entorhinal and transentorhinal layer. By stage V-VI the pathology now also involves the remaining neocortex. These have been shown to correlate with clinical dementia.

loss is directly correlated to clinical presentation (Gómez-Isla et al., 1997), detection at this stage is critical for early intervention. Diagnoses is made by a series of cognitive assessments with cerebrospinal fluid (CSF) biomarkers, such as total tau, phosphorylated tau (p-tau181 and p-tau231) and β-amyloid1–42 being increasingly used (Fortea et al., 2018; Niemantsverdriet et al., 2017). The only current treatment options are cholinesterase inhibitors and glutamate receptor antagonists (Parsons et al., 2013). They offer some delay in clinical progression but any symptomatic improvement is often short lived (Danysz and Parsons, 2003; Winblad et al., 2007). There is no cure.

1.2.2.2 Progressive supranuclear palsy

PSP also known as Steele-Richardson- syndrome is a progressive condition usually presenting in the mid-60s (Daniel et al., 1995). It is predominantly a movement disorder classified as a Parkinson plus syndrome (Stacy and Jankovic, 1992). There are three main subtypes; PSP-Richardson syndrome (PSP-RS), PSP-Parkinson's (PSP-S) and PSP-Cerebellar (PSP-C) (Ling, 2016). PSP-RS is the classical and most common subtype presenting with supranuclear gaze palsy, axial dystonia, postural instability, parkinsonism and cognitive impairment. In PSP-C there is marked truncal and limb ataxia, amongst other cerebellar signs, and PSP-P is defined by prominent parkinsonism (Ling, 2016; Williams, 2006).

While in AD tau deposits are predominantly neuronal, PSP is characterised by the accumulation of tau aggregates in neurons as well as in glial cells (Kahlson and Colodner, 2015). The presence of the so called 'tufted astrocytes', containing dense packed tau fibrils, is pathognomonic of PSP (Iwasaki et al., 2004; Kahlson and Colodner, 2015). In addition to 'tufted astrocytes', there is neurodegeneration accompanied by the presence of neurofibrillary tangles, neuoropil threads and gliosis. Ultrastructural

assessment of neurofibrillary tangles in PSP show these to consist of straight filaments and also identifies globose neuronal tangles (Tellez Nagel and Wiśniewski, 1973). The principle regions affected by tau pathology are the globus pallidus, subthalamic nuclei and substantia nigra within the basal ganglia, the dentate nucleus of the cerebellum and the brainstem nuclei, particularly within the mid brain (Williams et al., 2007). At later stages dementia can appear and it is associated with the presence of neurofibrillary tangles with 6 tau isoforms in cortex and hippocampus. A high density of neurofibrillary tangles and neuritic threads within the basal ganglia and brainstem is crucial for a diagnosis of PSP (Hauw et al., 1994). The National Institute for Neurological Disorders and Stroke (NINDS) criteria require a moderate to severe density of neuronal tangles and neuritic threads in three out of the following regions; pallidum, subthalamic nucleus, substantia nigra, or pons, and a low to high density in at least three of the following sites; striatum, oculomotor complex, medulla, or dentate nucleus (Hauw et al., 1994).

Clinical diagnosis is more challenging, due to similarities with other neurological conditions such as Parkinson's disease. MRI shows significant atrophy of the mid brain and is often used to support the diagnosis with some studies stating midbrain volume measurement provides a diagnostic accuracy of 99% in distinguishing PSP from Parkinson's disease (Zanigni et al., 2016). Management is symptomatic only with most parkinsonian features unresponsive to levodopa (Höglinger et al., 2017). Variations in the *MAPT* gene have been shown to be associated with PSP. The *MAPT* gene is located in a region of extended linkage disequilibrium that is determined by two main haplotypes, H1 and H2 (Baker et al., 1999; De Silva et al., 2001; Evans et al., 2004; Liu et al., 2001). Various studies, including genome wide association studies, have shown a significant association between PSP and the H1 haplotype, and in particular

the subhaplotype H1c (Baker et al., 1999; Conrad et al., 1997; De Silva et al., 2001; Höglinger et al., 2011; Myers et al., 2007; Pittman et al., 2005). The H1c haplotype has been shown to increase *MAPT* expression, in particular the 4R tau isoform, supporting a pathological role for tau in PSP (Myers et al., 2007)

1.2.2.3 Corticobasal degeneration

CBD, also part of the Parkinson plus syndromes, can present in a similar manner to PSP usually between the 5th and 7th decade of life (Williams and Litvan, 2013). There are progressive movement abnormalities accompanied by higher cortical dysfunction. Cortical basal syndrome (CBS) consists of patients presenting with motor symptoms such as akinesia, parkinsonism and rigidity alongside signs of cortical dysfunction and basal ganglia deficits (Parmera et al., 2016). Several disorders can present with CBS such as AD and PSP-RS (Parmera et al., 2016). Given the clinical heterogeneity with several other disorders, a diagnosis of CBD is usually only confirmed at postmortem. Pathologically there are neuronal and glial tau inclusions including astrocytic plaques distributed in the cortex, basal ganglia, diencephalon and brainstem (Kouri et al., 2011). Similar to PSP, CBD is associated with the *MAPT* H1 haplotype (Pittman et al., 2005) and tau aggregates formed by filaments containing 4R tau isoforms. Given the clinical and pathological similarities, it has been proposed that PSP and CBD are the same disease on different ends of a disease spectrum.

1.2.2.4 Pick's disease

Pick's disease is a sporadic disorder characterised by severe atrophy of the frontal, parietal and temporal lobes and is thus classified as a frontotemporal dementia (Weder et al., 2007). Patients often present with language impairment and personality change. There are three main clinical syndromes, which reflect the initial distribution of

pathology; frontal variant, progressive fluent aphasia and progressive non-fluent aphasia (Hodges, 2001; Olney et al., 2017; Weder et al., 2007). Pathologically there are spherical tau neuronal inclusion consisting of straight filaments known as Pick bodies as well as ballooned neurons known as Pick cells, present in the dentate gyrus and cortex (Olney et al., 2017; Probst et al., 1996). Pick bodies consist uniquely of 3R tau isoforms (Probst et al., 1996) as recently confirmed by cryo-electron microscopy (Falcon et al., 2018). Diagnosis is made by detailed cognitive testing supported by imaging but can only be truly confirmed post mortem (Hodges, 2001).

1.2.2.5 FTDP-17T

FTDP-17T is a familial disorder which presents usually around the 5th to 6th decade of life. Alongside Pick's disease, it is a cause of frontotemporal lobe degeneration (Olney et al., 2017; Spillantini et al., 1998; Wszolek et al., 2006). Clinically there is evidence of frontal lobe dysfunction with behavioural or personality change, cognitive impairment and Parkinsonism (Wszolek et al., 2006). Inheritance is predominantly autosomal dominant and due to genetic mutations in the *MAPT* gene (Hutton et al., 1998; Spillantini et al., 1998). To date over 60 different mutations in MAPT have been identified (Strang et al., 2019). The discovery of tau mutations in FTDP-17T demonstrate that abnormalities in tau alone are sufficient to cause pathology and disease (Spillantini and Goedert, 2013). Most of the mutations identified in FTDP-17T fall into three major categories, mutations that affect regulation of tau alternative splicing, missense or deletion mutations that impact on tau interaction with microtubules, or, favour tau assembly (Ghetti et al., 2015; Goedert et al., 2000; Strang et al., 2019). Splicing mutations disrupt the alternative splicing of exon 10 resulting in an imbalance of 3R and 4R tau isoforms, which are normally expressed in equal amounts in the normal human adult brain (Goedert and Jakes, 2005).

Missense mutations within the microtubule binding domain impair tau's ability to promote microtubule assembly, with some promoting tau aggregation, resulting in a loss of function and impaired axonal transport (Ingram and Spillantini, 2002; Strang et al., 2019). Deleterious effects of these mutations have also been attributed to their causing conformational changes in tau increasing its propensity to form a β sheet conformation enhancing misfolding and aggregation (Lathuilière et al., 2017).

All the cases with FTDP-17T where the brain has been examined, show tau aggregates that, depending on the *MAPT* mutation, contain different tau isoforms and resemble the tau pathology observed in sporadic tauopathies such as PSP, CBD, AD and Pick disease supporting the involvement of tau in the neurodegenerative process also in sporadic tauopathies. Although identifying these mutations has increased our understanding of possible mechanisms in tauopathies, the exact events leading to formation of neurofibrillary tangles is still poorly understood.

1.2.2.6 Mechanisms in tau pathology

Since hyperphosphorylated tau was identified as a key component of neurofibrillary tangles (Grundke-Iqbal, 1986), this has been one of the most well studied post translational modifications. Hyperphosphorylation of tau is thought to be a critical and early event leading to tau misfolding and aggregation (Dorard et al., 2016; Jin et al., 2015; Wang et al., 2013). Under normal physiological conditions, 80% of tau is bound to microtubules (Šimić et al., 2016). In AD brains, the level of tau phosphorylation is increased 3-4 fold, causing dissociation of tau from the microtubules, altering tau function and increasing the amount of tau free to aggregate (Wang et al., 2013). Some of the main kinases in the brain that have been found to phosphorylate tau include glycogen synthase

kinase 3β (GSK3β) (which has been strongly implicated in the pathology of AD), cyclin dependent kinase 5 (Cdk5), stress activated protein kinase c-Jun N-terminal kinase (SAPK/JNK) and mitogen activated protein kinases (MAPK) (Dolan and Johnson, 2010; Engmann et al., 2011; Medina and Avila, 2014). The pathogenic events leading to aberrant tau phosphorylation however remain unknown, although targeting kinase activity remains an important therapeutic strategy. There is evidence to suggest that in tauopathies there is a downregulation in phosphatase activity, leading to an imbalance between phosphorylation and dephosphorylation contributing to aberrant tau phosphorylation (Gong et al., 2000; Park et al., 2018; Sontag et al., 2004). Cleaved tau fragments have also been identified in regions associated with tau pathology in various tauopathies with an observed upregulation of caspase 3 (Delobel et al., 2008; Rohn et al., 2002). Proteolytic cleavage by caspase 3 has been shown to promote tau aggregation with caspase 3 cleaved tau species being more prone to phosphorylation (Gamblin et al., 2003; Means et al., 2016; Rissman et al., 2004; Zilka et al., 2006). It has been proposed that cleavage is an early event in disease development as the presence of truncated tau has been noted prior to the appearance of significant aggregation (Rissman et al., 2004).

The mechanism by which tau phosphorylation and aggregation lead to neuronal death is not understood. The loss of tau function, and subsequent dysregulation of axonal transport, is thought to be only part of the mechanism by which tau pathology results in neuronal death. Tau knockout mice display a mild phenotype of muscle weakness (Lei et al., 2014) which suggest that in addition to a loss, there is also a toxic gain of function involved. In addition to tau, the neuronal microtubule network is supported by other microtubule associated proteins (MAPs), such as MAP1 and MAP2 (Kapitein and Hoogenraad, 2015). These should compensate for the loss of functional tau, however, hyperphosphorylated tau has been shown to not only sequester

normal tau, but also both MAP1 and MAP2 (Alonso et al., 1997). The resulting transport defects are known to be a key feature of tauopathies contributing to neurodegeneration, although other mechanisms are also likely to be involved (De Vos et al., 2008). Microtubules provide tracts for cellular transport of cargo from pre- and postsynaptic sites, including mitochondria. Both mitochondrial and synaptic dysfunction have been observed in tauopathies (Rodríguez-Martín et al., 2016; Vogels et al., 2019). Perturbations in axonal transport could very well be contributing to this and animal models provide evidence that tau mutations are sufficient to cause mitochondrial and synaptic dysfunction (Rodríguez-Martín et al., 2016; Yoshiyama et al., 2007).

1.3 Inflammation and neurodegeneration

Neuro-inflammation, in the context of neurodegeneration is typified by morphological changes in glial cells, astrocytes and microglia, which become activated secreting low to moderate levels of inflammatory mediators such as prostaglandins and cytokines (Woodling and Andreasson, 2016). As this reaction does not differ significantly between neurodegenerative diseases it was initially thought to be common to various disease processes, however, increasing evidence seems to show that neuro-inflammation in itself is pathogenic (Heneka et al., 2015; Heppner et al., 2015; Metcalfe and Figueiredo-Pereira, 2010). There is a strong correlation between peripheral inflammation and cognitive deficits (Walker et al., 2017). Systemic infections have long been associated with cognitive impairment with one study showing 60% of elderly patients admitted with infection related delirium going on to develop dementia at a 3 year follow up compared to 18.5% of elderly patients without delirium (Rockwood, 1999). Several autoimmune disorders, which display marked systemic inflammation, are associated with an increased risk of dementia that in one study was reported to be 51% in the case of systemic lupus

erythematosus (SLE) (Gendelman et al., 2018). Systemic inflammation damages the integrity of the blood brain barrier allowing inflammatory cytokines and peripheral immune cells to cross causing tissue injury and triggering brain specific inflammatory responses thought to contribute to disease pathology (Abbott et al., 2003; Nation et al., 2019). Compromises in blood brain barrier integrity secondary to inflammation have been observed in cardiovascular disease, obesity and type 2 diabetes mellitus, all risk factors for developing Alzheimer's (Gustafson et al., 2007; Hawkins et al., 2006; Pooja Naik et al., 2014). More recently Walker et al., in a longitudinal study spanning 20 years, showed that higher levels of circulating inflammatory markers in midlife is associated with cognitive deficits as well as smaller brain volumes later in life, particularly in regions associated with Alzheimer's pathology such as the hippocampus (Walker et al., 2019, 2017).

The pathogenic role of inflammation is further supported by genome wide association studies (GWAS) identifying several genes involved in immune system regulation and inflammatory signalling as being risk factors for Alzheimer's (Gandhi and Wood, 2010; Jonsson et al., 2013; Kim et al., 2009; Zhang et al., 2015). The first significant risk gene for late onset AD identified was the apolipoprotein E4 gene, APOE (Bertram and Tanzi, 2009), a cholesterol transporter which also plays a role in modulating the neuro-inflammatory response of glial cells (Guo et al., 2004). Since then, several other risk genes involved in microglial function have been identified, including TREM2, CD33, SHIP1, CR1 and ABCA7 (Hollingworth et al., 2011; Jonsson et al., 2013; Naj et al., 2011). Microglia are the resident macrophages in the brain, with numbers normally tightly regulated (Perry and Holmes, 2014). During chronic inflammation, as seen in neurodegeneration, there is microglial proliferation and a sustained microglial response due to microglial priming, leading to neuronal toxicity. Priming is the concept

that microglia, following exposure to an initial stimulus, subsequent stimuli cause an exaggerated release of inflammatory cytokine, mediators and prostaglandins (Cunningham, 2013; Heneka et al., 2015; Levi et al., 1998). In early Alzheimer's, it has been proposed that the presence of amyloid plaques leads to microglial activation and priming. In support of this, microglia and activated astrocytes are commonly observed in post-mortem brains derived from individuals with Alzheimer's dementia (Kitazawa et al., 2004). Evidence from mouse models and *in vitro* work suggests this initial inflammatory response is beneficial with microglia phagocytosing the abnormal protein aggregates (Lee and Landreth, 2010; Michaud et al., 2013). However, a sustained response results in large abnormal microglia that are no longer able to process the phagocytosed material (Caldeira et al., 2017). This has been demonstrated *in vitro*, where cultured microglial display impaired phagocytic function following prolonged exposure to aggregated amyloid (Caldeira et al., 2017). This reduction in microglial efficiency is coupled with an increase in inflammatory cytokines and prostaglandin production thought to aggravate pathology (Kinney et al., 2018; Wang et al., 2015). More microglia and peripheral macrophages are then recruited to assist the failing microglia further exacerbating the inflammatory response driving disease pathology through a feed-forward loop. This inflammatory response, involving the secretion of cytokines and increased prostaglandin production, has been proposed as a potential link between amyloid plaques and subsequent development of tau neurofibrillary tangles (Kinney et al., 2018; Kitazawa et al., 2004), although this has yet to be demonstrated in humans.

1.4 Prostaglandins

Prostaglandins are key inflammatory mediators belonging to the eicosanoid family, signalling molecules resulting from oxidation of arachidonic acids (Park et al., 2006).

Prostaglandins can be constitutively expressed at low levels but their synthesis is greatly increased in the presence of trauma or inflammatory stimuli resulting in several of the cardinal signs of acute inflammation. In response to mechanical, immunological and infectious stimuli, arachidonic acid is released from the cell membrane by phospholipase A where it is oxygenated by cyclo-oxygenase enzymes (COX) to form prostaglandin H2 (PGH2) (Ricciotti and FitzGerald, 2011). There are two isoforms of COX, COX-1 and COX-2. COX-1 is constitutively expressed and is responsible for prostaglandin production involved in normal physiology and housekeeping functions whereas, COX-2 is induced under inflammatory conditions and is thus involved in prostaglandin associated with injury and inflammation (Fitzpatrick, 2004). Non-steroidal anti-inflammatory drugs (NSAIDs), a group of drugs used to treat conditions characterised by pain and/or inflammation, function by inhibiting COX enzymes preventing downstream prostaglandin production (Cryer and Feldman, 1998) as PGH2, a common substrate for the production of 4 main terminal prostaglandins; PGD2, PGE2, PGF2 and PGI2. Thromboxane A2, a prostanoid, is also generated through this pathway (Fig. 1.4).

Prostaglandin E2 (PGE2) is the most abundant prostaglandin in the human body and has multiple physiological and pathological functions including pyrexia, vascular permeability, smooth muscle contraction and relaxation, mucosal protection and modulation of inflammation (Inoue et al., 2002; Ivanov and Romanovsky, 2004; Kalinski, 2012). PGE2 is considered an important link between the peripheral immune system and the brain. PGE2 exerts its effects through four E-prostanoid (EP) receptors, EP1, EP2, EP3 and EP4 (Andreasson, 2010) which are all G-protein coupled receptors (GPCR). The differences between each receptor in intracellular signalling pathways and tissue expression explains the versatility of PGE2 that can exert multiple effects, including anti- and pro-inflammatory actions, depending on the receptor and its location.

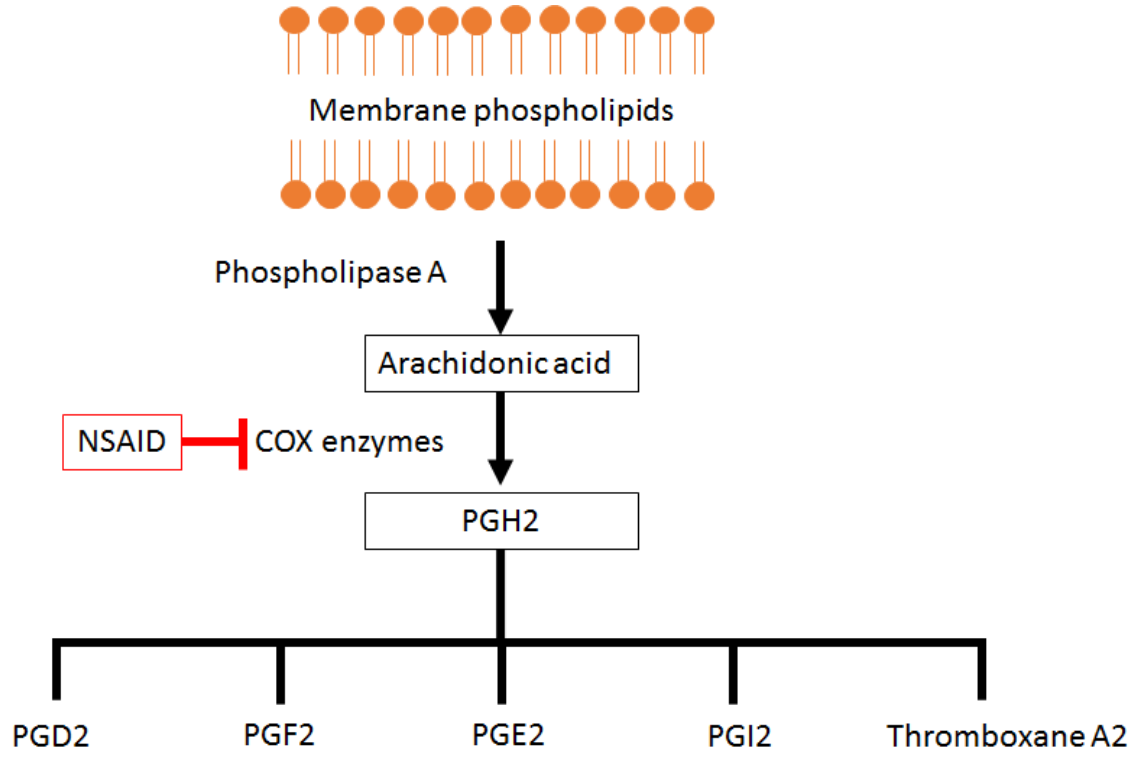


Fig. 1.4 In response to injury, phospholipase A releases arachidonic acid from the cell membrane. Arachidonic acid is then oxygenated by COX-1 or COX-2 to form PGH2 which is then converted by tissue specific isomerases to various prostanoids. NSAIDs work by inhibiting COX enzymes thus affecting all prostaglandin production. COX = cyclooxygenase, NSAID = non-steroidal anti-inflammatory drug, PGH2 = prostaglandin H2, PGD2 = prostaglandin D2, PGF2 = prostaglandin F2, PGE2 = prostaglandin E2, PGI2 = prostaglandin I2.

1.4.1 The PGE2 EP receptors

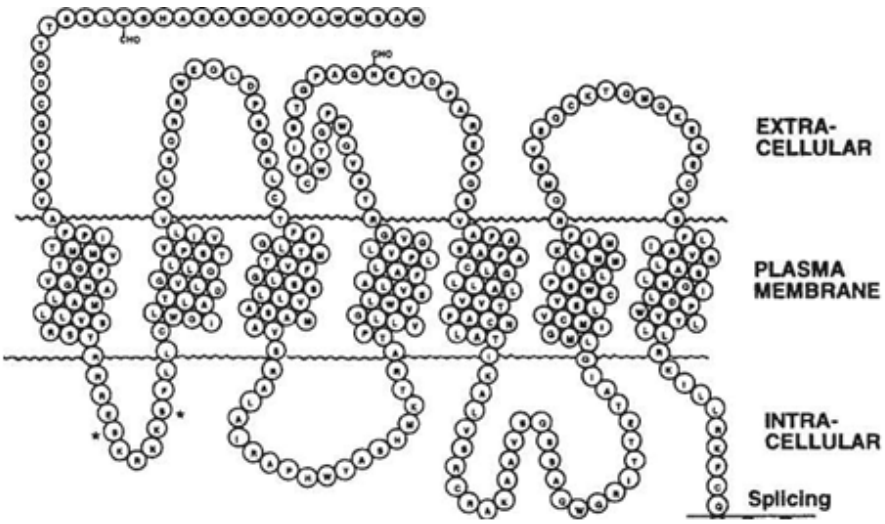
Reflecting the broad spectrum of pharmacological and physiological effects of PGE2, all 4 EP receptors are expressed throughout the body, with particularly high expression noted in the brain (Andreasson, 2010; Sugimoto and Narumiya, 2007). Each receptor has a different signal transduction pathways with signaling through the EP1 receptor increasing intracellular calcium levels, EP2 and EP4 increasing cyclic adenosine monophosphate (cAMP), and EP3 inhibiting this, although splice variants of the EP3 receptor do differ in their actions (Friis et al., 2005; Hatae et al., 2002a; Kim et al., 2002).

The EP1 receptor has been implicated in regulating impulsive behavior under stress. Matsuoka et al showed that EP1 deficient mice display abnormal behavior traits including impulsive and aggressive behavior (Matsuoka et al., 2005). This phenotype is reproducible by pharmacological inhibition of the EP1 receptor. EP1 deficient mice also display a significant increase in dopaminergic activity (Kitaoka et al., 2007; Matsuoka et al., 2005). Administration of a dopamine antagonist appeared to reverse the phenotype suggesting PGE2 modulates dopamine via EP1 (Kitaoka et al., 2007; Matsuoka et al., 2005). EP1 also functions to regulate cerebral blood flow inducing vasoconstriction in both peripheral and cerebral vasculature (Jadhav et al., 2004; Norel et al., 2004; Rutkai et al., 2009). The EP4 receptor, unlike EP1, is responsible for PGE2 induced vasodilation and smooth muscle relaxation (Thibodeau et al., 2016; Yokoyama et al., 2013). EP4 is expressed in endothelial cells, as well as neurons, mediating vasodilation under normal physiological conditions in various vascular beds in the peripheral and central circulation (Narumiya and FitzGerald, 2001). Although this is yet to be demonstrated in neurological disorders, in other systemic disorders such as sepsis and rheumatoid arthritis, EP4 has been shown to mediate anti-inflammatory effects of PGE2 through inhibition of inflammatory cytokines and downregulation of adhesion

molecules on monocytes (Fushimi et al., 2007; Sakamoto et al., 2004; Takahashi et al., 2002). The EP2 receptor within the CNS is involved in synaptic plasticity and long term potentiation (Yang et al., 2009). Expressed in the hippocampus, EP2 deficient mice show significant cognitive deficits and increased anxiety (Savonenko et al., 2009). In contrast to EP2, PGE2 signalling through the EP3 receptor has been shown to have detrimental effects on long term potentiation and synaptic plasticity with EP3 being implicated in postoperative cognitive impairment (Maingret et al., 2017; Xiao et al., 2018). The distribution of the EP receptors has been mapped in rodent brains, which show the EP3 and EP4 receptors as being the most widely expressed (Markovič et al., 2017; Sugimoto and Narumiya, 2007).

1.4.2 The EP3 receptor

The EP3 receptor is encode by the *PTGER3* gene which, has 12 coding exons and uniquely amongst the PGE2 receptors, generates multiple isoforms as a result of alternative splicing events (Fig. 1.5). These range in size from 365 to 431 amino acids. Each isoform differs in length due to variations in the carboxyl-terminal tail suggesting different intracellular downstream signalling effects. To date, 9 isoforms in humans have been identified, EP3Ia, EP3Ib, EP3II, EP3III, EP3IV, EP3V, EP3VI, EP3e and EP3f (Kotani et al., 1997). The EP3 receptor is thought to mediate several functions of PGE2 within the central nervous system, including modulation of neurotransmitter release through calcium inhibition (Nakamura et al., 2002) however the functional role of individual isoforms has never been explored. The e isoform has the shortest carboxyl-terminal tail (6 amino acids beyond the common region). EP3 is thought to play a role in energy homeostasis and metabolic regulation. EP3 deficient mice have been shown to not only develop an obese phenotype but also have elevated insulin and leptin levels with associated insulin resistance (Ceddia et al., 2016; Sanchez-Alavez et al., 2007).



EP3.I	IRYHTNNYASSSTSLPCQCSSTLMWSDHLER
EP3.II	VANAVSSCSNDGQKGQPISLSNEIIQTEA
EP3.III	EEFWGN
EP3.IV	MRKRRLREQEEFWGN
EP3.V	MRKRRLREQEMGPDGRCFCHAWRQVPRTWCSSHDREPCSVQLS
EP3.VI	EMGPDGRCFCHAWRQVPRTWCSSHDREPCSVQLS
EP3.e	MRKRRLREQLICSLRTLRYRGQLHIVGKYKPIVC
EP3.f	MRKRRLREQAPLLPTPTVIDPSRFCAQPFRWFLDLSFPAMSSSHPQLPLTLASFKLLREPCSVQLS

Fig. 1.5 The EP3 receptor. Alternative splicing of the *PTGER3* gene gives rise to species specific isoforms. Each isoform differs by the intracellular C-terminus (Kotani et al., 1997).

These mice are hyperphagic and obese on a chow diet. The mechanism is thought to be through impaired lipolysis and increased adipose tissue deposition. EP3 deficient mice display greater energy intake during their resting phase mimicking 'night time' eating. They show increased sleep fragmentation with inability to consolidate sleep in their rest periods (Sanchez-Alavez et al., 2007). PGE2 has been shown to play an important role in sleep regulation. PGE2 when directly injected into the histaminergic tuberomamillary nucleus in the posterior hypothalamus promotes wakefulness (Huang et al., 2003) which is thought to be mediated through histamine release from the medial preoptic area and frontal cortex (Huang et al., 2003; McKinley et al., 2015). The disrupted sleep/wake cycle noted in EP3 knock mice as well as the EP3 expression observed in the medial preoptic area, suggests an involvement of EP3 signalling. PGD2, another endogenous prostaglandin that has been shown to bind to the EPR3 receptor, also plays an important role in arousal and sleep (Hayaishi, 2000; Urade and Hayaishi, 2000, 2011).

The EP3 receptors are G-protein coupled receptors (GPCR) belonging to the rhodopsin-like family of GPCRs (class A), the largest class of GPCRs (Vassilatis et al., 2003). These are 7 trans-membrane receptor proteins that, upon ligand binding undergo a conformational change resulting in an intracellular signalling cascade transduced by a heterotrimeric G-protein. The heterotrimeric G protein consists of 3 main subunits, α, β and γ. GPCRs interact directly with the α subunit, of which there are 4 major classes, Gs, Gi, Gq and G12 (Oka et al 2009). They each interact with different second messenger molecules, such as cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG), inositol trisphosphate (IP3) or calcium. The EP3 receptor is unique among the E-prostanoid receptors due to its ability to couple to multiple Gα subunits. Which α subunit is determined by the carboxyl-terminal tail of EP3, which differs amongst each isoform. EP3 receptors signal predominantly via adenyl cyclase inhibition through Gi

coupling and calcium mobilisation by the G$\beta\gamma$ subunit of the Gi protein however they have also been shown to couple to Gs promoting cAMP production (Kozaki et al., 2007; Sugimoto and Narumiya, 2007). Gi coupling in the mouse EP3 receptor isoforms have been well characterised. Three different isoforms have been recognised, EP3α, EP3γ and EP3β with varying degrees of constitutive Gi activity thought to be determined by the length of the carboxy-terminal tail (Hizaki et al., 1997). Indeed, when this is completely truncated, the receptor remains constitutively active independent of its agonist (Hizaki et al., 1997). It has been proposed that it is the diversity of isoforms generated through alternative splicing that create the differing PGE2 dose response curves. The EP3γ isoform has also been shown to couple to Gs, promoting adenyl cyclase production. The diversity of isoforms generated through alternative splicing is thought to contribute to the wide variety of PGE2 effects, however, the physiological relevance of differing EP3 transduction pathways is yet to be determined. Studies in the bovine isoforms have shown EP3 Gs and Gi coupling, as well as their coupling to G13 resulting in neurite retraction and dendritic pruning that are essential components of memory formation (Katoh et al., 1996). This effect has been shown to be mediated through activation of small GTPase Rho, one of the few EP3 signalling cascades associated with a specific biological function.

Studies of EP3 signalling in COS-7 cells show that EP3 has also the ability to modulate other signalling cascades through intracellular cross talk. Hatae et al have shown that EP3 can increase adenyl cyclase production from PGE2-EP2 signalling (Hatae et al., 2002b). This occurs regardless of the carboxy-terminal tail structure and is believed to be mediated by EP3 induced calcium mobilisation and phospholipase C production resulting in EP2-induced cAMP formation (Hatae et al., 2002b; Yamaoka et al., 2009).

1.4.3 Prostaglandin signalling and Neurodegeneration

Inhibiting PGE2 signalling as a means of targeting inflammation in disease has been an important strategy for a long time but has only recently been seen as a potential therapeutic target for managing neurodegenerative disease. The role of inflammatory prostaglandin signalling was first highlighted through epidemiological data showing that patients taking NSAIDs had a 50% reduction in the risk of developing Alzheimer's compared to the general population (McGeer et al., 1996). PGE2, the most abundant prostaglandin in the CNS, became of particular importance when it was reported to be significantly elevated in the cerebrospinal fluid (CSF) of patients with early AD and declining as the disease progresses, suggesting a role in early pathology (Combrinck et al., 2006). It has also been reported to be elevated in other neurodegenerative diseases, in amyotrophic later sclerosis (ALS) in particular, where it has been found to be elevated in the CSF of patients as well as in mouse models of disease (Almer et al., 2002).

The COX-2 pathway and prostaglandin signalling have been shown to be neurotoxic in several experimental models of neurodegenerative disease where they promote synaptic toxicity and accelerate neuronal damage (Andreasson, 2010; Bazan et al., 2002; Liang et al., 2005a; Shi et al., 2012c). Di Giorgio et al demonstrated that astrocytes release PGE in amyotrophic lateral sclerosis which is directly toxic to motor neurons (Di Giorgio et al., 2008). In support of this finding, administration of COX-2 inhibitors has been shown to prolong survival in mouse models of ALS and delays the onset of motor symptoms (Pompl et al., 2003). The relationship between prostaglandins and Alzheimer's is complex with both neuroprotective and neurotoxic effects (Hatae et al., 2002b; Heppner et al., 2015). Whether PGE2 exerts a neuroprotective or neurotoxic effect is dependent on which receptor, as well as cell type it acts through (Hatae et al., 2002b). Wei L et al (Wei et al., 2010) propose that PGE2 signalling via EP1 and

EP3 exert neurotoxic effects while EP4 is neuroprotective. McChullogh et al showed that administration of PGE2 to hippocampal neurons protected them from glutamate toxicity in model of cerebral ischaemia, whilst administration of a specific EP1 and EP3 agonist elicited excitotoxicity and induced neuronal death (McCullough et al., 2004). This suggests the other EP receptors must have a neuroprotective role that is eliminated when PGE2 signals only through EP1 and EP3. The relationship between neuronal toxicity and signalling via the EP2 receptor is more complex with studies suggesting a protective role in acute inflammation but a more toxic one in models of chronic inflammation. In models of focal ischaemia, where neuronal death is induced through excitotoxicity, PGE2 signalling via EP2 is sufficient to rescue neurons (Liu et al., 2005). This ability is however eliminated on inhibition of protein kinase A (PKA) suggesting that this is mediated through EP2 coupling with Gs increasing cAMP production (Liu et al., 2005). In contrast to this, EP2 has been shown to mediate the inflammatory oxidative response driving secondary neuronal damage in the context of chronic inflammation (Liang et al., 2005a, 2008; Montine et al., 2002). In glial cells activated by an inflammatory stimulus such as LPS or amyloid peptides, EP2 promotes expression of pro-inflammatory genes leading to an increase in production of reactive oxygen species resulting in neuronal death (Liang et al., 2005a; Montine et al., 2002; Shie et al., 2005). In mouse models of ALS deletion of the EP2 receptor prolongs survival and improves motor symptoms (Liang et al., 2008).

The relationship between prostaglandin signalling and amyloid in Alzheimer's has been extensively looked at by the Andreasson Lab in Stanford (Andreasson, 2010; Bazan et al., 2002; Figueiredo-Pereira et al., 2016; Johansson et al., 2015; Liang et al., 2005a; Shi et al., 2012c; Wei et al., 2010; Wood, 2012; Woodling and Andreasson, 2016). They have demonstrated that deletion of the PGE2 EP2 receptor reduces amyloid

burden in a mouse model of Alzheimer's (Liang et al., 2005a). They suggest this may be through increased clearance and/or reduced generation of Aβ42. In the presence of EP2, they found an increase in β-CTF, a product of amyloid precursor protein (APP) cleavage by BACE1, suggesting EP2 modulates BACE1 cleavage increasing generation of Aβ42. EP2 has also been shown to impair microglial function resulting in reduced Aβ42 clearance (Johansson et al., 2015). Inhibiting microglial EP2 signalling not only decreases inflammation but in mouse models of disease also rescues memory (Johansson et al., 2015). The EP3 receptor has also been shown to increase BACE1 activity contributing to Aβ42 generation (Shi et al., 2012c). Post-mortem tissue of individuals with mild cognitive impairment and Alzheimer's show an upregulation of EP3 (Shi et al., 2012c). In control subjects EP3 is expressed at basally low levels in neurons whilst in individuals with Alzheimer's, not only is it upregulated, but EP3 is expressed in glial cells as well as neurons, particularly in areas surrounding amyloid plaques (Shi et al., 2012c). EP3 has also been implicated in synaptic dysfunction with Andreasson et al (2012) showing that deletion of the EP3 receptor in a mouse model of Alzheimer's rescues loss of pre-synaptic proteins (Shi et al., 2012c).

Unlike amyloid there is less published data on the interaction of prostaglandins and tau. It is generally accepted that neuro-inflammation plays an important role in the formation of tau tangles at the onset of the disease. In a review by Metcalfe et al. (Metcalfe and Figueiredo-Pereira, 2010), they propose a model by which an initial stimulus promotes inflammation. This activates glial cells releasing inflammatory markers, such as prostaglandins, which induce neurotoxicity. This then leads to impairment of the ubiquitin/proteasome pathway and activation of caspase which in turn cleaves tau, an important posttranslational modification which plays an important role in the generation of paired helical filaments and tangles. Arnaud et al. (2009) demonstrated this in

cell culture where they induced caspase mediated cleavage of tau using prostaglandin J2.

Results of intervention trials inhibiting PGE2 signalling in humans have been varied. The Alzheimer's Disease Anti-inflammatory Prevention Trial (ADAPT) trial randomised patients to either celecoxib (a COX-2 selective inhibitor), naproxen (a non-selective COX inhibitor) or placebo (ADAPT Research Group et al., 2009). The study unfortunately had to be terminated early due to adverse cardiac and gastric side effects, and no benefit was noted at the time the study was terminated with 7 patients who had been symptomatic at the start of the trial showing a deterioration in their symptoms. A four year follow up study was however done, which showed individuals who had been randomised to naproxen in the original study, or, who had previously been on naproxen prior to the study, had a 60% reduction in their risk of developing AD compared to the placebo group (Breitner et al., 2011). From this information they concluded that in order to be beneficial, NSAIDs must be taken for at least 3 years and early in the course of disease suggesting that prostaglandin signalling is likely to play a role in the early and pre-clinical stages of disease. This hypothesis is supported by epidemiological data as well as other intervention trials (McGeer et al., 1996; Szekely et al., 2004).

1.5 G Protein coupled receptors

As described above all EP receptors are G-protein coupled receptors (GPCRs), a large family of membrane bound receptors unique to eukaryotes. They have been remarkably conserved throughout evolution (De Mendoza et al., 2014) and share similar architecture, all containing seven alpha helical trans-membrane domains connected by three intracellular and three extracellular loops with an intracellular C-terminus (Kobilka, 2007). The domains form a pocket within the cell membrane and upon ligand

binding undergo a conformational change allowing the GPCR to act as a guanine nucleotide exchange factor (GEF) for an associated heterotrimeric guanine nucleotide binding protein (G-protein) resulting in an intracellular signalling cascade (Fredriksson et al., 2003; Kobilka, 2007). The G-protein complex consists of an α and a $\beta\gamma$ subunit. The G-protein is activated on exchange of guanine diphosphate (GDP) to guanosine triphosphate (GTP) allowing the α subunit to dissociate from the $\beta\gamma$ allowing both subunit to interact with second messengers as determined by the alpha subunit. There are four main classes of α subunit, Gαs, Gαi, Gαq, and Gα12/13. Gs, or stimulatory G-protein, stimulates adenyl cyclase converting adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) activating cAMP regulated proteins whilst Gi, has the opposite effect, inhibiting adenyl cyclase production, reducing cAMP levels (Gurevich and Gurevich, 2017). Gq activates phospholipase C which releases inositol-1,4,5-triphosphate (IP3) and diacyl glycerol (DAG) from the cell membrane by hydrolysing membrane phospholipids. These in turn increase intracellular calcium. The fourth class of G-protein, G12/13 function by activating GEFs for Rho small GTPases. Some GPCRs are promiscuous and can couple to several different classes of G-protein (Kostenis et al., 2005; Maudsley et al., 2005; Okashah et al., 2019). GPCRs are deactivated upon hydrolysis of GDP to GTP by intrinsic GTPase activity within the Gα subunit allowing the α and $\beta\gamma$ subunits to bind again terminating the signalling cascade. Regulators of G-protein signalling (RGS) are proteins that have the ability to accelerate this process by influencing the GTPase activity (De Vries et al., 2000). In the presence of a continuous stimulus, the receptor can be desensitised by binding to arrestin (Black et al., 2016). The receptor is first phosphorylated by G-protein coupled receptor kinases (GRK) allowing arrestins to bind to the cytoplasmic face of the receptor blocking any further G-protein coupling. Arrestin then directs the receptor for internalisation where it is packaged in endosomes and either recycled, back

to the cell membrane, or directed to lysosomes for degradation (Latorraca et al., 2018; Lohse and Hoffmann, 2014).

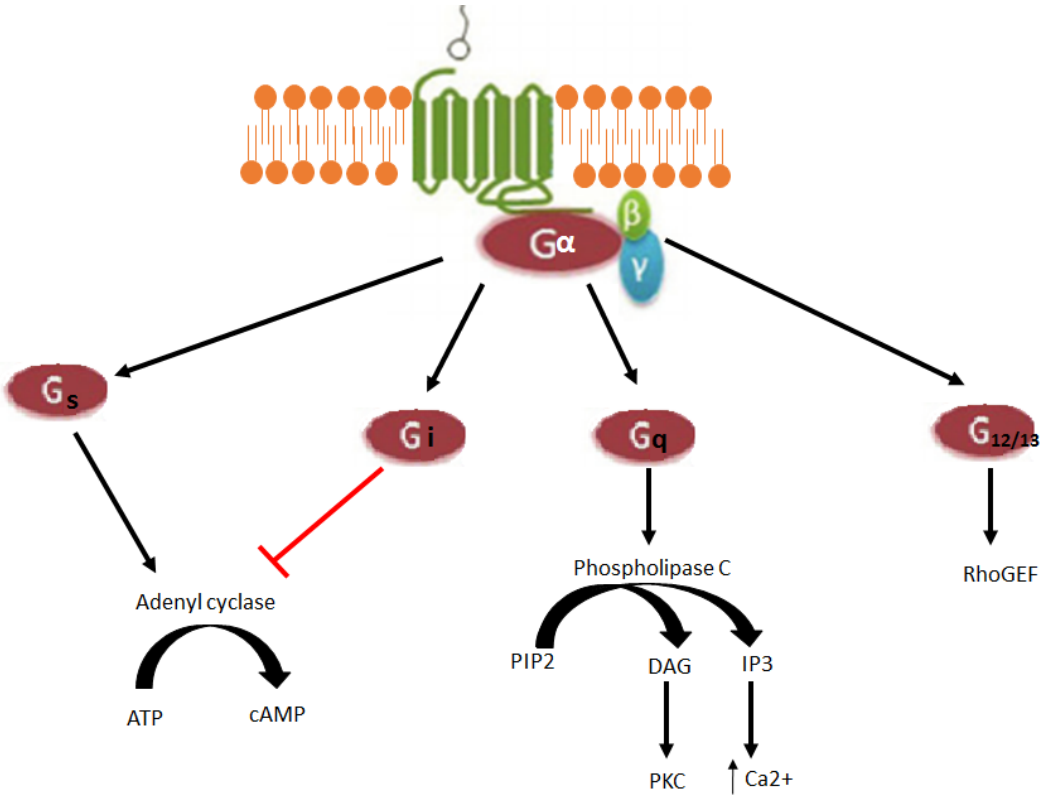


Fig. 1.6 GPCRs are coupled to a heterotrimeric G protein consisting of an α and $\beta\gamma$. On activation by ligand binding, the G-protein α subunit dissociates from the $\beta\gamma$ subunit allowing them to interact with second messengers signalling a signalling cascade. GPCRs couple to four main classes of G-protein determined by the α subunit; Gαs, Gαi, Gαq and Gα12/13. Gαs stimulates adenyl cyclase which converts ATP to cAMP. Gαi opposes this, inhibiting adenyl cyclase production. Gαq activates phospholipase C, hydrolysing PIP2 from the cell membrane releasing IP3 and DAG which in turn increase calcium levels and activate PKC which continues the signal transduction cascade regulating various downstream proteins. Gα12/13 activates RhoGEFs. ATP = Adenosine triphosphate, cAMP = cyclic Adenosine monophosphate, PIP2 = Phosphatidylinositol 4,5-bisphosphate, DAG = Diacylglycerol, IP3 = Inositol triphosphate, Ca^{2+} = Calcium, PKC = Protein kinase C, RhoGEF = Rho guanine nucleotide exchange factor.

GPCR's are responsible for multiple biological processes in humans mediating responses to hormones and neurotransmitters (Betke et al., 2012; Neubig and Siderovski, 2002). Their extensive involvement in pathophysiological processes make them one of

the most studied drug targets with GPCR modulators increasingly used as potential therapy for central nervous system disorders (Hauser et al., 2017; Neubig and Siderovski, 2002). Due to their expression on the plasma membrane they are easily accessible. Recent advances in structural biology and receptor pharmacology have also increased our ability to modulate these receptors making them a highly druggable and an ideal therapeutic target.

GPCRs are one of the most promising therapeutic targets for neurological disorders (Hauser et al., 2017; Neubig and Siderovski, 2002) and there is increasing awareness of the role they are likely to play in dementia and other neurodegenerative diseases (Hauser et al., 2017; Huang et al., 2017; Neubig and Siderovski, 2002; Thathiah and De Strooper, 2009). They play a wide role in the peripheral and nervous system with over 90% of non-sensory GPCRs being expressed in the brain (Gainetdinov et al., 2004). GPCRs fine tune many critical functions within the central nervous system and act as receptors to several neurotransmitters such as glutamate, GABA, serotonin and dopamine (Betke et al., 2012). Disrupted cholinergic, glutamatergic, adrenergic and serotonergic neurotransmission is seen in Alzheimer's and, to date, targeting this group of GPCRs has been the predominant focus of therapeutic intervention (Dall'Igna et al., 2007; Heaney and Kinney, 2016; Teng et al., 2010; Zhu et al., 2015).

The metabotropic glutamate receptors (mGluR), a family of GPCRs that modulate the neurotransmitter glutamate, have been implicated in the development of Alzheimer's (Thathiah and De Strooper, 2011) with pharmacological inhibition of mGluR5 alleviating amyloid pathology and improving cognitive function in a mouse model of Alzheimer's (Hamilton et al., 2014). The mGluR5 is now considered to act as a receptor for β-amyloid modulating toxicity and neuronal death (Beraldo et al.,

2016; Hamilton et al., 2014). The muscarinic acetylcholine receptors M1 and M3 when stimulated promote the secretion of soluble amyloid precursor protein (sAPP) with subsequent reduction in insoluble βamyloid generation (Lee et al., 1995). This effect is blocked by M1 and M3 receptor antagonists. Furthermore, M1 acetylcholine receptors play an important role in learning and memory consolidation making the muscarinic acetylcholine receptors an attractive target for therapeutic intervention (Scarpa et al., 2020). The β2 adrenergic receptor has also been implicated in disease. Deletion of the receptor in a mouse model of tauopathies results in amelioration of tau pathology (Wisely et al., 2014). This is associated, and thought to be mediated, by a reduction in tau kinase activity, specifically GSK3β and CDK5 (Wisely et al., 2014). Other GPCRs that have been implicated in disease pathology, and proposed therapeutic targets, include the serotonin receptors (Woods et al., 2012; Yamazaki et al., 2015; Zhang et al., 2013, 2015) and orphan receptors, such as G-protein coupled receptor 3 (GPR3), where the endogenous ligand is unknown (Huang et al., 2015; Thathiah and De Strooper, 2009). The presence of amyloid pathology has also been shown to generate a toxic environment thought to induce GPCR oligomerisation and sequestration of the Gα subunit impairing GPCR signalling (AbdAlla et al., 2009; Suo et al., 2004). This has been shown with the angiotensin 2 receptor, with the resulting reduction in receptor activity correlating well with hippocampal neurodegeneration and tau phosphorylation (AbdAlla et al., 2009). Alterations in gene expression profiles between controls and patients with Alzheimer's for a number of GPCRs has been shown (Blalock et al., 2004). Alongside GPCRs that regulate neurotransmission, differences are noted in expression profiles of GPCRs involved in hormone regulation and inflammation (Blalock et al., 2004). Changes observed correlated with the pathological severity of disease (Blalock et al., 2004).

GPCRs have been traditionally targeted with agonists and antagonists but with recent advances it has become increasingly clear that allosteric ligands altering selective downstream signals, but not all, will allow for precisely targeted interventions making GPCRs particularly attractive therapeutic targets (Conn et al., 2009; Nickols and Conn, 2014; Zhao et al., 2016).

1.6 Data leading to project

This project emerged from a collaborative study between the Farooqi (Metabolic Research Laboratories, Institute of Metabolic Science, University of Cambridge) and Spillantini (Department of Clinical Neuroscience, University of Cambridge) laboratories.

The PGE2 EP3 receptor is involved in multiple biological processes including energy homeostasis. Selective deletion of the EP3 receptor in mice leads to obesity (Ceddia et al., 2016). As there is a high degree of conservation between the pathways that regulate energy balance across species, as part of the genetics of obesity study, it was hypothesised that disruption of EP3 in humans might also lead to obesity. To identify genetic variants in the gene encoding EP3, *PTGER3*, intron/exon boundaries were sequenced in 300 individuals of mixed European descent with severe, early onset obesity (mean body mass index (BMI) standard deviation score 3.5; age of onset<10 years) who had been recruited to the Genetics of Obesity Study (GOOS) (Farooqi et al., 2003). The data on individuals with severe obesity was compared to 1400 control individuals from a large UK population-based study, the Ely study (Williams et al., 1995) and publicly available exome sequencing data. A frameshift mutation affecting a primate specific isoform of the EP3 receptor, EP3Re, was identified. As EP3 localises to neurons, select regions within the human brain were stained with the EP3Re antibody to determine the receptors expression. Whilst staining what had

been identified as a control brain, it was noted that some of the neurons contained tau neurofibrillary tangles which were being identified by the EP3Re antibody suggesting a possible association between the EP3Re isoform and tau tangles. Although the frameshift mutation in severely obese individuals was later also identified in control subjects, the finding of a PGE2 receptor identifying with tau tangles warranted further investigation and is the main focus of this project.

1.7 Aim of the project

With epidemiological and experimental data suggesting a role for PGE2 in Alzheimer's and primary tauopathies, this incidental finding could suggest a mechanism linking prostaglandin signalling with tau pathology through this primate specific EP3Re isoform and thus needed to be further explored. The hypothesis was that in early stages of Alzheimer's and tauopathies, following an initial inflammatory insult, such as generation of amyloid or abnormal tau processing, PGE2 signals via EP3Re, which in turn interacts with tau influencing tau hyperphosphorylation, aggregation and tangle formation leading to disease progression.

To determine this, we set out with 3 main objectives:

1. To establish the distribution and function of EP3Re in the human brain
2. To explore the physical interaction between EP3Re and tau. Does EP3Re co-localise with tau neurofibrillary tangles in disease?
3. To understand the functional role of EP3Re in tauopathies. Is the receptor contributing to tau pathology?

This study will show the distribution of the human specific EP3Re receptor not previously described as well as identify the second messenger through which it functions.

In Alzheimer's disease tau pathology has been shown to correlate better with the level of cognitive impairment (Braak and Braak, 1991; Brier et al., 2016) suggesting that targeting neurofibrillary tangles, has the potential to alter disease progression. As there is little published data on the pathway linking PGE2, EP3 and tau, this makes the project novel with significant implications for future treatment strategies.

Chapter 2

MATERIAL AND METHODS

2.1 Materials

2.1.1 Antibodies

Antibodies used for immunohistochemistry, immunofluorescence, immunoblotting and immunoprecipitation can be found below in Tables 2.1 and 2.2.

Primary antibody	**Target**	**Type**	**Working dilution**	**Application**
Phospho AKT (Ser473) (Cell Signaling #9271)	Detects endogenous levels of Akt1 only when activated by phosphorylation at Ser473.	Polyclonal	1:1000	Western Blotting
4G8 Anti-β-Amyloid, 17-24 Antibody (Biolegend SIG-39200)	Recognises amino acid residues 17-24 of βamyloid. The epitope lies within amino acids 18-22 (VFFAE). 4G8 β-amyloid antibody reacts to abnormally processed isoforms, as well as precursor forms	Monoclonal	1:1000	Immunohistochemistry
Beta-actin (Abcam 8227)	Recognises beta actin, a ubiquitously expressed protein. Raised against synthetic peptide within human beta actin amino acids 1-100	Polyclonal	1:5000	Western Blotting

Beta III tubulin (TUJ1) (BioLegend 845502)	Antibody raised against rat microtubules to recognise tubulin beta III, a major microtubule component. It is primarily expressed in neurons and is commonly used as a neuronal marker	Monoclonal	1:50	Immunocytochemistry
Anti-FLAG (M2) (Sigma, F1804)	Antibody directed against the N-terminus, Met N-terminus and C-terminus of FLAG sequence. It is also able to recognise FLAG at an internal site	Monoclonal	ELISA 1:1000, ICC 1:200	ELISA, Immunocytochemistry
GAPDH (ProSci 3781)	Antibody directed against 16 amino acid sequence near C-terminus of human GAPDH	Polyclonal	1:1000	Western Blotting
Phospho-GSK3 alpha/beta (Ser21/9) (Cell signaling #9331s)	Phospho-specific antibody directed against GSK3 that has been inactivated by phosphorylation at Serine 21 of GSK-3alpha and Serine 9 of GSK-3beta	Polyclonal	1:1000	Western Blotting

Phospho-GSK3 alpha/beta (Tyr279/216) (BioSource 44-604G)	Phospho-specific antibody directed against GSK3 activated by phosphorylation at Tyrosine 279 of GSK-3alpha and Tyrosine 216 of GSK-3beta	Polyclonal	1:1000	Western Blotting
Anti-LAMP 1 (Abcam 25630)	Antibody directed against lysosomal-associated membrane protein 1. Specific antigen not specified by manufacturer	Monoclonal	1:100	Immunocytochemistry
p44/42 MAPK (ERK1/2) (Cell Signaling #9102)	Antibody directed against C-terminus of p44 mitogen-activated protein kinase. Used to detect total endogenous MAPK enzymes ERk1 and ERK2	Polyclonal	1:1000	Western Blotting

Anti-Active MAPK pAb (pTEpY) (Promega V803A)	Antibody directed against dually phosphorylated Thr/Glu/Tyr region (pTEpY) derived from the catalytic core of the active form of the MAPK enzymes, ERK1 and ERK2, which corresponds to Thr183 and Tyr185 of the mammalian ERK2 enzyme.	Polyclonal	1:5000	Western Blotting
Anti- PTGER3 (Abcam 94496)	Antibody directed against human EP3Re receptor. Recognises the internal sequence amino acids 360-409 (MRKRRLREQLICSLQNSQIQRATAHCGQVQTYRVLNREEMEVLVSSINVY)	Polyclonal	IP 1:10 WB 1:500, IHC 1:250, PLA 1:250	Immunoprecipitation, Immunohistochemistry, Immunocytochemistry, Western blotting, Proximity ligation assay
Anti-PTGER3 (Abcam 115667)	Antibody directed against all human EP3 receptor isoforms. Recognises region from 3rd cytoplasmic domain of EP3 receptor shared by all isoforms	Polyclonal	WB 1:500, IHC 1:500	Immunohistochemistry, Western blotting

Synaptobrevin (Abcam 24735)	Antibody recognises native, denatured and recombinant synaptic vesicle proteins Synaptobrevin 1 (aa 1-118) and Synaptobrevin 2 (aa 33-96)	Monoclonal	1:500	Immunocytochemistry
Anti-alpha synuclein (LB509) (Abcam 27766)	This antibody recognizes human alpha-synuclein. Clone LB 509 recognizes amino acids 115-122 of alpha-synuclein which has been reported as specific to human alpha-synuclein	Monoclonal	1:500	Immunohistochemistry
Anti-human tau (Dako A0024)	Phosphorylation independent antibody against human tau. Recognises C-terminal part of human tau (amino acids 243-441)	Polyclonal	IP 1:200 WB 1:5000	Immunoprecipitation, Western blotting
Anti-tau (HT7) (Thermoscientific MN1000)	Phosphorylation independent antibody against human tau. Recognises residues 159-163 of human tau	Monoclonal	WB 1:1000 IP 1:10 PLA 1:1000	Immunoprecipitation, Western blotting, Proximity ligation assay

Anti- Phospho-Tau (AT8) (Thermoscientific MN1020)	Phosphorylation dependent antibody recognises human and murine phosphorylated-tau (Ser202,Thr205)	Monoclonal	1:1000	Immunohistochemistry, Western blotting
Anti-Phospho-Tau (PHF1) Gifted from Prof Peter Davies, Litwin-Zucker Center for the Study of Alzheimer's Disease and Memory Disorders, Feinstein Institute for Medical Research	Phosphorylation dependent antibody directed against tau phosphorylated at Ser396 and Ser404	Monoclonal	1:1000	Immunohistochemistry, Immunocytochemistry, Western blotting

Table 2.1 List of primary antibodies

2.1.2 Human brain samples

Post mortem paraffin embedded sections were obtained from 5 Alzheimer's patients, 5 PSP patients and 5 age and gender matched controls from the Cambridge Brain Bank. Diagnosis was made based on the clinical presentation as well as the pathology on autopsy. Three serial sections from 15 different brain regions from each subject were used for this study. Frozen tissue was also obtained from the same regions.

Frozen tissue for a Parkinson's disease patient was obtained from the Oxford brain bank.

Ethical approval was obtained for the use of all human tissue presented in this thesis. Clinical characteristics of patients can be seen in Tables 2.3 and 2.4.

Table 2.2 List of secondary antibodies

Secondary antibody	**Target**	**Working dilution**	**Application**
Biotinylated horse anti-mouse IgG antibody (Vector laboratories BA-2000)	To detect monoclonal AT8 antibody	1:250	Immunohistochemistry
Biotinylated horse anti-rabbit IgG antibody (Vector laboratories BA-2001)	To detect polyclonal EP3Re antibody	1:250	Immunohistochemistry
Goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody Alexa fluor plus 647 (Invitrogen A327733)	To detect polyclonal EP3Re antiserum	1:1000	Immunofluorescence
Goat anti-mouse IgG (H+L) HRP conjugated (BioRad)	Used to detect monoclonal FLAG antibody	1:1250	ELISA
ECL anti-mouse IgG, HRP linked (GE Healthcare life sciences NA931V)	To detect monoclonal antibodies AT8 and HT7 tau	1:5000	Western blotting
ECL anti-rabbit IgG, HRP linked (GE Healthcare life sciences NA934V)	To detect EP3Re and Tau (dako) polyclonal antisera	1:5000	Western blotting

Table 2.3 Characteristics of control brain samples

Case reference number	Gender	Age (y)	Cause of death[1]	
NP16.28	M	68	Pneumonia	IHC, WB
NP16.59	F	60	Influenza A	IHC
NP16.154	M	35	Hodgkin's lymphoma	IHC
NP16.184	F	69	Congestive Cardiac Failure	IHC, WB
NP16.202	M	73	Rectosigmoid carcinoma	IHC, WB

Table 2.4 Characteristics of case brain samples

Case reference number	Gender	Age (y)	Diagnosis	
NP16.42	M	78	PSP	IHC, WB, IP, PLA, EM
NP16.69	F	79	PSP	IHC, WB, IP, PLA, EM
NP16.102	F	73	PSP	IHC, WB, IP, PLA
NP16.178	F	73	PSP	IHC, WB
NP15.293	F	65	PSP	IHC, WB
NP16.41	F	91	AD	IHC, WB, IP, PLA, EM
NP16.115	M	78	AD	IHC, WB
NP16.116	M	66	AD	IHC, WB, IP, PLA, EM
NP16.216	M	82	AD	IHC, WB
NP16.242	M	89	AD	IHC, WB, IP, PLA, EM
NP133.2014	M	80	PD	EM
NP11.85	M	70	PD	IHC
NP13.74	M	60	MSA	IHC
NP16.163	F	70	Pick's disease	EM
NP11.85	M	70	FTLD-TDP43	IHC

PSP - Progressive supranuclear palsy, AD - Alzheimer's dementia, PD - Parkinson's disease, FTLD - Frontotemporal lobar degeneration, TDP43 - TAR DNA-binding protein 43, MSA - Multiple system atrophy, IHC - immunohistochemistry, WB - western blotting, IP - immunoprecipitation, PLA - proximity ligation assay, EM - electron microscopy.

[1]Cause of death as listed on the death certificate.

2.1.3 P301S mouse sections

Paraffin embedded sections taken from homozygous P301S tau transgenic mice and wild type C57/BL6J mice were kindly provided by Dr Jack Brelstaff. P301S tau mice are a transgenic model of tauopathy that express mutant P301S tau under a Thy1 promoter (Allen et al., 2002). Pathologically, the P301S mice display widespread tau pathology both in the peripheral and central nervous system. The clinical phenotype consists of progressive motor impairment, neuronal death and cognitive decline which starts at around 3 months and progresses up to 5 months when the mice have to be killed due to the motor phenotype (Hampton et al., 2010). All brain sections used were from mice sacrificed at 5 months of age.

2.1.4 cDNA constructs

The following cDNA constructs were used for experiments:

2.1.4.1 PTGER3

cDNA constructs for PTGER3 were created and provided by Elena Avale from the Farooqi laboratory (IMS, University of Cambridge).
cDNA encoding the wild type splice variant 8 (e isoform) of the human *PTGER3* gene was synthesised by GenScript (Piscataway, NJ, USA). This was N-terminally fused with FLAG-tag and ligated into pcDNA3.1 using EcoRI/XhoI sites cloned into pCMV-scipt (Stratagene, CA, USA). All constructs were verified by DNA sequence analysis by MWG-Biotech AG (Ebersberg, Germany).

2.1.4.2 MAPT and MAPT with the P301S point mutation

cDNA constructs for MAPT (0N4R) with a C-terminal venus tag and MAPT with the P301S point mutation were kindly donated from the McEwan laboratory (Dementia

Research Institute, University of Cambridge). These were created as described in McEwan et al. (2017).

2.1.5 Reporter gene vectors

Constructs used for Luciferase reporter gene assays were provided by Jacek Mokrosinski (IMS, University of Cambridge) and originally obtained from Agilent.

- pNFAT-Luc: construct encoding Nuclear Factor of activated T cells (NFAT) binding sequence upstream of a luciferase reporter
- pFA2-CREB: Plasmid expressing the N-terminal 280 amino acid of cAMP Response Element Binding protein (CREB) fused to Gal4 DNA binding domain
- G$\alpha\delta$6qi4myr: Construct encoding chimeric G-protein as described by Kostenis et al 2001. Mutant Gαq construct lacking the characteristic highly conserved six amino acid extension bearing four residues of G4αi sequence at the extreme C-terminus, replaced with the corresponding Gαq sequence.
- p-FR-Luc: Plasmid containing a synthetic promoter with 5 tandem repeats of the yeast GAL4 binding sites that control expression of the *Photinus pyralis* (American firefly) luciferase gene.

For plasmids used in Nanobit® protein-protein interactions please see section 2.2.18.

For full sequences of constructs see Appendix 1.

2.2 Methods

2.2.1 Immunohistochemistry

Human and mouse brain paraffin embedded sections were treated in xylene overnight to remove the paraffin. They were then rehydrated through an ethanol series 100%, 95%, 70%, 50% and washed 3x in PBS (pH7.4). Sections that were destined to be stained for EP3e at this stage underwent heat induced epitope retrieval in 10mM citrate buffer (pH6) (Hussaini et al., 2013). The sections were then quenched for 20min in buffer containing 20% methanol, 10% hydrogen peroxide and 70% PBS for 1hr, and, the non-specific background blocked with 1hr incubation in 5% horse serum (Vector Laboratories) at room temperature. Sections were then incubated in the appropriate primary antibody in PBS with 0.3% Triton x100 overnight at 4°C. Sections were then washed 3 times in PBS containing 0.3% Triton x100, incubated in appropriate biotinylated secondary antibody at room temperature for 2hrs, then incubated for 1hr at room temperature with Avidin Biotin Complex (ABC) staining kit reagent (Thermoscientific), washed 3 times in PBS containing 0.3% Triton x100, and, finally developed using DAB peroxidase (HRP) (Vector laboratories) according to substrate kit instructions. Some sections were counterstained with Cresyl Violet to visualise tissue morphology, rehydrated through an ethanol series then cleaned in xylene, and, mounted in DPX mounting medium. Sections were examined using an Olympus BX50 microscope and images were captured by a QImaging Retiga 2000R CCD camera.

2.2.2 Antibody pre-adsorption test

Anti-PTGER3 antibody was incubated in 1mg/ml PTGER3 blocking peptide (Abcam 151180) or tau aggregates obtained by sarkosyl extraction, followed by guanidinium

treatment and de-phosphorylation (see 2.2.4 and 2.2.5) for 2hr at room temperature. The adsorbed antibody was then used as the primary antibody for immunohistochemistry and western blotting to ensure specificity of the staining and of the antibody. Other controls performed to ensure staining specificity were omission of primary antibody and staining of mouse brain tissue sections considering that the antibody was considered to recognise specifically human PTGER3.

2.2.3 Protein extraction

Total protein was extracted from different brain regions or cells for experiments in radioimmunoprecipitation (RIPA) buffer (50mMTris-HCl, 150mM Sodium chloride, 1% Nonidet P-40, 0.5% 10%Sodium deoxycholate, 0.1% 10%SDS) containing protease inhibitors (Roche Diagnostics) and phosphatase inhibitors (Sigma). For cell extraction, cells were placed on ice, rinsed in PBS and lysed in RIPA buffer. For brain tissue, 100mg of tissue was homogenised in RIPA buffer on ice. The cell lysate, or brain homogenate was then centrifuged for 20min at 4°C at 22000g. The supernatant was then collected and stored at -20°C or used immediately for immunoblotting.

2.2.4 PHF tau extraction

Insoluble filamentous tau extraction was done using the method described by Goedert et al (Goedert et al., 1992). Briefly, 100-200mg of post mortem AD or PSP frozen brain tissue was homogenised in 1-2ml of A68 buffer (10mM TRIS-HCl pH7.4, 0.8M NaCl, 1mM EGTA, 10% sucrose) on ice. The homogenate was then spun at 22000g for 20min at 4°C and the supernatant collected. The pellet was then re-suspended in half the original volume of A68 buffer, spun again at 22000g for 20min at 4°C, and the 2 supernatants combined. The pooled supernatant was then incubated with N-Lauryl-sarkosine (Sigma), at a final concentration of 1%, for 1hr with rotation at

room temperature before being spun at 100000g for 1hr at 4°C. The supernatant was removed and kept as the soluble fraction, whilst the pellet was reconstituted in 50mM TRIS-HCl pH7.4 using 0.1ml/mg of initial tissue weight. The insoluble tau was either used immediately or stored at -20°C.

2.2.5 Alkaline phosphatase and guanidinium treatment

4 M Guanidine hydrochloride (Applichem) was added to sarkosyl-insoluble tau in equal volumes and the solution was left for 1hr at room temperature to unravel the tau filaments. The mixture was either dialysed overnight at 4°C against 100ml 50mM TRIS-HCl pH8.2, 0.1mM EDTA and 1mg/ml PMSF or centrifuged at 100000g for 1hr at 4°C with the pellet re-suspended in 50mM TRIS-HCl pH 7.4, in order to remove the guanidine hydrochloride. E.Coli Alkaline phosphatase 18U/ml (Sigma-Aldrich) in 50mM TRIS-HCl pH 7.4 and 5mM MgCl2 was added to the solution which was left for 4hr at 65°C to de-phosphorylate tau from the disassembled filaments. The solution was either used immediately or stored at -20°C.

2.2.6 Immunoprecipitation

Immunoprecipitation was done using Dynabeads^{TM} protein G (Invitrogen) following the manufacturers protocol. Antibodies recognising either total tau (HT7 or Dako tau – see 2.1.1) or the EP3e receptor (see 2.1.1) were bound to the Dynabeads^{TM} by adding 10 µg of antibody diluted in 200 µl of PBS with 0.02% Tween20 to the beads and incubating the solution for 2hr at 4°C with rotation. For the negative controls, appropriate sera, based on the origin of the antibody, was added to the beads with no antibody present. To avoid co-elution of the antibody, the antibody was crosslinked to the beads using 100mM BS3 (Thermoscientific) in conjugation buffer (20mM sodium phosphate, 0.15mM NaCl pH 7-9) for 30min at room temperature. The solution was

then quenched in 12.5 µl of quenching buffer (1M TRIS-HCl pH 7.5) for 15min at room temperature before the beads were washed in PBS with 0.02% Tween20 and 50 µg of PHF tau or soluble protein extract from AD brain added. The sample was left at 4°C overnight with rotation. The target antigen was then eluted by heating the beads-antibody-antigen complex for 10mins at 70°C in 20 µl of premixed NuPage sample buffer with NuPage sample reducing buffer (Invitrogen). The supernatant was then loaded onto a polyacrylamide gel (10%) for western blot (see 2.2.7), protein transferred onto a nitrocellulose membrane, and the blot probed for either the presence of tau or EP3Re.

2.2.7 Immunogold negative stain electron microscopy

2.5 µl of insoluble filamentous tau, extracted as described in 2.2.4, was placed on glow discharged carbon coated copper grids (Electron Microscopy Sciences). Some filaments were treated with pronase at this stage. All samples were then blocked for 10min in blocking buffer (0.1% cold water fish skin gelatin in PBS). Primary antibody recognising the EP3Re receptor was prepared (1:50 diluted in blocking buffer) and applied to the carbon grids for 1hr. Excess antibody solution was blotted with filter paper and grids were washed in blocking buffer. Grids were then placed in secondary antibody (goat anti-rabbit IgG conjugated to 10nm gold diluted 1:20 in blocking buffer) for 1hr, washed in water and 4 µl of 2% of uranyl acetate placed on each grid. Excess uranyl acetate was blotted with filter paper leaving a thin film of solution and grids left to air dry. Electron microscopy images were acquired on a Tecnai G2 Spirit at 120kV with a Gatan Orius SC200B detector.

2.2.8 Cell culture

2.2.8.1 SHSY5Y cells

SHSY5Y cells are a human neuroblastoma cell line derived from a subclone of the parental cell line, SK-N-SH generated from a bone marrow biopsy from a metastatic neuroblastoma (Shipley et al. 2016). When undifferentiated they express markers of immature neurons and have been used in both differentiated and undifferentiated states to study neurodegenerative disease (Shipley et al. 2016). SHSY5Y cells were maintained in Gibco^{TM} Roswell Park Memorial Institute medium (RPMI) 1640 (Life technologies) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% L-glutamine (Life Technologies), 100U/ml penicillin and 100 µg/ml streptomycin. Cells were passaged upon reaching 90% confluence (approximately every 3 days). Cells were kept in T75 flasks (Thermoscientific) and plated onto 6 or 12 well plates for experiments. SHSY5Y cells were used for microscopy and western blotting.

In some experiments differentiated SHSY5Y cells with a neuronal phenotype were required. Differentiation of cells was induced by lowering FBS in maintenance media to 1% and addition of 10μM all trans retinoic acid (Sigma). Cells were kept in differentiation media for 10 days prior to performing experiments with media changed every 3 days.

2.2.8.2 HEK293 cells

Human embryonic kidney 293 cells are a line originally derived from a human embryonic kidney cell transformed with sheared fragments of human adenovirus type 5 DNA (Graham et al., 1977). They have been extensively used for expressing recombinant proteins due to their ease of transfection and efficient translation and protein processing

(Thomas and Smart, 2005). These made them an ideal cell line for functional studies of the EP3e receptor. They display a neuronal lineage phenotype and are most likely of adrenal origin (Stepanenko and Dmitrenko, 2015). HEK293 cells were maintained in Dulbelco's modified Eagle Media (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen), 1% L-glutamine (Life Technologies), 100U/ml penicillin and 100 µg/ml streptomycin. HEK293 cells were passaged upon reaching 90% confluence (approximately every 3 days) and maintained in T75 flasks (Thermoscientific). For experiments cell were plated onto 6, 12 or 96 well plates (Perkin Elmer). HEK293 cells were used for signaling assays, microscopy and western blotting.

2.2.8.3 COS7 cells

COS7 (**C**V-1 in **O**rigin with **S**V40 genes) are a cell line derived from CV-1 simian cells transformed by an origin defective mutant of SV40. They are commonly used as a mammalian production cell for recombinant proteins. They display a fibroblast phenotype. COS7 cells were maintained in low glucose DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen), 1% L-glutamine (Life Technologies), 100U/ml penicillin and 100 µg/ml streptomycin. COS7 cells were passaged on reaching 70% - 80% confluence and maintained in T75 flasks (Thermoscientific). For experiments cells were plated onto 96 well plates. COS7 cells were used for cAMP assays.

2.2.8.4 Induced Pluripotent stem cells (iPSC)

Human iPSC derived neurons were used for experiments to understand the functional significance of EP3Re signalling and its possible relationship with tau. iPSCs generated from individuals with familial Alzheimer's and other tauopathies have been shown in several studies to recapitulate several of the pathological features of the disease making

them an ideal way of studying the EP3e receptor in pathological conditions in vitro (Iovino et al., 2015; Muratore et al., 2014; Shi et al., 2012b).

CRL and N279K lines had already been established in the lab from a healthy newborn male's fibroblasts (Iovino et al., 2015; Vallier et al., 2009) and from a 43 year old male respectively.

The following iPSC derived neural stem cells were obtained from Axol generated from the dermal fibroblast of a 64 year old female;

- hN9 (ax0019) control line
- hN9 MAPT P301L (ax0325) isogenic line edited with CRISPR-Cas9 technology to introduce the P301L mutation (CCG>CTG) into the *MAPT* gene. This line is heterozygous for the P301L mutation where one allele contains the mutation and the other allele is wild type at the locus.

Maintaining neural stem cells

Maintenance and neuronal differentiation were done according to the Shi protocol (Shi et al., 2012b). Neural stem cells were maintained in neural maintenance media (N2B27 media) consisting of a 1:1 mixture N-2 and B-27 containing media. N2 medium consists of Dulbecco's Modified Eagle Medium Nutrient mixture F-12 (DMEM/F-12) GlutaMAX (Gibco^{TM}), N2, 5 µg/ml insulin (Sigma), 1mM L-glutamine (Gibco^{TM}), 100μM non-essential amino acids (Gibco^{TM}), 100μM 2-mercaptoethanol (Gibco^{TM}), 50U/ml penicillin (Gibco^{TM}) and 50mg/ml streptomycin (Gibco^{TM}). B-27 containing medium consists of Neurobasal medium (Gibco^{TM}), 1%B-27 with vitamin A (Gibco^{TM}), 200mM L-glutamine (Gibco^{TM}), 50U/ml penicillin (Gibco^{TM}) and 50mg/ml streptomycin (Gibco^{TM}). Media was supplemented with fibroblast growth factor (FGF2) (20ng/ml) and epidermal growth factor (EGF) (20ng/ml) (Peprotech) to prevent cells differentiating. Cells were plated in 12 well plates coated with 0.01%poly-L-ornithine (Sigma) followed by 20 µg/ml laminin (Sigma) and media changed every 2 days. Cells

were passaged when confluent, approximately every 4 days using accutase and plated in N2B27 media supplemented with 10μM ROCK inhibitor (Tocris Bioscience).

Neuronal differentiation

Once cells had been expanded sufficiently to perform experiments, FGF and EGF were withdrawn from N2B27 medium and cells treated with μM N-[N-3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT)(Tocris Bioscience) for 2 media changes. Neurons were maintained in N2B27 media which was changed every other day.

2.2.9 Transfection

Cells were plated in either 96 or 12 well poly-l-lysine coated plates upon reaching 70-90% confluence. Twenty four hours later, normal plating media was replaced with Opti-MEM (Gibco^{T}M). Plasmid DNA-lipid complexes were prepared consisting of a 1:1 ratio of Lipofectamine 2000 (Invitrogen) in Opti-MEM at a concentration of 20 µl/ml and 30pmol - 150pmol of cDNA, depending on the DNA construct, diluted in Opti-MEM media. The DNA-lipofectamine complex was left to incubate for 5min before being added to cells. Transfection was terminated 5hr later by changing from Opti-MEM to serum free plating media and cells were used for experiments 24hr later.

2.2.10 siRNA

In order to establish if any changes observed in the SHSY5Y cells following activation of the EP3 receptor were due to the EP3Re isoform, EP3Re was silenced using siRNA delivered in lipofectamine.

The following siRNA duplexes were obtained from Qiagen;

- PTGER3 – Predesigned siRNA directed against human PTGER3 (NM_198718) (Hs_PTGER3_17)

  target sequence 5'-CAGCTGAGTTTGTGACTCTAA-3'

sense strand 5'-GCUGAGUUUGUGACUCUAATT-3'

antisense strand 5'-UUAGAGUCACAAACUCAGCTG-3'

- All stars negative control – validated non-silencing siRNA with no known homology to any mammalian gene and of unknown sequence

For siRNA reverse transfection was performed using Lipofectamine RNAiMAX (Invitrogen). To establish the ideal concentration of siRNA, 30pmol to 150pmol of siRNA was mixed with 6µl of Lipofectamine RNAiMAX in 500µl of Opti-MEM medium (Invitrogen) in a 6 well plate. The complex was left to incubate for 10-20mins at room temperature before 2.5ml of RPMI media without antibiotics containing 0.3 x 10^6 SHSY5Y cells was added to each well making a final concentration of 10-50nM siRNA per well. Seventy-two hours after transfection, cells were lysed in RIPA buffer and expression of the EP3e receptor analysed using western blotting. If experiments were to be performed with the selective EP3R agonist Sulprostone (Cayman Chemicals), 72hrs after transfection the media was changed to serum free media and cells were exposed to Sulprostone 24hrs later.

2.2.11 Immunofluorescence

SHSY5Y, HEK293 or human iPSC derived neurons were plated onto 12 well plates on coverslips coated in Poly-D-lysine (Sigma) at a concentration of 10 µg/ml. Prior to staining, cells were washed in Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich) and fixed on ice using methanol. They were then gently rinsed in PBS followed by PBS containing 0.3% Triton x100 to permeabilise cells. For reducing non-specific background, cells were incubated in 5% horse serum (Vector Laboratories) for 1hr and then incubated with the appropriate primary antibody overnight at 4°C. Cells were then washed in PBS containing 0.3% Triton x100 before being incubated with the appropriate Alexa fluor conjugated secondary antibody for 2hrs at room

temperature followed by a 10min incubation in 4,6-diamidino-2-phenylindole (DAPI) (Roche diagnostics) to visualise nuclei. Coverslips were mounted onto glass slides using FluorSave mounting medium (Calbiochem) and visualised using Leica DMI4000B and images captured using the Leica DFS3000G camera or Leica SP8 Confocal microscope. For immunofluorescent staining of paraffin embedded human tissue, sections were treated in autofluorescence eliminator reagent (Merck) as per the manufacturers protocol to eliminate lipofuscin-like autofluorescence. Briefly, following immunofluorescence histochemistry, sections were immersed 5min in PBS, 70% ethanol for 5min and finally autofluorescence eliminator reagent (Merck) for 5 min. Sections were then immersed in 3 changes of 70% ethanol for 1min each time and then mounted using FluorSave mounting medium (Calbiochem).

2.2.12 Proximity ligation assay (PLA)

Proximity ligation assays were performed on paraffin embedded tissue and human iPSC derived neurons to determine the extent of the interaction between EP3Re and Tau. Primary antibodies are applied to detect EP3Re and tau followed by a secondary probe to detect the primary antibodies. If the two proteins are within close proximity to one another, a signal is detected. PLA were done using a Duolink^{TM} PLA kit as per manufacturers protocol. iPSC derived neurons were fixed on ice in methanol and permeablised with PBS-0.3%Triton x100 whilst paraffin embedded sections underwent heat induced antigen retrieval in citrate buffer pH6 before being permeablised with PBS-0.3%Triton x100. Samples were blocked for 1hr at 37°C with Duolink^{TM} blocking solution then incubated in primary antibodies diluted in Duolink^{TM} antibody diluent overnight. Samples were washed in wash buffer, provided by the manufacturer and incubated in PLUS and MINUS probes directed against mouse or rabbit antibodies for 1hr at 37°C. Following incubation in ligase for 30min at 37°C, the signal was

amplified using a polymerase. Samples were kept for 100min in polymerase diluted in amplification buffer as provided by the manufacturer at 37°C, washed in wash buffer and mounted in Duolink™ In Situ mounting media with DAPI. Slides were left at 37°C for at least 15min before being visualised using Leica DMI4000B and images captured using the Leica DFS3000G camera or Leica SP8 Confocal microscope.

2.2.13 Western Blot

Following protein extraction from brain tissue or cell lysis, protein concentration was determined using a BCA assay kit (Novogen) and concentrations were then normalised using the original extraction buffer. With the exception of the immunoprecipitated samples, 10 µg of protein was then mixed in sample buffer, Laemmli 3x concentrated (6.3% SDS, 21% glycerol, 19.6% 1M TRIS pH6.8, 0.035% bromophenol blue, 15.7% β-mercaptoethanol, 62.6% H2O), boiled for 5min then run on either a pre-prepared NuPAGE™ 4-12% Bis-Tris gradient gel (Invitrogen) or 10% Tris-glycine gel alongside a SeeBlue™ Plus2pre-stained protein ladder (Invitrogen) or Precision Plus Protein Dual Colour Standards (Biorad) as a protein molecular weight marker. Electrophoresis was performed using a constant voltage of 100V for 2-3hr. Proteins were then transferred from the gel onto a 0.45 µm Nitrocellulose membrane (Thermoscientific) at a constant amplitude of 300mA for 3hr. The non-specific background on the membrane was then blocked by incubating the membrane for 1hr either in 5% milk or 5% BSA (Europa) in TBS with 0.1% Tween20, and, incubated overnight in appropriate primary antibodies diluted solutions containing 5% milk or 5% BSA. The membrane was then rinsed in TBS with 0.1% Tween20 and incubated for 3hr in appropriate HRP-linked secondary antibody. The blot was then developed using SuperSignal® West Dura Extended Duration Substrate (Thermoscientific) and visualised using a ChemiDoc XRS+ system (Biorad).

2.2.14 Luciferase reporter gene assays

Luciferase reporter gene assays have been well described by Cheng et al. (2010) for the study of G-protein signalling. Due to the reporter gene being significantly downstream of the initial binding event, there is significant amplification of the binding related signal, making luciferase reporter gene assays an excellent screening tool for deciphering EP3Re signalling pathways.

For detection of Gαs and Gαi transduction pathways the PathDetect *trans*-reporting system (Agilent) was used, whilst detection of Gαq was done using the PathDetect *cis*-reporting system (Agilent). HEK293 cells were plated into poly-l-lysine (sigma) coated 96 well plates at a density of 40000cells/well and transiently transfected with the EP3Re construct at concentrations varying from 0 - 10ng/well or empty vector alongside the reporter vector and destablised luciferase gene (with the exception of pNFAT-Luc which already has the destablised luciferase gene incorporated into the construct). The following concentrations were used per well for the following vectors:

- pNFAT-Luc 50ng
- pFA2-CREB 5ng
- G$\alpha\delta$6qi4myr 50ng
- p-FR-Luc 25ng

Transfection was terminated 5hr later by replacing Opti-MEM with serum free DMEM. Cells were incubated in ligands targetting the receptor (final concentrations varying from 0 - $10^{-}6$M) at 37°C. After 5hr, media was aspirated and cells were rinsed twice in PBS containing calcium and magnesium (Gibco^{TM}). Cells were then incubated

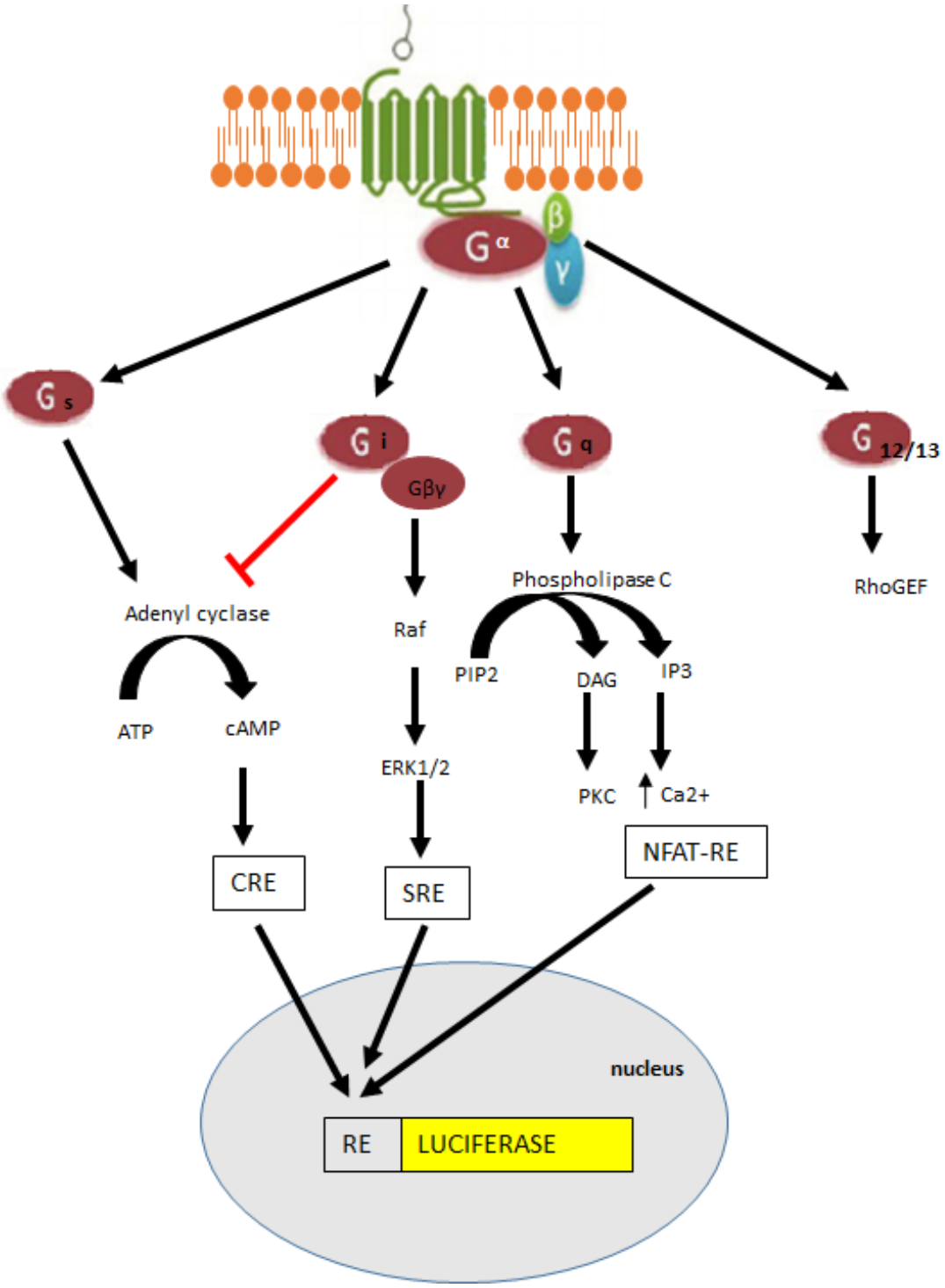


Fig. 2.1 Reporter gene assay for deciphering GPCR signal transduction pathways. Schematic explaining the use of reporter assays to determine G-protein signalling pathway. On GPCR activation, the G protein α subunit dissociates from $\beta\gamma$ subunit, initiating a cascade of downstream second messenger pathways, eventually inducing transcription of the luciferase gene by various response elements including cAMP response element (CRE), nuclear factor of activated T-cells response element (NFAT-RE) and serum response element (SRE), emitting a detectable signal that can be measured.

in steadylite (PerkinElmer) for 20min at room temperature on a shaking platform. Luminescence was then measured using a microplate scintillation counter (TopCount).

2.2.15 cAMP assay

cAMP levels were measured using HitHunter® (DiscoverX) according to manufacturer's instructions. HEK293A cells were plated in 96 well plates at a density of 20000 cells/well and transiently transfected with EP3e receptor constructs or empty vectors. The following day cells were washed twice in phosphate buffered saline (PBS), incubated in assay buffer (DiscoverX) for 30min and cells stimulated by addition of the EP3 specific agonist, Sulprostone (Cayman chemicals) for 30min at 37°C. Cells were subsequently incubated in cAMP antibody and detection solution then placed on a shaking platform for 1hr. Following a 3hr incubation in enzyme solution, luminescence was measured using a microplate scintillation counter (TopCount). To inhibit Gαi signalling that could be masking a rise in cAMP, assays were repeated with the addition of Pertussis toxin (Invitrogen, 1:100).

2.2.16 Inositol phosphate assay

A cell-based radiometric inositol phosphate accumulation assay was performed to determine the presence of Gq and Gi coupling. The assay incorporates $[^3H]$inositol into membrane-associated inositol phospholipids and measures GPCR-mediated phospholipase Cβ activation by the measurement of generated $[^3H]$inositol phosphate. All stages requiring use of radioactive reagents were done by Jacek Mokrosinski (IMS, University of Cambridge).

HEK293 cells were plated in 96 well plates at a density of 40000 cells/well and transiently transfected with EP3Re and G$\alpha\delta$6qi4myr, to asses Gαi coupling, or, EP3Re

alone to assess Gαq. G$\alpha\delta$6qi4myr is a chimeric G-protein that converts Gαi coupling into a Gαq signalling pathway allowing Gαi activity to be measured by Inositol monophosphate (IP1) accumulation. Transfection was terminated after 5hr by replacing Opti-MEM media with DMEM supplemented with *Myo*-[$2\,{}^{-3}H$]-inositol (5 µl/ml) and cells cultured overnight. They were then washed in HBSS and kept in HBSS containing 10mM LiCl (assay buffer) for 30min at 37°C in 5% CO2 and stimulated by further incubation for 1hr with varying concentrations of Sulprostone. The assay buffer was removed and 10mM ice cold formic acid dispensed over the cells which were left for 30min on ice. Twentyμl/well of cell lysate (formic acid) was transferred onto a new 96 well plate containing polylysine YSi SPA beads (Perkin Elmer). Plates were then sealed and manually shaken for 10-15 sec. Radioactivity measuring generated inositol phosphate was measured using a microplate scintillation counter (TopCount) following an 8hr delay to allow for a more stable signal. Data was analysed using GraphPad Prism 7.0.

2.2.17 ELISA for FLAG tagged EP3Re

To determine if the presence of wild type or tau with the P301S mutation influenced EP3Re cells surface expression, we performed ELISA for FLAG. COS-7 or HEK293 cells were plated into 96 well plates at a density of 30-35000 cells/well and transfected with FLAG tagged EP3Re or empty vector with either constructs for wild type tau or tau with the P301S mutation. Twenty-four hours after transfection, media was aspirated and cells were washed in PBS, fixed in 3.7% formaldehyde and incubated in blocking buffer (3% milk in PBS with 50mM TRIS) for 1hr at room temperature to reduce non-specific staining. Anti-FLAG primary antibody diluted in blocking buffer was applied to cells and left overnight at 4°C. The following day, the primary antibody was aspirated, cells washed in PBS and HRP conjugated goat anti-mouse

IgG(H+L) diluted in 1.5% milk applied to cells for 2hr at room temperature. The secondary antibody was then removed, cells washed in PBS and then incubated in 90 µl/well of the HRP substrate 3,3',5',5 - Tetramethylbenzidine (Sigma) for 5 - 20min at room temperature. This reaction was terminated on addition of 60 µl per well of 0.5M Sulfuric acid. One-hundredμl from each well was transferred to a new 96 well plate and absorbance measured at 450nm using a microplate reader (Tecan M1000).

2.2.18 NanoLuc® Binary Technology (NanoBIT ®) Protein-Protein interaction (PPI)

NanoBIT® PPI systems allow for detection of protein interactions in live cells and was used to assess the dynamic interaction between EP3Re and tau with and without the P301S mutation. NanoBIT® is a structural complementation reporter system composed of a Large BiT (LgBiT; 18kDa) subunit and a small complimentary peptide. For the study of protein:protein interactions, the complimentary peptide is a Small BiT (SmBiT; 11 amino acid peptide), which has been optimized to have low affinity for LgBiT. The LgBiT and SmBiT subunits are expressed as fusions to target proteins of interest and expressed in cells. When the two proteins interact, the subunits come together to form an active enzyme and generate a bright luminescent signal in the presence of substrate.

2.2.18.1 Creation of constructs for NanoBIT® experiments

All constructs used for this assay were developed with the assistance of Dr Edson Mendes de Oliveira (Farooqi lab, Institute of Metabolic Science, University of Cambridge) and sequences verified by PCR, performed with the assistance of David Clarke (Farooqi lab, Institute of Metabolic Science, University of Cambridge).

The following primers were used to introduce restriction sites at either the N-terminus or C-terminus of MAPT and EP3Re to create NanoBIT expression vectors:

- MAPT_EcoRI_3_(R) (5'-TCCTGAATTCTCACAAACCCTGCTTGGCCA)
- MAPT_XhoI_5_(F)(5'-ATTACTCGAGTGCTGAGCCCCGCCAGGAGTT)
- MAPT_XhoI_3_(R)(5'-ATTACTCGAGATCAAACCCTGCTTGGCCAGGG)
- MAPT_EcoRI_5_(F)(5'-ATATGAATTCATGGCTGAGCCCCGCCAGGA)
- EP3e_NheI_3_(R)(5'-GGGTGCTAGCTTATTCTGTCTTTACTGTTG)
- EP3e_XhoI_5_(F)(5'-AACACTCGAGTAAGGAGACCCGGGGCTACGG)
- EP3e_NheI_5_(F)(5'-TTAAGCTAGCATGAAGGAGACCCGGGGCTA)
- EP3e_XhoI_3_(R)(5'-TCTACTCGAGCGTTCTGTCTTTACTGTTGAGA)

Each reaction contained 10ng of cDNA, 1 µl of 10mM dNTP, 2μl each of appropriate forward and reverse primer, 0.25μl of One Taq DNA polymerase (New England BioLab) and nuclease-free water to make a final volume of 50 µl per reaction. PCR was performed in a thermal cycler under the following conditions: 94°C for 2min followed by 94°C for 50s, 64°C for 50s, 68°C for 3min for a total of 30x cycles, and finally 68°C for 7min. The resulting PCR products were run on a 1% agarose gel, extracted from the gel and cleaned using the NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel) according to manufacturers instructions. The PCR products then underwent restriction enzyme double digestion alongside C-LgBIT, N-LgBIT, C-SmBIT and C-LgBIT constructs readily available in the lab. The reaction was set up as follows; 1μg DNA, 5μl of 10X cut smart buffer (New England BioLabs), 10 units XhoI (New England BioLabs), 10 units EcoRI-HF (New England BioLabs) or 10 units of NheI-HF (depending on the construct) and 50 µl nuclease-free water, and incubated at 37°C for 2hr. The product

of enzyme digestion was then run on an agarose gel, extracted and cleaned using the NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel) according to manufacturers instructions. The MAPT and EP3e digested constructs were then ligated with the appropriate LgBIT or SmBIT construct at a molar ratio of 3:1 respectively, 10x T4 DNA ligase buffer (New England BioLabs), T4 DNA ligase (New England BioLabs) and nuclease-free water to make a final volume of 20 µl per reaction. This was left for 10min at room temperature.

Following transformation, the successful insertion of EP3Re and MAPT into NanoBit® system LgBit and SmBit vectors was assessed by sequencing using the following primers:

For detection of insertion into C-SmBiT and N-SmBiT constructs:

- Forward primer 5' ATATTAAGGTGACGCGTGTG
- Reverse primer 5' CATTCTAGTTGTGGTTTGTCC

For detection of insertion into N-LgBiT constructs:

- Forward primer 5' CATGCTGTTCCGAGTAAC
- Forward primer 5' ATATTAAGGTGACGCGTGTG
- Reverse primer 5' CATTCTAGTTGTGGTTTGTCC

For detection of insertion into C-LgBiT constructs:

- Forward primer 5' CATGCTGTTCCGAGTAAC
- Reverse primer 5' GAAGACACCTGACGACCCTC
- Reverse primer 5' CATTCTAGTTGTGGTTTGTCC

Plasmid maps for constructs used in experiments can be seen in figures 2.2 and 2.3.

Constructs with the MAPT P310S mutation were created by site directed mutagenesis of MAPT constructs.

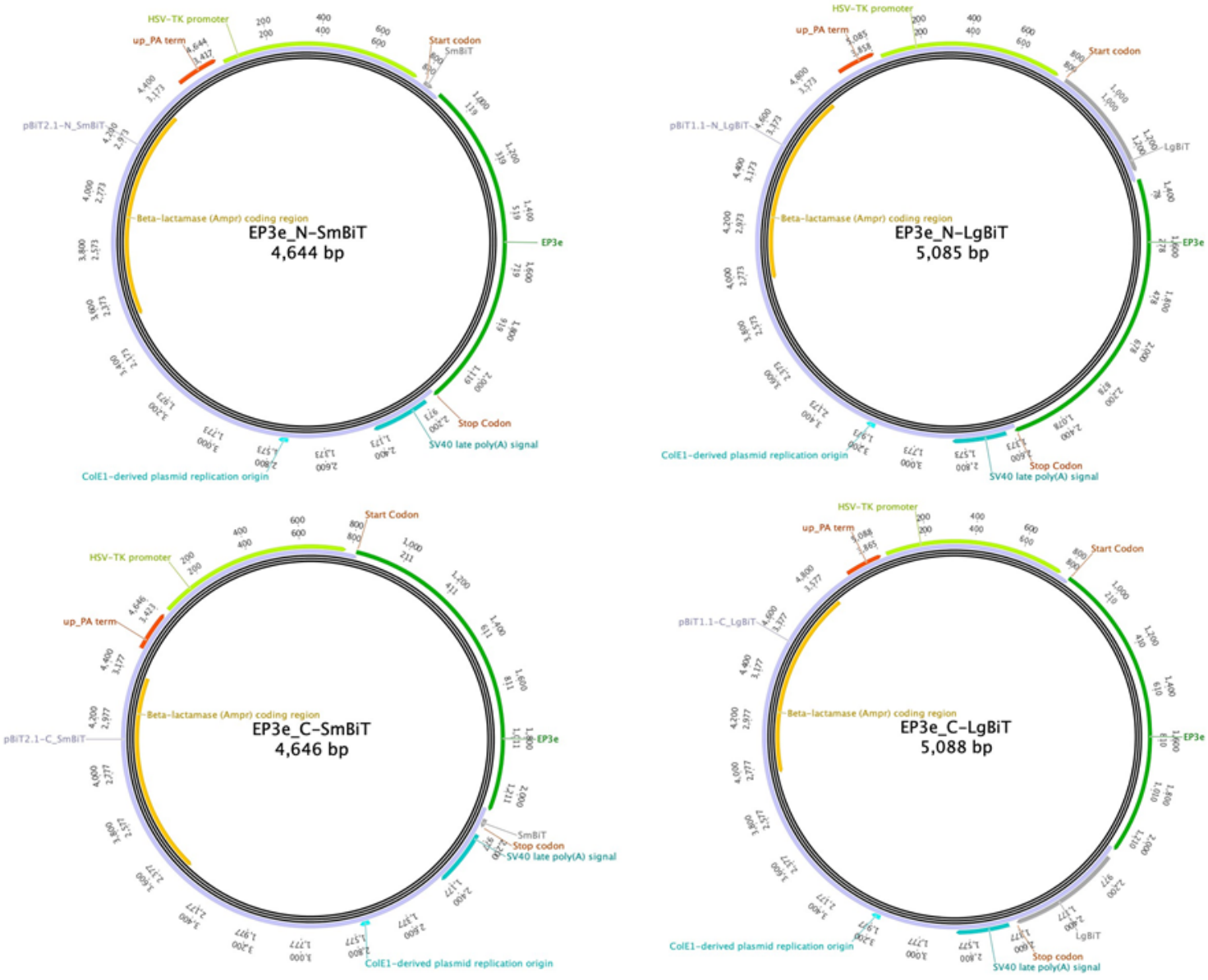


Fig. 2.2 Circular maps of plasmids containing EP3Re used in interaction assays

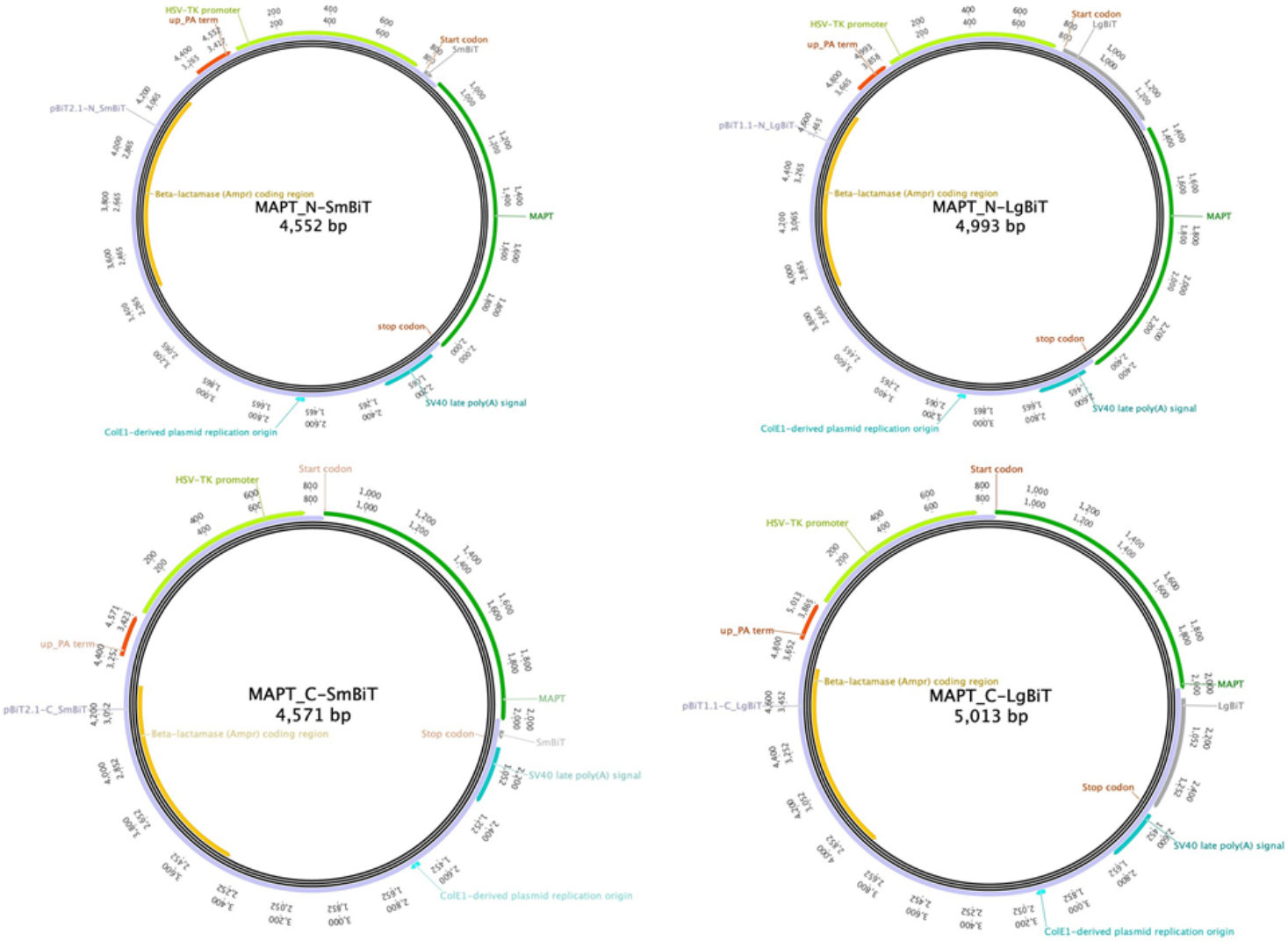


Fig. 2.3 Circular maps of plasmids containing MAPT used in interaction assays

2.2.18.2 Protein:protein interaction assay

HEK293 cells were seeded into 96 well plates at a density of 40000 cells/well. Cells were transiently transfected with the two constructs of interest. Transfection was terminated after 5 hrs by replacing Opti-MEM media with normal growth media. Prior to performing assays, media was changed back to Opti-MEM and baseline luminescence measured for 5 min at 37°C by a luminometer (Tecan). Keeping the solution at 37°C, 25 µl of Nano-Glo Live cell reagent (1:20 Nano-Glo Live cell substrate in NanoGlo LCS dilution buffer) was added to each well. Following a repeat baseline measurement of luminescence over 20min with the administered substrate, 10^{-3}M Sulprostone was added to each well and changes in luminescence recorded over 20min.

2.3 Statistics

All results were analysed using Graphpad prism 7.0 and statistical tests applied as stated in each chapter.

Chapter 3

DISTRIBUTION AND PATHWAY OF ACTION OF THE HUMAN EP3RE RECEPTOR

3.1 Summary

Prostanoid 3 receptor (EP3) mediated signalling is involved in multiple biological processes including the thermogenic response to systemic inflammation and blood pressure regulation. Targeted deletion of EP3 leads to obesity in mice, suggesting an additional role in energy homeostasis. EP3 is unique amongst the PGE2 receptors as it is alternatively spliced giving rise to species specific isoforms. Each isoform differs in the carboxy-terminal part that determines its second messenger and intracellular activity. The EP3Re isoform is unique to higher primates including man and consists of the shortest C-terminus. Its localisation in the human brain is not known. In this chapter we examine the distribution of the EP3Re receptor in the adult human brain using immunohistochemistry and western blotting and identify the receptors second messenger system through which it signals. The receptor is found to be ubiquitously expressed with particularly high levels within the brainstem nuclei and the hypothalamus and it signals through Gαi coupling with some evidence of Gαq mediated activity. Identifying the distribution and signalling transduction pathway of the receptor, will allow us to gain a better understanding of its function in physiological and pathological conditions.

3.2 Introduction

The human specific EP3Re receptor has not been previously characterised. In this chapter we aim to establish the distribution and signalling transduction pathways of EP3Re. The expression of the non-human specific EP3 receptor has previously been characterised in mouse and rat brains (Ek et al., 2000; Nakamura et al., 2000; Sugimoto et al., 1994). In these studies antibodies or probes directed against the common coding region for all isoforms were used and therefore do not distinguish between individual isoforms. In both the mouse and rat brains, similar patterns of expression are described. EP3 was found to be present throughout the brain, highly enriched in the hypothalamus and preoptic areas, the locus coeruleus, raphe nucleus and brainstem nuclei involved in processing and modulating pain. Based on their findings, Nakamura et al. concluded that the receptor is likely to play a function in conveying peripheral signals from the systemic circulation to the brain. This is supported by its enrichment in regions that lack an effective blood brain barrier such as the median preoptic nucleus, a region involved in thermoregulation, sleep homeostasis and osmoregulation (Cerri et al., 2017; McKinley et al., 2015). However, it can be found also enriched in regions within the blood brain barrier where it may regulate several neuromodulatory functions of locally synthesised PGE2. Studies in both rat and mouse brain have reported strong EP3 mRNA expression and immunoreactivity in monoamine containing nuclei such as the raphe nucleus and locus coeruleus implying its role in serotonergic and noradrenergic systems. PGE2 binding sites in rat brain mapped using quantitative autoradiography (Matsumura et al., 1992) correlate with the EP3 receptors distribution (Ek et al., 2000; Nakamura et al., 2000) suggesting in the rat brain, PGE2 effects within the CNS are largely mediated by the EP3 receptor.

In addition to immunohistochemical analysis, several functional animal studies have examined the role of EP3 signalling in the central nervous system. One of the most established functions of EP3 mediated signalling is its role in thermoregulation and febrile response (Hatae et al., 2002b; Lazarus et al., 2007; Morrison, 2016; Oka et al., 2003). The preoptic area regulates the dorsomedial hypothalamic thermogenesis promoting neurons by balancing GABAergic inhibition and glutamatergic excitatory signals (Cao et al., 2004; Nakamura et al., 2005). The excitatory input is thought to arise from EP3 positive neurons in the median preoptic nucleus that project to the dorsomedial hypothalamus (Fig. 3.1). Animal studies show selective deletion of EP3 receptors within the median preoptic nucleus results in loss of the febrile response as elicited by lipopolysaccharide (LPS) (Lazarus et al., 2007). Selective deletion of EP1 and EP4 receptors, also found within the preoptic area, does not result in loss of the febrile response (Lazarus, 2006) suggesting this is predominantly mediated by EP3 signalling.

Studies exploring the signal transduction pathways of the EP3 receptor have identified that most EP3 isoforms couple to Gi, although, as described in Chapter 1, other transduction pathways have been demonstrated based on variations in its C terminus (Hatae et al., 2002a; Kotani et al., 2000). Similar to the studies of EP3 distribution and expression, most of the signalling assays have examined bovine or mouse isoforms (Hatae et al., 2002a; Irie et al., 1994; Katoh et al., 1996). A few studies however, have looked at signalling of isoforms identified only in humans. Kotani et al examined the human EP3 isoforms V and VI using Chinese Hamster Ovary cells (CHO) by measuring cAMP accumulation, IP3 accumulation and MAPK activity. Stimulation of EP3 in cells transfected with both isoforms V and VI inhibited forskolin induced cAMP accumulation and MAPK activity. Both these responses were eliminated on treatment

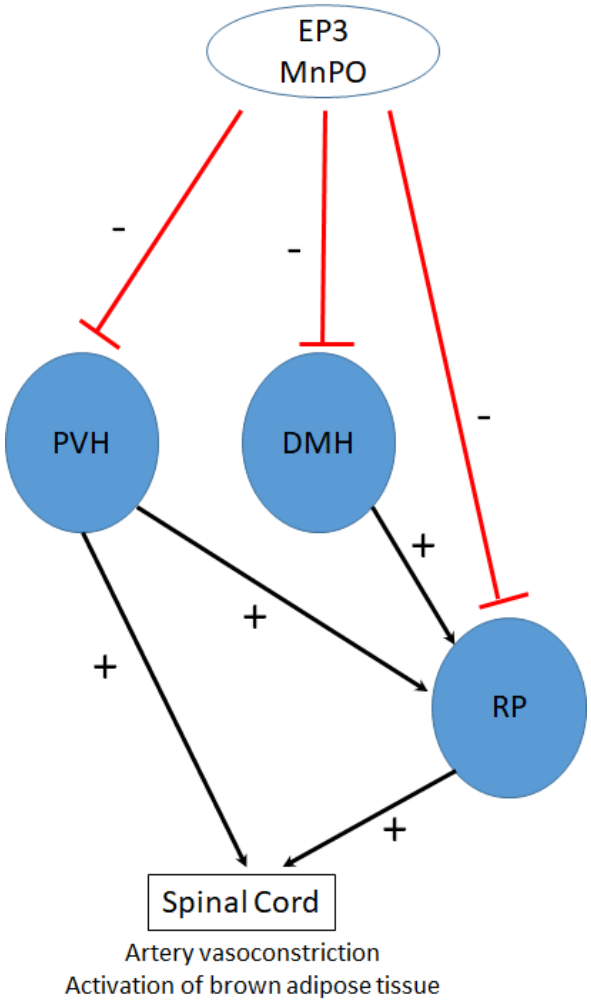


Fig. 3.1 EP3 and the pyrexial response. Hyperthermia is generated by a system of neurons including the PVH, DMH, and the RP, which activate thermogenic brown adipose tissue and heat conservation (vasoconstriction) responses. This thermogenic system is normally restrained by inhibitory neurons in the MnPO. Some MnPO neurons project to the RP; there are also projections to neurons in the PVH and the DMH, which in turn provide excitatory inputs to the RP. During fever, hyperthermic neurons in the MnPO are inhibited by EP3 receptors, thus disinhibiting, i.e., releasing, thermogenesis. MnPO - Median preoptic area, RP - Raphe pallidus nucleus, PVH - Paraventricular hypothalamus, DMH - Dorsomedial hypothalamus (Lazarus 2006).

with pertussis toxin, suggesting predominant Gi activity (Kotani et al., 2000). When CHO cells were transfected with a single isoform, cAMP accumulation was observed in the presence of EP3 VI but not V, demonstrating that differences in the isoforms at the C terminus results in activation of different transduction pathways (Kotani et al., 2000). The brain distribution and signalling pathways of the human EP3Re isoform has not been previously characterised.

The main aim of the study presented in this chapter was to:

1. Identify the areas within the human brain that express the EP3Re isoform
2. Understand the main EP3Re signalling pathways under normal physiological conditions

This study, by characterising the human specific EP3Re receptor distribution and signalling pathways will lead to a better understanding of the EP3Re receptor function in physiological and pathological conditions.

3.3 Materials and Methods

3.3.1 Antibodies

All primary and secondary antibodies used are listed in **Table 2.1** and **Table 2.2** (Section 2.1.1.).

3.3.2 Human brain samples

Four μm paraffin embedded and frozen sections from 5 healthy human control brains were obtained by ethical-committee approved procedures from the Cambridge Brain Bank. Three serial sections from 11 different brain regions (midbrain, pons, medulla,

basal ganglia, hypothalamus, hippocampus, cerebellum, frontal, temporal, occipital and parietal cortices) from each subject were used for this study. Full clinical details of each subject can be found in **Table 2.3** (Section 2.1.2).

3.3.3 Immunohistochemistry

The distribution of the receptor was characterised by immunohistochemistry using a human EP3Re specific antibody (Abcam 94496) as well as an antibody directed against all EP3 isoforms (Abcam 115667). Staining was performed on paraffin embedded sections using the protocol described in **2.2.1**. Prior to mapping the receptor's distribution, the specificity and optimum conditions for using the antibody needed to be established. To this end, following removal of paraffin as indicated in **2.2.1**, sections were stained with and without prior antigen retrieval performed with 10mM citrate buffer at 95° for 20mins or left at 60° overnight. Specificity of the staining was determined by pre-adsorbing the EP3Re antibody with PTGER3 blocking peptide (Abcam 151180) as described in **2.2.2**. This was performed on 2 adjacent sections from the medulla of 3 different patients, one section was stained with the pre-adsorbed antibody while the other was stained using the non-preadsorbed antibody at the same dilution. In this way it was possible to determine the specificity of the antibody and its suitability to be used for the remaining experiments. Having determined the specificity and optimal dilution and staining conditions immunohistochemistry was performed on paraffin embedded sections taken from 11 different regions (3 adjacent sections from each region) from 5 control brains using the antibody directed against EP3 and EP3Re.

3.3.4 Immunohistochemical evaluation of EP3Re staining

To quantify the relative expression of EP3Re a semi-quantitative five-point rating scale was used determined as a product of the percentage of positive cells and intensity of

staining. The percentage of positive cells were rated as follows; 0 points = 0%, 1 point = 5-25%, 2 points = 25-50%, 3 points = 50-75% and 4 points = 75-100%. The intensity of the staining was then assessed as follows; 0 = no staining, 1 = weak staining (yellow), 2 = moderate staining (light brown), 3 = strong staining (dark brown). A final score, from 0 - 12, was calculated based on the product of the number of positive cells x the intensity of the staining. The ratings were then allocated as follows; 1 - 3 = +, 4 - 6 = ++, 7 - 9 = +++ and 10 - 12 = ++++. Each slide was assessed in this way and the average score across 5 control brains used for each region.

3.3.5 Western Blot

The distribution of the EP3Re receptor was then quantified by western blotting as described in **2.2.10**. Fresh frozen tissue was obtained from the various brain regions investigated by immunohistochemistry from 3 of the control subjects who had one brain hemisphere fresh frozen and the other, used for immunohistochemistry fixed in formaldehyde. Loading of samples in western blot was normalised using GAPDH. 3 technical replicates were performed for each subject.

3.3.6 Luciferase reporter gene assays

Reporter gene assays are a well described method for determining the second messenger signalling pathway of G-protein coupled receptors (GPCRs). This is done by following a signal transduction pathway from ligand binding at the cell surface to nuclear gene transcription. GPCRs signal via coupling to an intracellular trimeric G-protein. Activation of the trimeric G-protein results in the dissociation of the Gα subunit from the $\beta\gamma$ dimeric subunit initiating a signalling cascade inducing gene transcription by various response elements. Response elements associated with G-protein signalling include cAMP response element (CRE), nuclear factor of activated T-cells response

element (NFAT-RE), serum response element (SRE), and, serum factor response element (SRF-RE), which is determined by the subfamily of the Gαs subunit being Gs, Gi, Gq or G12. Reporter vectors are available with specific response elements for Gs, Gi, Gq and G12 pathways making them an excellent assay to initially screen the pathway involved in EP3Re signalling. Luciferase reporter assays were performed as described in **2.2.12**. Human embryonic kidney cells (HEK293) cells were transiently transfected with either the reporter vector CRE, NFAT or NFAT co-transfected with the chimeric G-protein G$\alpha\delta$6qi4myr, alongside the luciferase construct to determine the presence of Gs, Gq and Gi coupling respectively. These were co-transfected with either the EP3Re construct or the empty vector pCDNA3.1 as a negative control. The receptor was then stimulated with the EP3 specific agonist Sulprostone. Assays were performed in triplicates. Reporter assays were repeated twice for general screening and three times when verifying results of second messenger assays.

3.3.7 Second messenger assays

Second messenger assays were then performed to confirm the results observed from the reporter gene assays. Gs coupling was further explored by measuring cAMP (see **2.2.13** for detailed protocol) in response to ligand stimulation in HEK293 cells transiently transfected with EP3Re. As no elevation in cAMP was observed, pertussis toxin was added to cells to inhibit any Gi activity that could be masking a cAMP rise in response to Gs coupling. The original reporter gene assays were then repeated using HEK293A cells with the gene encoding the Gs alpha subunit (GNAS) deleted.

Gi and Gq coupling was assessed by measuring Inositol monophosphate (IP1) accumulation in response to ligand stimulation as described in **2.2.14**. IP1 is a breakdown product of Inositol triphosphate, a second messenger produced in the presence of

Gq coupling. The chimeric G protein, G$\alpha\delta$6qi4myr, converts Gi coupling into a Gq signalling pathway allowing for a positive readout of IP1 accumulation. HEK293 cells were transiently transfected with EP3Re and the chimeric G protein, G$\alpha\delta$6qi4myr, to measure Gi coupling and with the EP3Re receptor alone to measure Gq coupling. Cells were then stimulated with Sulprostone and IP1 accumulation measured.

The EP3 receptor has been shown to bind to 3 different ligands, sulprostone, PGE2 and PGD2. Affinity for the EP3Re receptor was then determined using the IP1 assay and stimulating the receptor with each ligand. All second messenger assays were done in quadruplicates and repeated 3 times.

All steps in the IP1 assay requiring use of radioactive material were carried out by Dr Jacek Mokrosinski (Farooqi laboratory, MRC Institute of Metabolic Science)

3.3.8 Statistics

Statistical analysis was carried out using GraphPad prism 7.0. For signalling assays, half maximal effective concentration (LogEC50) was calculated from data normalised and fitted to a non-linear regression curve in GraphPad prism 7.0.

All materials and methods are described in detail in Chapter 2.

3.4 Results

3.4.1 Optimisation of immunohistochemistry and antibody specificity

3.4.1.1 Staining of brain tissue sections for EP3Re requires antigen retrieval

Optimum conditions for use of the EP3Re antibody needed to be verified, due to lack of published literature on previous use of the antibody. Sections obtained from the brain bank were paraffin embedded using neutral buffered formalin as a fixative. In order to map out the distribution of the receptor, I had to establish if antigen retrieval, the process of unmasking the epitope, was required. Antigen retrieval of 4μm paraffin embedded sections was performed in 10mM sodium citrate buffer and stained with the antibody against EP3Re. Figure 3.2 shows brain sections stained for EP3Re with and without antigen retrieval. No staining can be seen without antigen retrieval suggesting unmasking of the epitope is required for EP3Re staining. It was noted that antigen retrieval at 95° over 20 minutes appeared to affect the tissue integrity however this was not the case when the tissue was left at 60° overnight. All further immunohistochemistry was therefore performed with antigen retrieval in 10mM citrate buffer at 60° overnight.

3.4.1.2 EP3Re antibody is specific to the EP3Re receptor

Prior to mapping the distribution of the receptor in the human brain, we decided to test the specificity of the antibody. In order to do this we used the pre-adsorption method, a well described technique for testing antibody specificity (Holmseth et al., 2012) and previously used in the lab for other antibodies. This involves saturating an

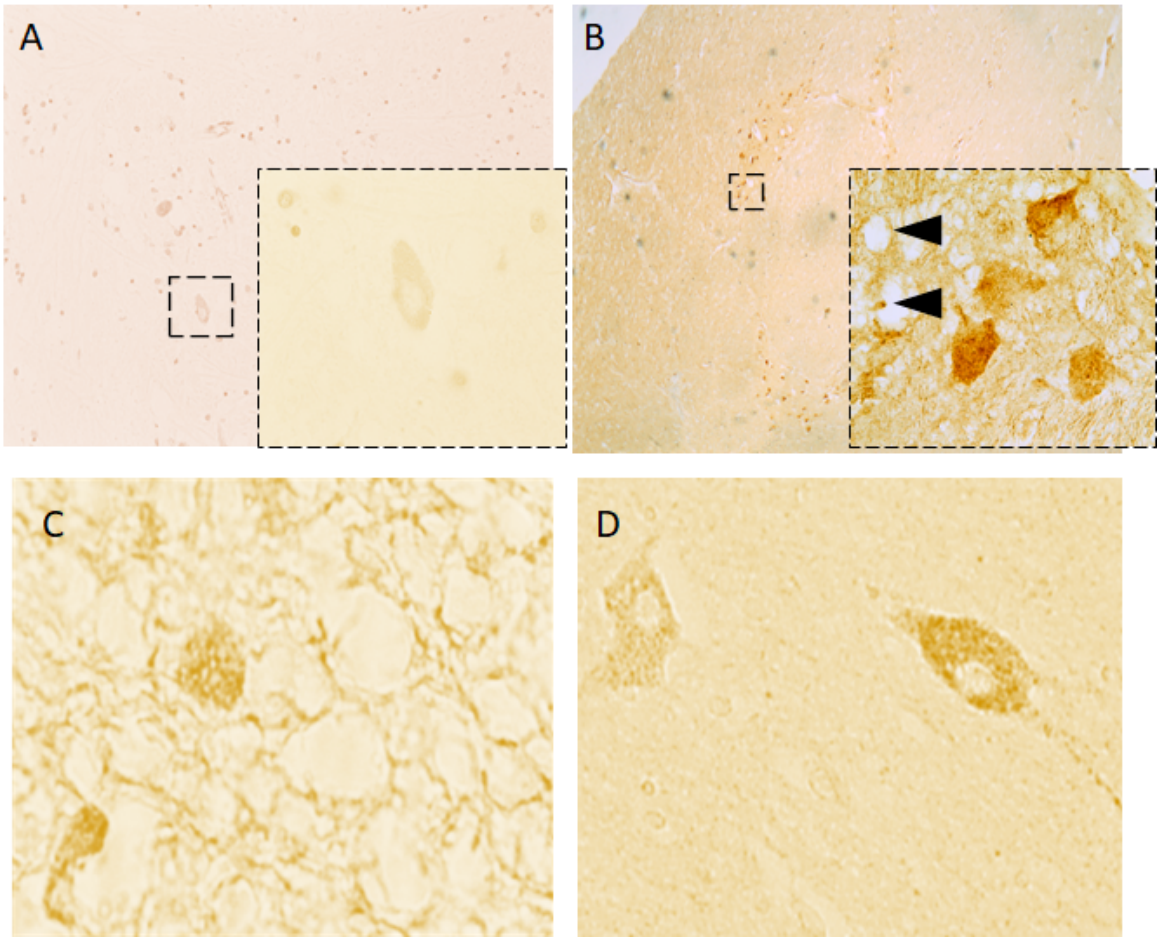


Fig. 3.2 Staining of 4 µm paraffin embedded brain tissue sections for EP3Re requires antigen retrieval. (*A*) Brain tissue section stained with EP3Re. (*B*) Brain section stained with EP3Re following antigen retrieval in 10mM sodium citrate buffer for 20 min at 95°. At higher magnification vacuolation is noted in the tissue (black arrow heads). Conditions for antigen retrieval were further optimised by performing antigen retrieval at different temperatures for differing lengths of time to see if tissue integrity could be better maintained during the process. (*C*) and *D* show optimisation of antigen retrieval conditions with *C* showing a section of brain tissue following antigen retrieval at 95° and *D* at 60° overnight in a waterbath. Antigen retrieval performed overnight resulted in improved tissue integrity.

antibody with its antigen and using this pre-adsorbed antibody for staining. If any staining is seen following this, it suggests that the antibody is recognising another epitope other than the antigen used for pre-adsorption. Paraffin embedded sections were taken from the medulla of a control patient and stained with the EP3Re antibody pre-adsorbed with the EP3Re antigen. The results can be seen in figure 3.3. Antigen pre-adsorption appears to block any staining suggesting that the antibody does not recognise any other epitope other than the EP3Re receptor in these sections.

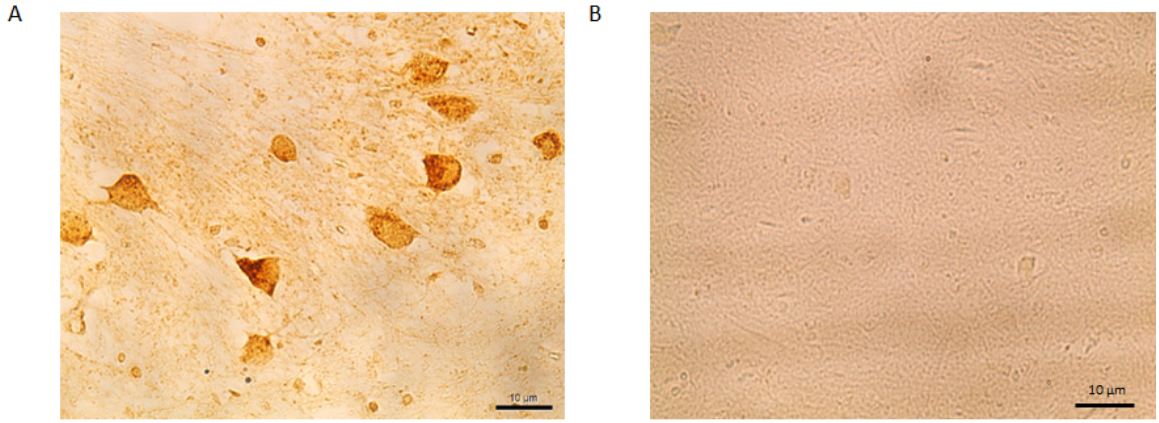


Fig. 3.3 Testing of antibody using the using the pre-adsorption method. Paraffin embedded sections stained with antibody preadsorbed with EP3Re antigen (*A*) and without preadsorption (*B*). No staining is noted when the preadsorbed antibody is using. The EP3Re antibody does not recognise any other epitope in these sections.

3.4.2 Distribution of the EP3Re receptor in the human brain

The distribution of EP3Re within the brain was determined by immunohistochemistry using a human EP3Re-specific antibody on post-mortem human brains obtained by ethical committee-approved procedures by the Cambridge Brain Bank. Fourμm sections were taken from 15 different regions from 5 control brains. Three adjacent sections were taken from each region.

EP3Re immunoreactivity was widely observed throughout the brain displaying characteristic punctuate cytoplasmic staining. The receptor was mainly observed in cells displaying a neuronal cellular morphology and granular staining of the neuropil was also often noted. In contrast, when sections were stained with an antibody recognising all EP3 isoforms, staining was observed in both neurons and glial cells (Figure 3.4).

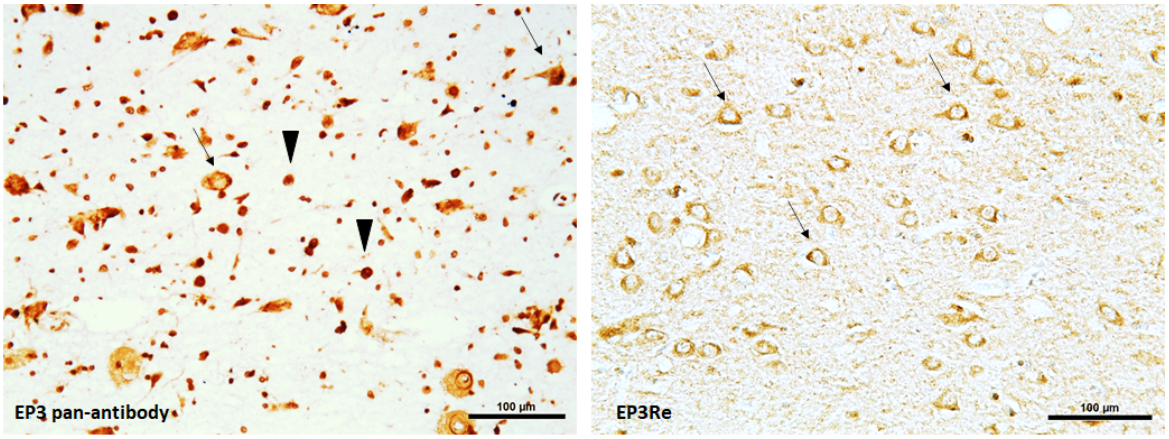


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Fig. 3.4 EP3Re immunoreactivity is observed predominantly in cells with a neuronal morphology. Brain sections showing EP3 (left panel) and EP3Re (right panel) staining. EP3Re immunoreactivity is only observed in cells with a neuronal phenotype (arrow) while EP3 staining is also noted in cells with a glial morphology (arrowhead)

The strongest EP3Re immunoreactivity observed was in the brainstem nuclei and hypothalamus with weaker staining noted in the basal ganglia nuclei, and moderate staining noted in the cortex. A summary of EP3Re staining in the various brain regions can be seen in table 3.1 and described below.

3.4.2.1 Medulla

Transverse sections taken at the level of the middle portion of the inferior olive showed intense staining in all 3 nuclei within the inferior olive, the primary olivary nuclei, the medial accessory olivary nucleus and the dorsal olivary accessory nucleus (figure 3.5). The staining within the cells had a compact and granular appearance with a dotted

Table 3.1 Summary of EP3Re staining with relative intensity of staining; + weak, ++medium, +++ strong ++++ very strong

REGION	EP3Re IMMUNOREACTIVITY	
Medulla	Inferior olivary nuclei	++++
	Reticular formation	+++
	Raphe nucleus	+++
	Cochlear nuclei	+++
	Vestibular nuclei	++
	Hypoglossal nucleus	+++
Pons	Pontine nuclei	++++
	Pontocerebellar fibres	++++
	Locus coeruleus	+++
Midbrain	Raphe nucleus	+++
	Superior colliculus	+++
	Oculomotor nucleus	+++
	Red nucleus	++
	Substantia nigra	+++
Hypothalamus	Mamillary bodies	+++
	Mammillothalamic tract	+++
	Paraventricular nuclei	+++
	Ventromedial nuclei	+++
	Median preoptic area	+++
Hippocampus	Dentate granular layer	+++
	CA1	+++
	CA4	+++
	CA2	++
	CA3	++
	Subiculum	+++
Cerebellum	Dentate nucleus	+++
	Purkinje cell layer	++++
	Granular cell layer	+++
	Molecular layer	++
Basal ganglia	Putamen	++
	Globus pallidus	++
	Substantia nigra	+++
Cerebral neocortex	Frontal	+++
	Parietal	+++
	Temporal	+++
	Occipital	++

appearance within the neuropil. Moderate staining could also be seen in the reticular formation and raphe nucleus. Moving further up towards the medulla-pons junction, cells strongly positive for EP3Re could be seen within the cochlear and vestibular nuclei. Other regions noted to have moderate EP3Re immunoreactivity included the hypoglossal nucleus and lateral and medial cuneate nuclei.

3.4.2.2 Pons

Within the pons, at the level of the decussation of the trochlear nerves, strong immunoreactivity was noted across the pontine nuclei and along the pontocerebellar fibres (figure 3.6). Neurons strongly positive for EP3Re are also noted within the locus coeruleus. Staining appears to be within neurons with a dotted, granular appearance both within the cytoplasm and the neuropil with weaker staining noted in the neuropil.

3.4.2.3 Midbrain

At the pons-midbrain junction, strong immunoreactivity in neurons was seen at the raphe nucleus. Moving more rostral, strong to moderate immunoreactivity was found in neurons within the superior colliculus, the oculomotor nucleus, and the red nucleus, with weaker staining in the substantia nigra pars reticulata and pars compacta. The neuro-melanin within the dopaminergic cells of the substantia nigra, made it difficult to fully assess for the presence of EP3Re in addition to neuro-melanin within these neurons. Staining of neuro-melanin was however, distinguishable from EP3Re as it appeared darker, more intense appearing as larger brown globules in contrast with the finer granular staining of EP3Re. In some of the pigmented neurons, this lighter granular EP3Re staining could also be identified. The staining of the neuropil in the substantia nigra took on slightly more diffuse fibrillary background in contrast to the oculomoter and red nucleus where the staining appeared more granular (Figure 3.4.2.3).

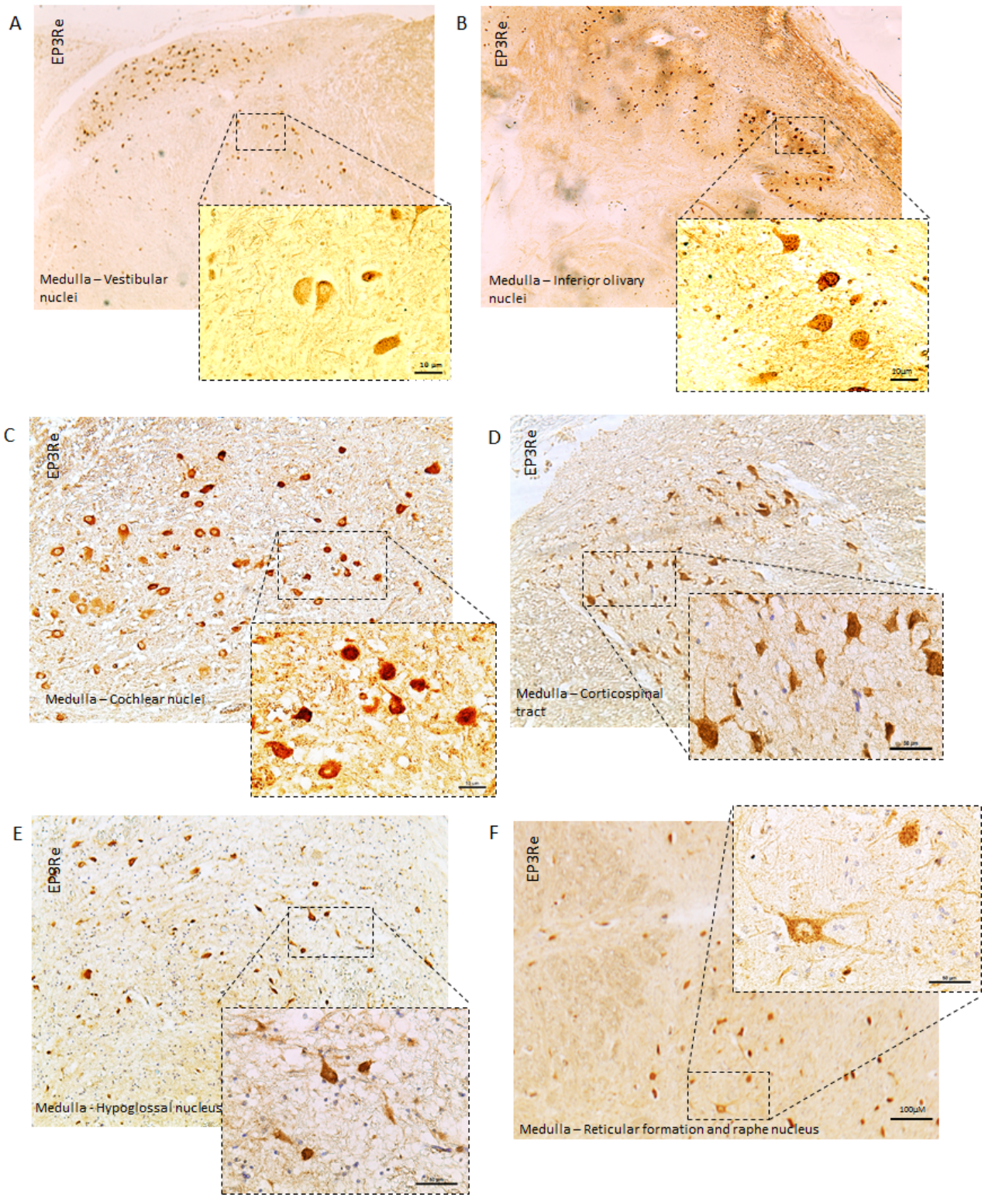


Fig. 3.5 EP3Re distribution in the medulla. A) Vestibular nuclei B) Inferior olivary nuclei C) Cochlear nuclei D) Corticospinal tract E) Hypoglossal nucleus F) Reticular formation and raphe nucleus obscura

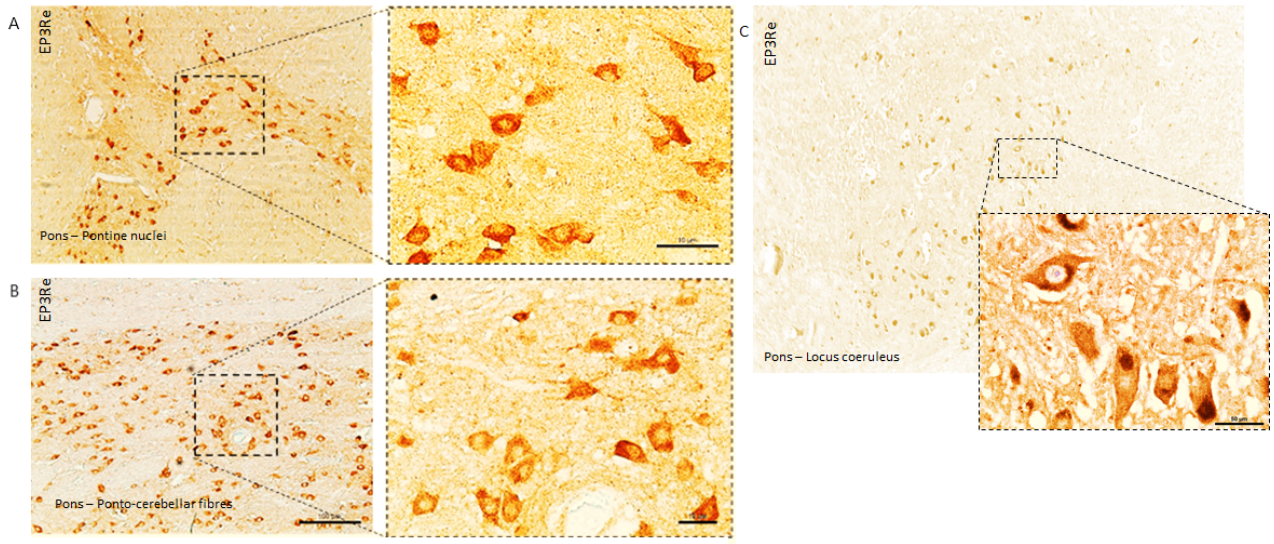


Fig. 3.6 EP3Re distribution in the Pons. A) Pontine nuclei B) Pontocerebellar fibres

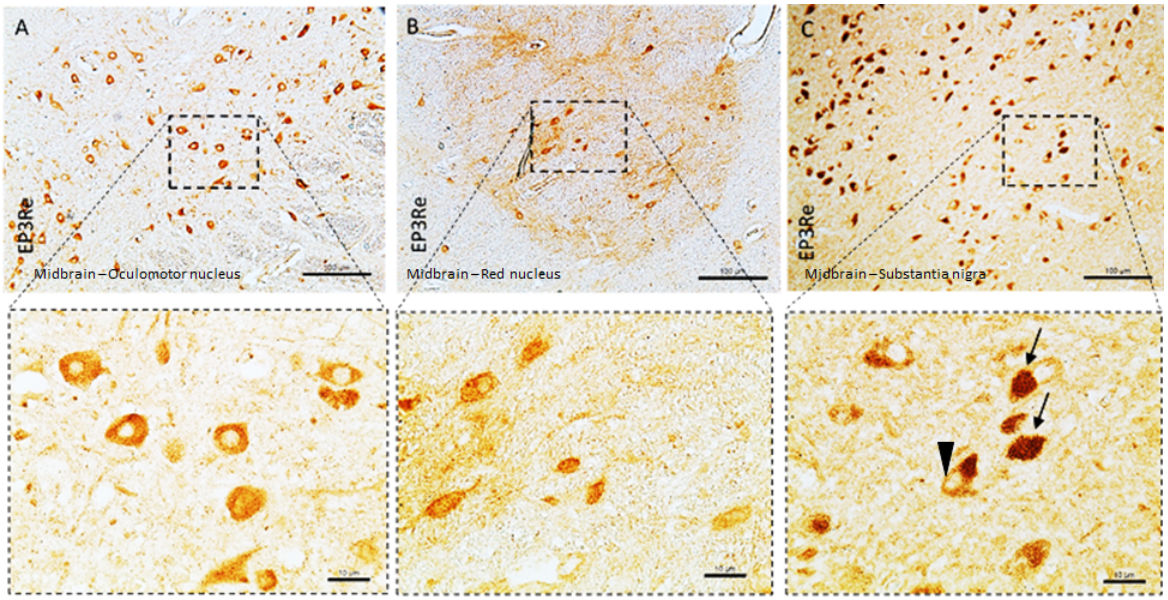


Fig. 3.7 EP3Re distribution in the midbrain. *A*) The oculomoter nucleus *B*) The red nucleus *C*)Substantia nigra, where pigmented neurons are also noted (highlighted with arrows). EP3Re staining in the same neurons is noted with an arrowhead.

3.4.2.4 Hypothalamus/Pre-optic area

The hypothalamus is part of the limbic system and serves a key role in neuroendocrine function, regulating hormonal function, circadian rhythms and playing an important role in regulating homeostatic mechanism. EP3Re positive neurons appeared more numerous within the periventricular and medial zones with minimal staining seen within the lateral zone. Intense staining was noted in the mammillary bodies, mammillothalamic tract, paraventricular nuclei, and, ventromedial nuclei with some intensely labelled neurons distributed along the wall of the third ventricle within the periventricular nuclei (figure 3.8). Strong immunoreactivity was noted in the median preoptic area, a region associated with thermogenesis thought to be mediated by EP3 signalling.

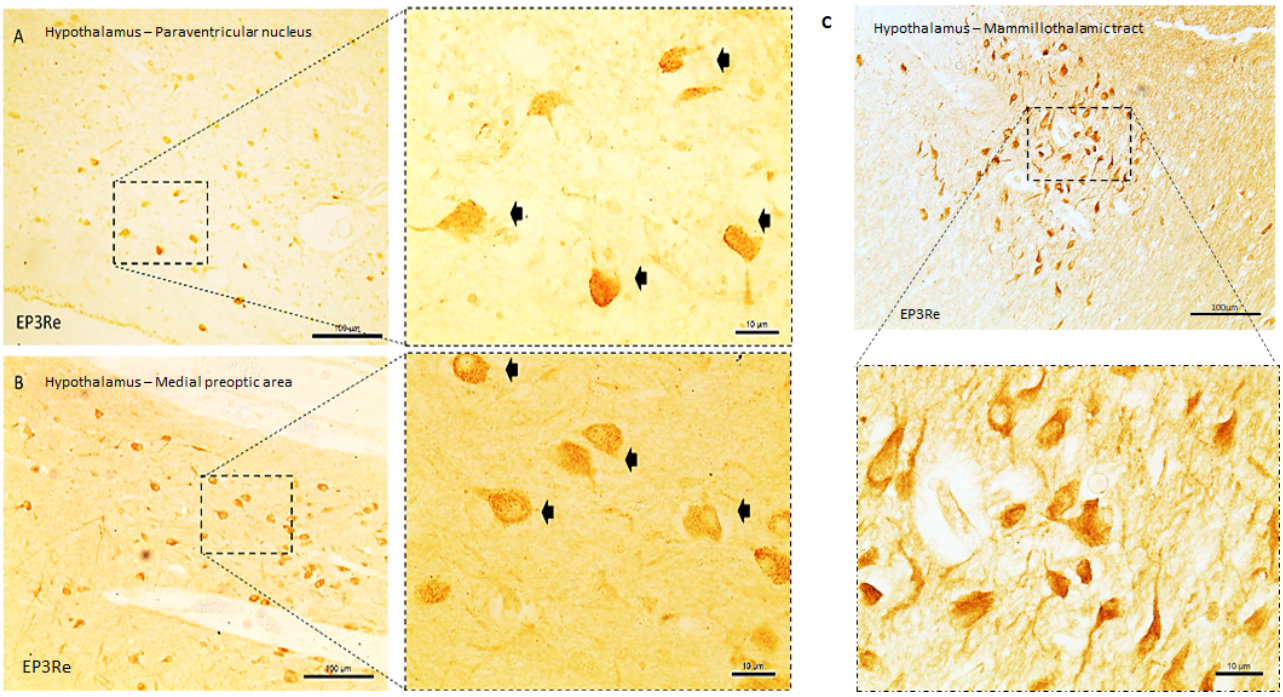


Fig. 3.8 EP3Re distribution in the hypothalamus. (A) Paraventricular nuclei (B) Medial preoptic area (C) Mammillothalamic tract

3.4.2.5 Hippocampus

The hippocampus is a major component of the limbic system and plays an important role in consolidation of short and long term memory. The hippocampal formation showed strong EP3Re immunoreactivity in neurons in CA1 and CA4 regions with weaker staining in CA2 and CA3. The granular layer neurons of the dentate gyrus were strongly positive for EP3Re (Figure 3.9).

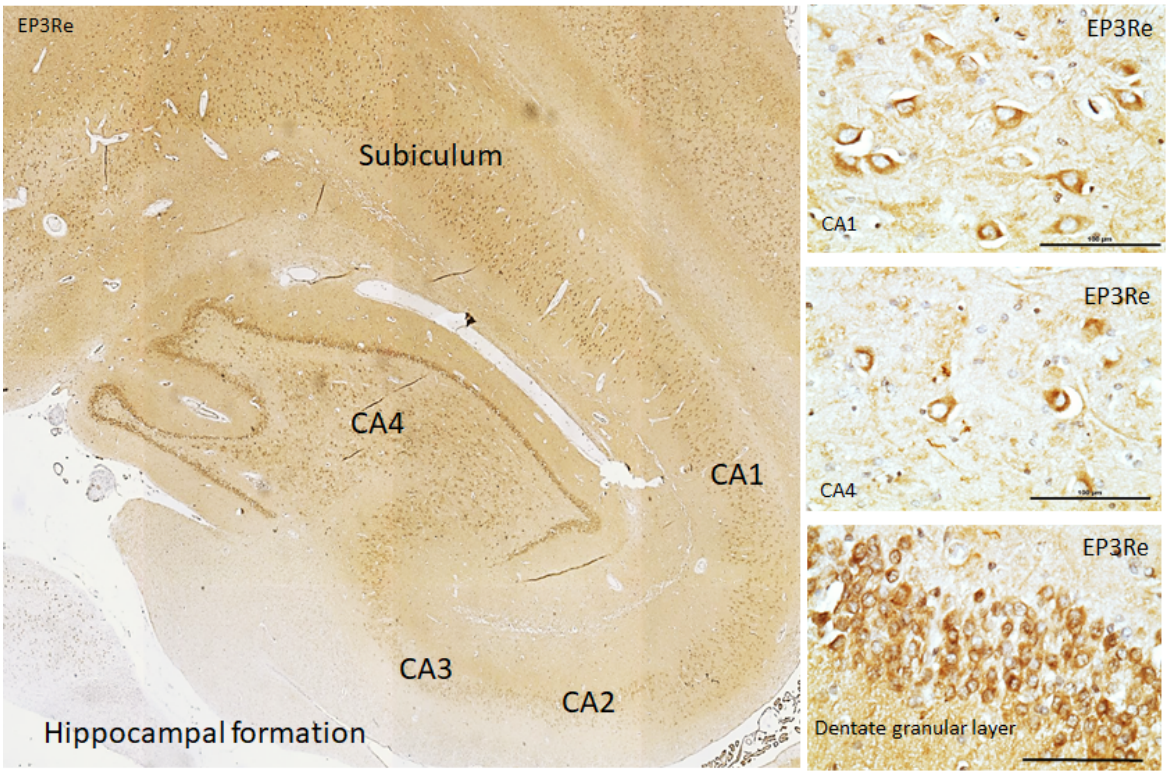


Fig. 3.9 EP3Re distribution in the hippocampal formation. The CA1, CA4 and granular layers show the highest level of immunoreactivity with moderate staining in CA2 and weak staining in CA3 regions

3.4.2.6 Cerebellum

The cerebellar cortex consists of three main layers, the outer molecular layer, the middle Purkinje cell layer and the densely packed granular layer. The EP3Re receptor appeared to be present in all three with the Purkinje cells showing some of the strongest

immunoreactivity. There was also EP3Re staining within the deep nuclei. Figure 3.10 shows the dentate nuclei, one of the four main deep nuclei within the cerebellum.

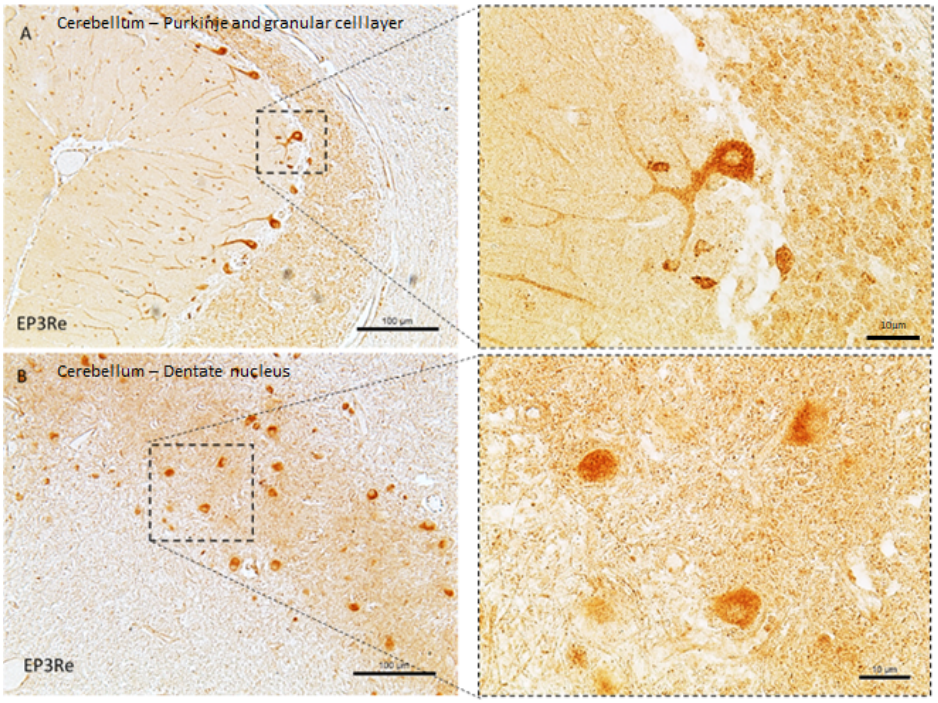


Fig. 3.10 EP3Re distribution in the cerebellum. *A*) Purkinje and granular cell layer *B*) Dentate nucleus

3.4.2.7 Basal ganglia

Consisting of several subcortical nuclei, the basal ganglia is involved in control of voluntary movement, eye movement, procedural learning, emotion and cognition. The main nuclei of the brain are the caudate nucleus, the putamen, the nucleus accumbens, the globus pallidus, the substantia nigra and the subthalamic nucleus. EP3Re positive staining was present in the associated nuclei particularly within the putamen and globus pallidus (Figure 3.11). Staining within the substantia nigra is shown in figure 3.4.2.3.

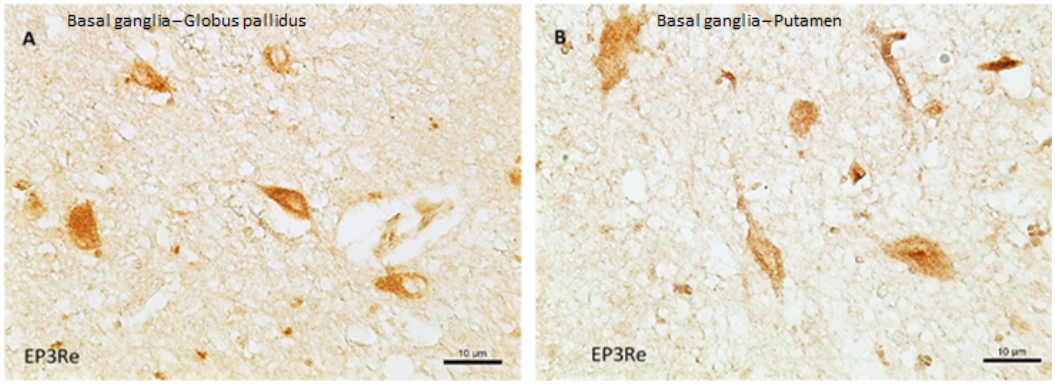


Fig. 3.11 Sections taken from the basal ganglia stained with the EP3Re antibody. Images show neurons stained in *A*) Globus pallidus internal segment *B*) Putamen.

3.4.2.8 Cerebral cortex

Sections taken from the cerebral cortex of frontal, parietal, temporal and occipital lobes all show neuronal EP3Re immunoreactivity. Pyramidal neurons of layer III within the neocortex showed more intense immunoreactivity for EP3Re than neurons within granular layers.

3.4.2.9 Biochemical evaluation of EP3Re expression

In addition to the immunohistochemical analysis, western blot was performed to validate the immunohistochemical findings and quantify protein expression (figure 3.13). Protein lysate was extracted from 3 of the control subjects, selected due to their similar age (average age 70, range 68-73yrs) and western blotting performed examining protein expression across 10 different brain regions. Although there was significantly more variability in expression across the three individuals, that immunohistochemical analysis would suggest, the highest level of EP3Re protein expression was in the medulla with the lowest being in the basal ganglia and occipital cortex.

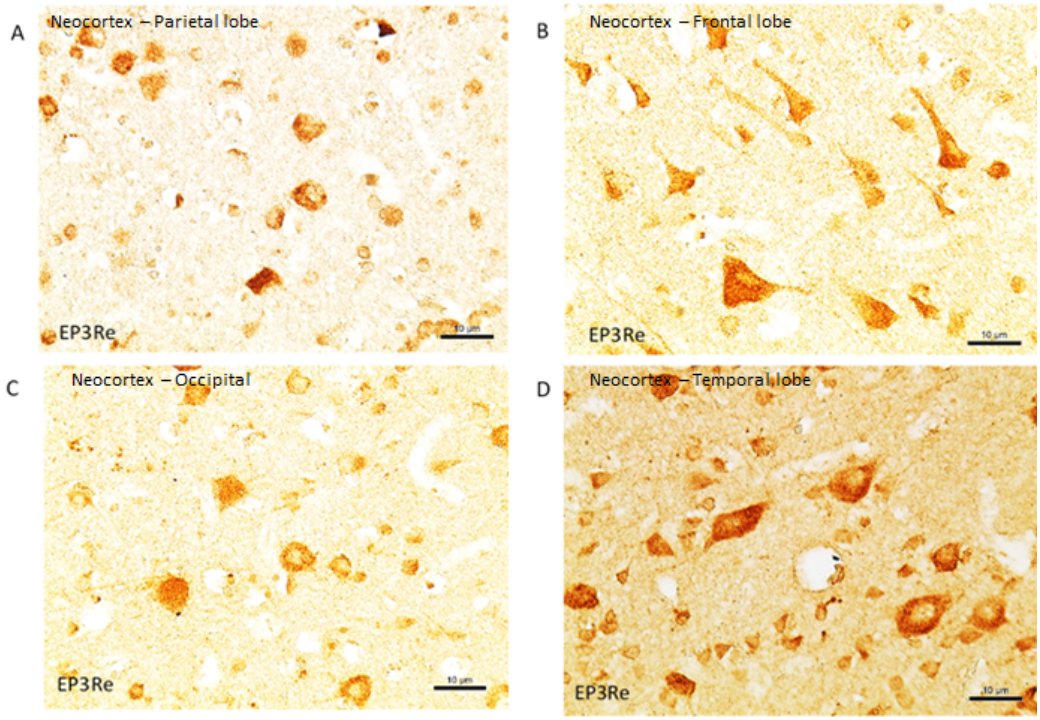


Fig. 3.12 EP3Re distribution in the cortex. Staining in *A*) Parietal *B*)Frontal *C*) Occipital *D*) Temporal cortices.

3.4.3 Characterisation of EP3Re signalling

The EP3R receptor is one of four E-prostanoid receptors that mediates signalling of PGE2. These are all G-protein coupled receptors. The EP3Re isoform has never previously been characterised but based on studies on the bovine and rodent EP3R isoforms, it has been shown to couple intracellularly to three different Gα subunits, Gs, Gi and Gq. Using a combination of reporter gene assays and second messenger assays we explored the possibility that EP3Re couples to all three Gα subunits.

3.4.3.1 Coupling of EP3Re with Gs

In response to ligand binding to cell surface GPCRs that couple to Gs, the activated intracellular Gαs subunit dissociates from the trimeric G-protein complex, where it is normally bound to the $\beta\gamma$ subunit, and binds to adenyl cyclase producing the

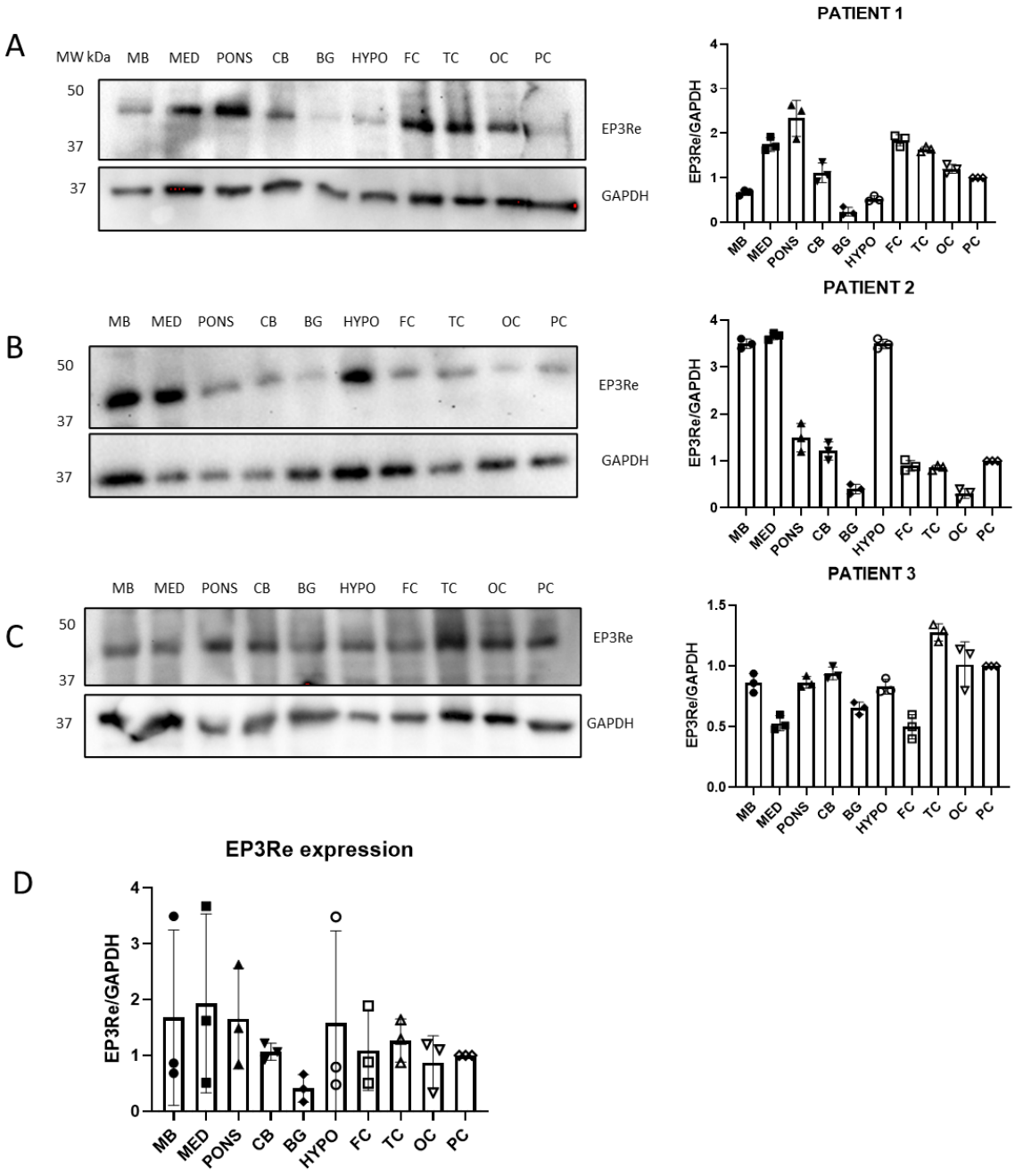


Fig. 3.13 EP3Re protein expression. Western blot showing protein expression of EP3Re by brain region across 3 different control subjects can be seen in (A), (B) and (C). (D) Graphical representation of EP3Re expression across all 3 subjects. There is significant variability in protein expression across the patients in contrast to immunohistochemical findings, which showed more consistent staining across individuals. n=3. OC= Occipital Cortex. TC=Temporal cortex, PC=Parietal cortex, FC=frontal cortex, MB = Midbrain, Med = Medulla, P = Pons, Hy = Hypothalamus, BG = basal ganglia, Cer = Cerebellum.

second messenger cyclic adenosine monophosphate (cAMP). This in turn activates the cAMP-dependent protein kinase A (PKA) with multiple cellular effects including downstream activation of cAMP response element binding protein (CREB). CREB acts as a transcription factor binding to a cAMP response element (CRE) sequence within the promoter of target genes leading to their transcription (Kobilka, 2007). (Fig. 3.14A) .

To determine if EP3Re signalling is mediated by Gs binding, various points along this signalling cascade can be examined. Initial screening of EP3Re-Gs coupling was performed by measuring CREB activity using a luciferase reporter gene assay. The signal amplification, due to the cumulative nature of the assay, makes reporter gene assays an ideal screening tool as even the smallest amount of Gs binding would be detected. CRE was built upstream of a luciferase gene and transfected into HEK293 cells alongside EP3Re. If EP3Re couples to Gαs, there should be a subsequent rise in CREB which in turn binds to the CRE region within the luciferase gene promoter, increasing luminescence allowing us to quantify EP3Re-Gs coupling. For the purpose of these experiments the EP3R specific PGE2 analogue, Sulprostone was used. This was done to minimise any signal from other E-prostanoid receptors. Initial screening were performed using a fixed dose of EP3Re cDNA (5ng) and Sulprostone (100nM), as shown in Fig. 3.14B. In response to EP3Re activation by Sulprostone, an increase in luminescence was noted suggesting EP3Re signals through Gαs coupling. No signal was seen in the absence of the receptor. The optimal EP3Re cDNA dose was then determined by repeating assays using varying concentrations of EP3Re cDNA, ranging from 0-10ng. Optimal receptor activity was detected at 5ng with a reduction in Gs mediated signalling at higher concentrations. No significant intrinsic receptor activity is noted, as determined by changes in receptor activity, based on increasing cDNA, in

the absence the ligand (Fig. 3.14C). Experiments were then repeated using varying concentrations of Sulprostone. A dose response curve shows an increase in Gs mediated signalling in a Sulprostone dose dependent manner with an average half maximal response (EC50) detected at a concentration of 1 x $10^{-9.3}$M (Fig. 3.14D).

Having determined that EP3Re couples to Gs by measuring production of CREB, it was important to validate this by measuring the second messenger, cAMP, directly. The cumulative nature of reporter gene assays means there is significant signal amplification, making them an ideal tool for general screening, however gene transcription occurs significantly downstream from the ligand binding to the receptor, allowing for the possibility of other confounding factors which may interfere in the signalling pathway. Measuring the second messenger directly reduces this. HEK293 cells were transfected with EP3Re cDNA and cAMP measured following Sulprostone exposure. This was performed with and without the addition of pertussis toxin, which inhibits any inhibitory Gi activity which might mask any rise in cAMP. No increase in cAMP was detected in response to EP3Re stimulation both in the absence and presence of pertussis toxin (Fig. 3.14E and Fig. 3.14F). This suggested that the previously noted rise in CREB as measured via the reporter gene assay was not being mediated by cAMP or Gs coupling.

In order to understand this discrepancy, I revisited the reporter gene assay using HEK293 cells lacking the guanine nucleotide binding protein alpha stimulating (GNAS) gene, which encodes the αs subunit. An increase in luciferase activity was still noted, despite there being no Gαs subunit present (Fig. 3.14H). The rise in CREB was not being mediated through Gs coupling and with no measurable rise in cAMP, there was

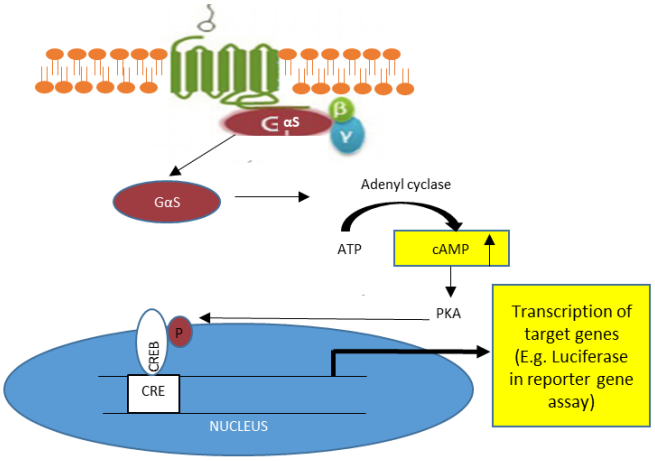
no evidence to support that EP3Re interacts with Gαs. EP3Re was not signalling through Gαs coupling.

3.4.3.2 Coupling of EP3Re with Gq

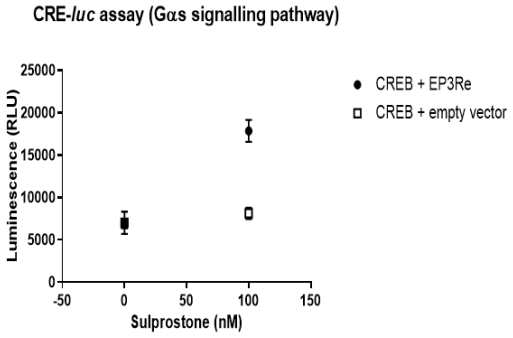
In response to ligand binding to cell surface GPCRs that couple to Gq, the activated intracellular Gαq subunit dissociates from the trimeric G-protein complex, and activates phospholipase C (PLC) which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 acts as a second messenger releasing calcium into the cytosol which in turn activates the calcium/calmodulin dependent phosphatase calcineurin which dephosphorylates the transcription factor nuclear factor of activated T-cells (NFAT) allowing it to translocate to the nucleus binding and transcribing target genes containing an NFAT-response element (RE) sequence (Mizuno and Itoh, 2009)(Fig.3.15A). To investigate EP3Re-Gq coupling any one of the second messenger molecules generated as a result of Gq activity as well as gene transcription instigated by NFAT could be measured.

In this study, to detect the presence of EP3Re-Gq coupling, I first performed a luciferase reporter gene assay using a luciferase gene with an NFAT-RE built upstream as the reporting vector. If EP3Re coupled to Gαq activating NFAT this would result in transcription of the luciferase gene emitting a luminescent signal that could be measured. HEK293 cells were transfected with the NFAT-luciferase gene alongside either EP3Re or an empty vector. The cells were then treated with the EP3R agonist sulprostone and luminesce measured. The results are shown in figure 3.15B. No significant increase in signal was noted in response to EP3Re activation suggesting EP3Re was not signalling via this pathway. The reporter gene assay did not detect EP3Re-Gq coupling.

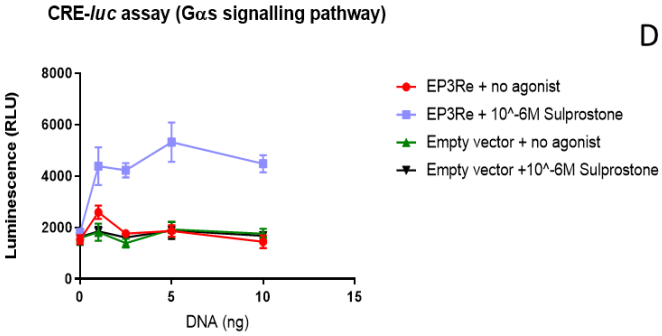
A


Gαs
β
γ
GαS
Adenyl cyclase
ATP
cAMP
PKA
P
CREB
CRE
NUCLEUS
Transcription of target genes (E.g. Luciferase in reporter gene assay)

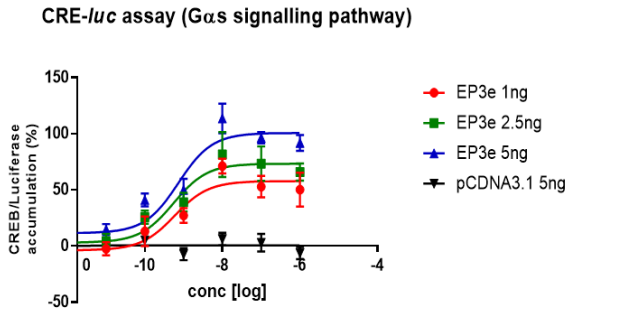
B


CRE-luc assay (Gαs signalling pathway)
Luminescence (RLU)
25000
20000
15000
10000
5000
0
-50
0
50
100
150
Sulprostone (nM)
CREB + EP3Re
CREB + empty vector

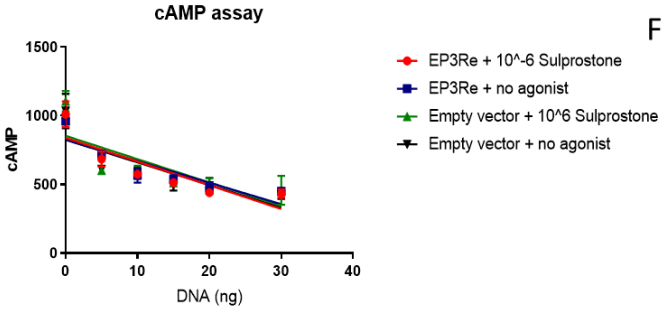
C


CRE-luc assay (Gαs signalling pathway)
Luminescence (RLU)
8000
6000
4000
2000
0
0
5
10
15
DNA (ng)
EP3Re + no agonist
EP3Re + 10^-6M Sulprostone
Empty vector + no agonist
Empty vector +10^-6M Sulprostone

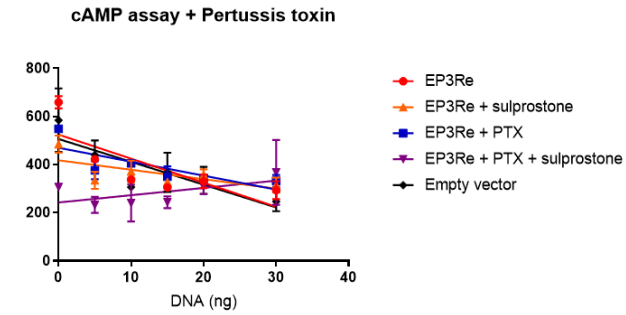
D


CRE-luc assay (Gαs signalling pathway)
CREB/Luciferase accumulation (%)
150
100
50
0
-50
0
-10
-8
-6
-4
conc [log]
EP3e 1ng
EP3e 2.5ng
EP3e 5ng
pCDNA3.1 5ng

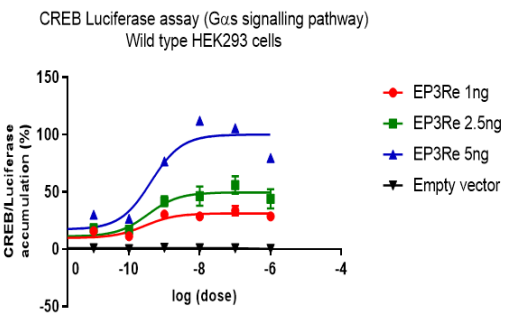
E


cAMP assay
cAMP
1500
1000
500
0
0
10
20
30
40
DNA (ng)
EP3Re + 10^-6 Sulprostone
EP3Re + no agonist
Empty vector + 10^6 Sulprostone
Empty vector + no agonist

F


cAMP assay + Pertussis toxin
800
600
400
200
0
0
10
20
30
40
DNA (ng)
EP3Re
EP3Re + sulprostone
EP3Re + PTX
EP3Re + PTX + sulprostone
Empty vector

G


CREB Luciferase assay (Gαs signalling pathway)
Wild type HEK293 cells
CREB/Luciferase accumulation (%)
150
100
50
0
-50
0
-10
-8
-6
-4
log (dose)
EP3Re 1ng
EP3Re 2.5ng
EP3Re 5ng
Empty vector

H

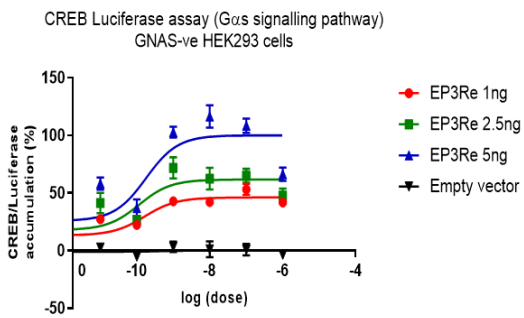

CREB Luciferase assay (Gαs signalling pathway)
GNAS-ve HEK293 cells
CREB/Luciferase accumulation (%)
150
100
50
0
-50
0
-10
-8
-6
-4
log (dose)
EP3Re 1ng
EP3Re 2.5ng
EP3Re 5ng
Empty vector

Fig. 3.14 Coupling of EP3Re with Gs. A) Gs signalling cascade. Upon ligand binding the αs subunit dissociates from the $\beta\gamma$ subunit increasing cAMP levels by promoting adenyl cyclase activity. This begins a signalling cascade that results in protein kinase A (PKA) activation. The PKA catalytic units translocate into the nucleus phosphorylating CREB which binds to the CRE region within the promoter of target genes instigating gene transcription. B) Luciferase reporter gene assay using a CRE- reporting vector showed a positive signal in response to sulprostone suggesting EP3Re coupling with Gs. Assays were run in quadruplicate. Each point represents the mean +/- standard deviation of 2 independent experiments. C) Receptor activity is measured at increasing doses of EP3Re cDNA with and without ligand stimulation. Optimum receptor activity is seen at 5ng of the receptor. No intrinsic receptor activity is noted in the absence of the ligand, despite increasing the amount of EP3Re cDNA. D) Dose response of EP3Re expression and luciferase activation, confirming data shown in (C). Optimum receptor activity is noted at 5ng of EP3Re cDNA with an average LogEC50 of -9.3. E) Gene transcription, as measured through reporter gene assays, in response to Gs coupling is significantly downstream of ligand binding. We therefore needed to directly assess the second messenger, cAMP, that is produced in response to receptor Gs coupling. No increase in cAMP was seen in response to EP3Re stimulation by sulprostone suggesting EP3Re does not couple to Gs. F) To exclude the possibility that no accumulation in cAMP was seen due to cAMP inhibition by simultaneous Gi activity, pertussis toxin was used. Pertussis toxin inhibits Gi protein coupling and was added to cells at a concentration of 100nM. No increase in cAMP is noted to suggest simultaneous Gi activity is preventing cAMP accumulation. There is no evidence to support the initial findings of the luciferase reporter gene assay to suggest EP3Re coupling to Gs. We varied the concentration of pertussis toxin from 100nM up to 1mM with no change in results (data not shown). The original reporter gene assay was then repeated using wild type HEK293 cells (G) and HEK293 cells lacking the Gαs subunit (GNAS –ve). CRE activity was detected despite there being no Gαs subunit suggesting transcription of CRE is not being mediated by Gs coupling. EP3Re does not signal by coupling to Gs. Unless otherwise stated all assays shown were run in triplicate. Each point represents the mean +/- standard deviation of 3 independent experiments. Data from dose response assays were fitted to a non-linear regression curve and normalised in GraphPad Prism 7.0.

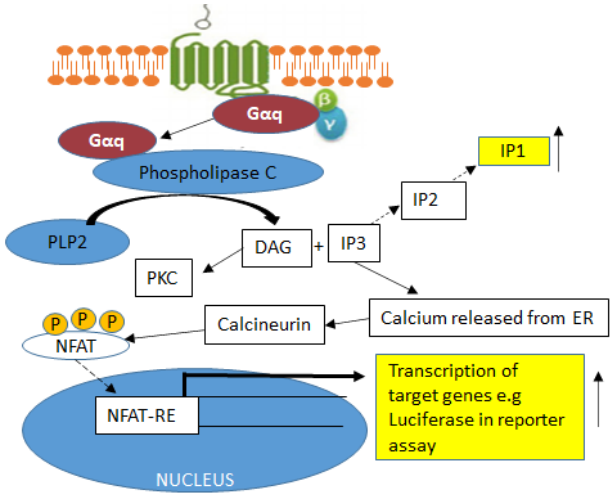
As the reporter gene assay measured gene transcription occurring significantly downstream from ligand binding, it was important to also assess the presence of EP3Re-Gq coupling by measuring accumulation of a second messenger, upstream of gene transcription such as IP3. IP3 however, is unstable and present only briefly before being rapidly degraded to produce inositol phosphate (IP1), for which there are readily available assays. IP1 accumulation is therefore a reflection of PLC activity confirming the presence of Gq coupling. IP1 accumulation assays were performed in HEK293 cells transfected with EP3Re or an empty vector. Cells were treated with the EP3R agonist sulprostone at concentrations ranging from 0 – $1x10^{-6}$M and IP1 accumulation measured. Figure 3.15C shows the dose response curve and a small increase in IP1 is noted with a half maximal effect seen at $1x10^{-9.3}$M. This suggested that some EP3Re-Gq coupling was occurring. The potency of the endogenous EP3R ligands prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2) were also examined and can be seen in figures 3.15D and E. Sulprostone has the highest affinity for EP3Re followed by PGE2 and then PGD2.

3.4.3.3 Coupling of EP3Re with Gi

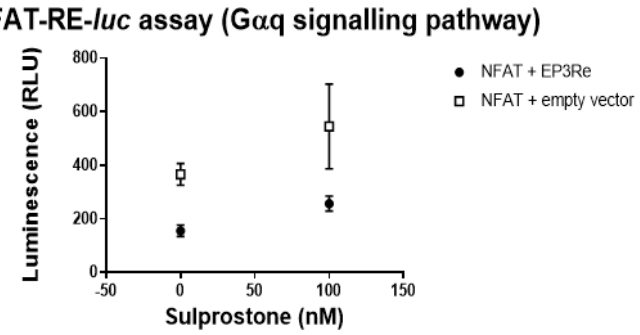
Gi signalling, in contrast to Gs, inhibits cAMP dependent pathways through inhibition of adenylyl cyclase. Determining the presence of EP3Re-Gi coupling by measuring a reduction in cAMP dependent pathways can be difficult to assess and quantify. To address this, we conducted experiments by co-expressing a chimeric G-protein, consisting of a Gαi/Gαq chimeric subunit, G$\alpha\delta$6qi4myr, as described by Kostenis et al. (Kostenis, 2001). This confers Gq-effector coupling on Gi linked receptors allowing us to measure NFAT mediated transcription and IP1 as markers of Gi receptor coupling.

Initial screening was performed using the luciferase reporter gene assay. HEK293

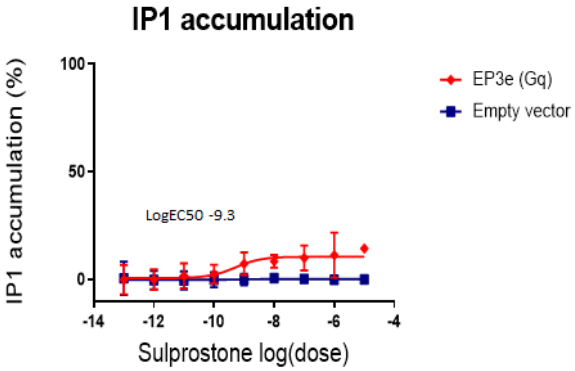
A


Gαq
β
γ
Gαq
Phospholipase C
IP1
IP2
PLP2
DAG
+
IP3
PKC
P P P
NFAT
Calcineurin
Calcium released from ER
Transcription of target genes e.g Luciferase in reporter assay
NFAT-RE
NUCLEUS

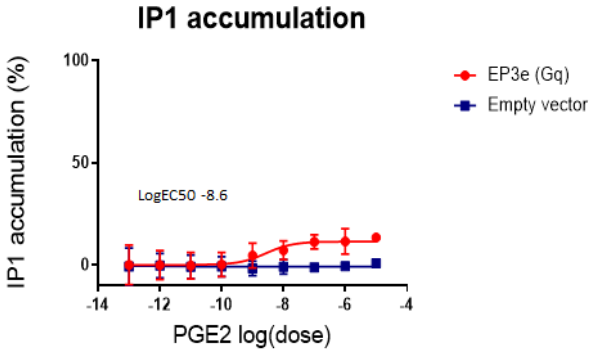
B


NFAT-RE-luc assay (Gαq signalling pathway)
Luminescence (RLU)
800
600
400
200
0
-50
0
50
100
150
Sulprostone (nM)
NFAT + EP3Re
NFAT + empty vector

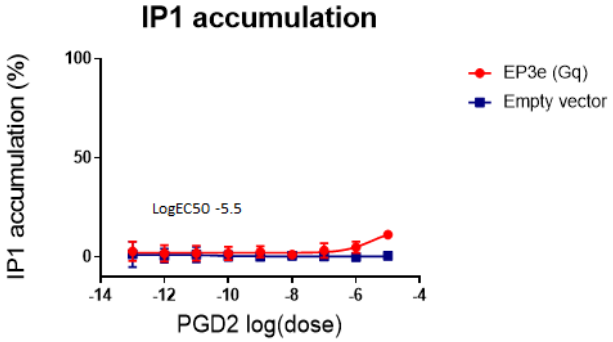
C


IP1 accumulation
IP1 accumulation (%)
100
50
0
LogEC50 -9.3
-14
-12
-10
-8
-6
-4
Sulprostone log(dose)
EP3e (Gq)
Empty vector

D


IP1 accumulation
IP1 accumulation (%)
100
50
0
LogEC50 -8.6
-14
-12
-10
-8
-6
-4
PGE2 log(dose)
EP3e (Gq)
Empty vector

E


IP1 accumulation
IP1 accumulation (%)
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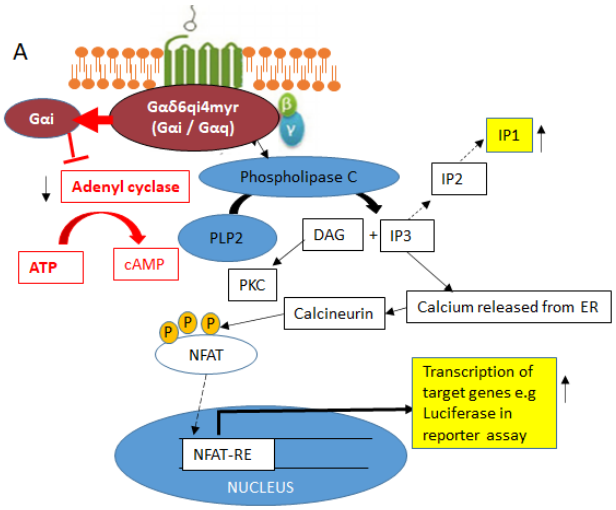
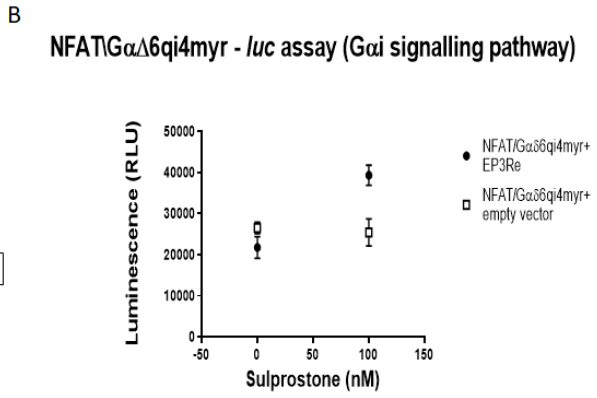
50
0
LogEC50 -5.5
-14
-12
-10
-8
-6
-4
PGD2 log(dose)
EP3e (Gq)
Empty vector

Fig. 3.15 Coupling of EP3Re with Gq. A) Gq signalling pathway. Receptor coupling through Gq results in the activation of phospholipase C by the dissociated Gαq subunit. This cleaves the membrane phospholipid PIP2 into DAG and IP3 which act as important second messengers. DAG recruits and activates PKC whilst IP3 instigates the opening of IP3 calcium channels, releasing calcium from the endoplasmic reticulum. This regulates several signalling cascades including activation of the calcium/calmodulin dependent phosphatase calcineurin which activates the transcription factor NFAT by dephosphorylating it, leaving it free to translocate to the nucleus where it is able to instigate gene transcription of target genes. Outputs measured for reporter gene assays (NFAT) and second messenger assays (IP1) highlighted in yellow. B) The luciferase reporter gene assay measuring NFAT activity showed no increase in signal in sulprostone treated cells in the presence of EP3Re compared to controls. EP3Re was not activating a Gq signalling cascade. Assays were run in quadruplicate. Each point represents the mean +/- standard deviation of 2 independent experiments. C) IP1 accumulation assays, a breakdown product of the second messenger IP3 produced on Gq coupling, were performed to further assess EP3Re-Gq coupling. HEK293 cells were transfected with EP3Re or an empty vector and treated with varying concentrations of sulprostone. The dose response curve shows some IP1 being produced in a dose dependent manner suggesting EP3Re-Gq coupling. The potency of the endogenous ligands PGE2 (D) and PGD2 (E) were then assessed and compared to the synthetic EP3R agonist sulprostone. Although PGD2 signals through DP1 and DP2 receptors, some EP3Re activity was noted at higher concentrations. Sulprostone had the strongest affinity to EP3Re (LogEC50 -9.3) followed by PGE2 (LogEC50 -8.6) and then PGD2 (LogEC50 -5.5). All IP1 assays were run in triplicate. Each point represents the mean +/- standard deviation of 3 independent experiments. Data from dose response assays were fitted to a non-linear regression curve and normalised in GraphPad Prism 7.0.
PIP2 - phosphatidylinositol-4,5-bisphosphonate; IP3 - Inositol 1,4,5-triphosphate; DAG - diacylglycerol; PKC - Protein kinase C; ER - Endoplasmic reticulum; NFAT - Nuclear factor of activated T-cells.

cells were transfected with EP3Re alongside the chimeric G protein, G$\alpha\delta$6qi4myr and a construct containing an NFAT-RE built upstream of a luciferase gene. The receptor was then activated by exposing cells to the EP3R agonist sulprostone and luminescence measured as a marker of luciferase transcription. An increase in NFAT-mediated luciferase activity was noted but only in the presence of G$\alpha\delta$6qi4myr suggesting EP3Re-Gi coupling (Fig. 3.16B). Cells were then transfected with increasing doses of EP3Re cDNA to determine the optimal gene dose concentration and presence of intrinsic receptor activity. Optimal activity was noted between 2.5ng – 5ng (Fig. 3.16C). Any further increase resulted in reduced receptor activity both in the presence and absence of stimulation. To validate the findings of the luciferase reporter gene assay, we went on to measure IP1 accumulation, in the presence of G$\alpha\delta$6qi4myr, as a more upstream target of EP3Re-Gi coupling. Cells were stimulated with the EP3R agonist sulprostone and in the presence of G$\alpha\delta$6qi4myr we noted an increase in IP1 accumulation in a dose dependent manner confirming the results of the reporter gene assay further supporting EP3Re-Gi coupling. We then compared the potency of the endogenous ligands PGE2 and PGD2 to the synthetic EP3R agonist sulprostone and similar to our study of EP3Re-Gq coupling, we found sulprostone to have the strongest affinity to EP3Re, followed by PGE2 and PGD2.

Our present study has shown EP3Re to signal through Gi and Gq signalling pathways. There is no evidence to support EP3Re-Gs coupling.


A
Gαi
Gαδ6qi4myr
(Gαi / Gαq)
β
γ
Adenyl cyclase
Phospholipase C
IP1
IP2
ATP
cAMP
PLP2
DAG
+
IP3
PKC
Calcineurin
Calcium released from ER
P
P
P
NFAT
Transcription of target genes e.g Luciferase in reporter assay
NFAT-RE
NUCLEUS


B
NFAT\GαΔ6qi4myr - luc assay (Gαi signalling pathway)
Luminescence (RLU)
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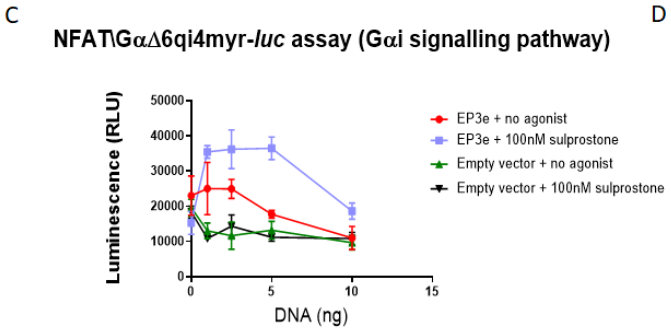
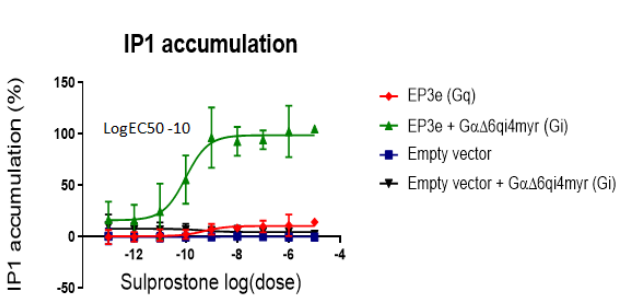
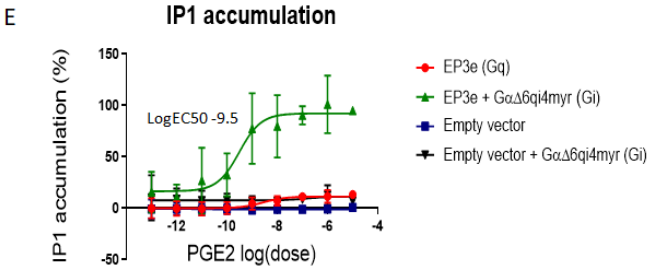
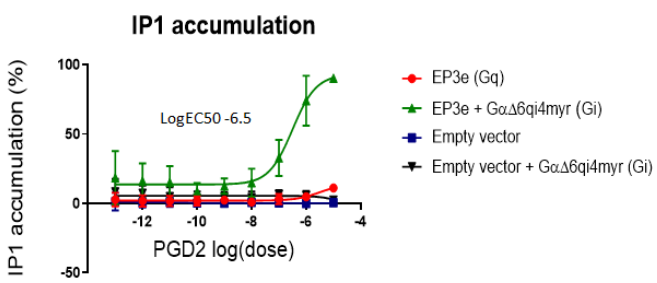
40000
30000
20000
10000
0
-50
0
50
100
150
Sulprostone (nM)
NFAT/Gαδ6qi4myr+ EP3Re
NFAT/Gαδ6qi4myr+ empty vector


C
NFAT\GαΔ6qi4myr-luc assay (Gαi signalling pathway)
Luminescence (RLU)
50000
40000
30000
20000
10000
0
0
5
10
15
DNA (ng)
EP3e + no agonist
EP3e + 100nM sulprostone
Empty vector + no agonist
Empty vector + 100nM sulprostone


D
IP1 accumulation
IP1 accumulation (%)
150
100
50
0
-50
LogEC50 -10
-12
-10
-8
-6
-4
Sulprostone log(dose)
EP3e (Gq)
EP3e + GαΔ6qi4myr (Gi)
Empty vector
Empty vector + GαΔ6qi4myr (Gi)


E
IP1 accumulation
IP1 accumulation (%)
150
100
50
0
-50
LogEC50 -9.5
-12
-10
-8
-6
-4
PGE2 log(dose)
EP3e (Gq)
EP3e + GαΔ6qi4myr (Gi)
Empty vector
Empty vector + GαΔ6qi4myr (Gi)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IP1 accumulation
IP1 accumulation (%)
100
50
0
-50
LogEC50 -6.5
-12
-10
-8
-6
-4
PGD2 log(dose)
EP3e (Gq)
EP3e + GαΔ6qi4myr (Gi)
Empty vector
Empty vector + GαΔ6qi4myr (Gi)

Fig. 3.16 Coupling of EP3Re with Gi. A) Gi signalling pathway. The red arrow indicates the usual signalling cascade instigated on Gi coupling. Upon ligand binding the Gαi subunit dissociates and inhibits adenyl cyclase activity resulting in a reduction in cAMP. To assess EP3Re-Gi coupling, cells were co-transfected with the G-protein alpha subunit G$\alpha\delta$6qi4myr, a chimera between Gαi and G*alpha*q. This converts a Gi-coupled receptor into a Gq signalling pathway allowing us to measure IP1 and NFAT mediated transcription to quantify Gi-coupling. B) Initial screening using the luciferase reporter gene assay showed an increase in signal in the presence of G$\alpha\delta$6qi4myr suggesting the signal was being instigated by EP3Re-Gi coupling. Assays were run in quadruplicate. Each point represents the mean +/- standard deviation of 2 independent experiments. C) The luciferase reporter gene assay was performed using increasing doses of EP3Re cDNA with and without ligand stimulation. Optimum receptor activity is seen at 5ng of the receptor. Any further increase results in reduced EP3Re activity as measure by Gi coupling. Intrinsic receptor activity is noted in the absence of the ligand at lower doses of EP3Re cDNA. D) IP1 accumulation was measured in the presence of G$\alpha\delta$6qi4myr to validate the results of the luciferase reporter gene assay. HEK293 cells were transfected with EP3Re and G$\alpha\delta$6qi4myr and stimulated with the EP3R agonist sulprostone. There is a dose dependent accumulation of IP1 which is significantly higher in the presence of G$\alpha\delta$6qi4myr. This suggests that EP3Re-Gi coupling is the predominant signalling pathway. E) IP1 accumulation assay performed in the presence and absence of G$\alpha\delta$6qi4myr in response to cell stimulation by the endogenous ligand PGE2. F) IP1 accumulation assay performed in the presence and absence of G$\alpha\delta$6qi4myr in response to cell stimulation by the endogenous ligand PGD2. Sulprostone showed the strongest affinity for the receptor (LogEC50 -10) followed by PGE2 (LogEC50 -9.5) and then PGD2 (LogEC50 -6.5).
Unless otherwise stated all assays were run in triplicate. Each point represents the mean +/- standard deviation of 3 independent experiments. Data from dose response assays were fitted to a non-linear regression curve and normalised in GraphPad Prism 7.0. ATP; Adenosine triphosphate, cAMP; cyclic adenosine monophosphate, PIP2; phosphatidylinositol-4,5-bisphosphonate, IP3; Inositol 1,4,5-triphosphate, DAG; diacylglycerol, PKC; Protein kinase C, ER; Endoplasmic reticulum, NFAT; Nuclear factor of activated T-cells.

3.5 Discussion

This is the first study to characterise the distribution of the EP3Re receptor in the human brain and the pathway through which it exerts its function. EP3Re appears to be ubiquitously expressed in the human brain with stronger staining noted in the brainstem nuclei, cerebellum, hippocampus and hypothalamus. In this study, we found EP3Re to signal predominantly through a Gi coupled signalling cascade with some evidence of Gq coupling under normal physiological conditions.

The widespread expression of EP3Re suggests that it could modulate several functions of PGE2 within the brain, including its possible involvement in thermoregulation, memory, and appetite control. The receptor enrichment in the hypothalamus and pre-optic areas supports EP3Re isoforms involvement in thermoregulation and the pyrexial response, a function known to be modulated by the EP3 receptor family. The role of individual isoforms is unclear, however as the isoforms differ only by the intracellular C-terminus, which determines the signalling cascade, it is possible that each isoform plays a different functional role, although this goes beyond the scope of this study. Strong staining within the hypothalamus was also noted within the mammillothalamic tract, which connects the mammillary bodies to the anterior thalamus which plays a role in spatial memory. PGE2 has previously been shown to be involved in long term synaptic plasticity (Yang et al., 2009) however in mice this is modulated via the EP2 receptor. In humans, however, EP3Re could be playing a similar role supported by its presence in the hippocampal formation. Strong immunoreactivity was noted in CA1, the main output pathway to the entorhinal cortex and subiculum. CA1 is thought to be important in autobiographical and spatial memory and is particularly vulnerable to neuronal loss in Alzheimer's disease (Padurariu et al., 2012). EP3Re

immunoreactivity was also observed within the cerebellum and the pons, an important relay point between the forebrain and the cerebellum. Strong immunoreactivity was also noted within the inferior olivary nuclei, a region that relays signals from the spinal cord to the cerebellum regulating motor control and learning. EP3Re positive neurons were present in regions consisting predominantly of monoaminergic neurons such as the serotonergic neurons of the raphe nuclei and noradrenergic neurons in the locus coeruleus. Monoaminergic systems are involved in cognitive regulation and the presence of EP3Re may suggest a possible role for EP3Re in cognitive function and monoamine release.

Our study observed by immunohistochemistry, the highest levels of EP3Re immunoreactivity within the brainstem, hypothalamus and cerebellum. When this was quantified using western blotting the highest levels were noted again in the brainstem with lower levels in the basal ganglia, however significant variation was noted between the 3 individuals used for the study, more variability than was suggested based on the immunohistochemical analysis. Data obtained from the human protein atlas (Uhlen et al., 2015), which combines RNA data from the genotype tissue expression project (GTEx) and the functional annotation of the mammalian genome project (FANTOM5) show levels of EP3 RNA within the human brain to be highest in the amygdala and hypothalamus with significant variability across the two data sets, where between 139 – 255 individuals were sequenced for each data set, depending on the region. This variation is similar to our findings examining protein levels and is likely to arise from variations in gene expression as suggested by the human protein atlas. A larger cohort would need to be studied to validate these findings. External individual factors resulting in upregulation of protein expression in certain regions or factors affecting fresh frozen tissue preservation could also have impacted our results. The study was

limited to characterising EP3Re distribution, although immunohistochemistry using the antibody directed against all EP3 isoforms was also performed. In subcortical structures EP3Re immunoreactivity was observed predominantly in neurons whilst the antibody directed against all EP3 isoforms showed staining in both neuronal and glial cells. These results confirm the presence of other isoforms in the brain but the lack of commercially available antibodies targeting individual isoforms, limited our ability to explore this further. Future studies using quantitative reverse transcription polymerase chain reaction (RT-qPCR) would allow us to better determine the expression of individual EP3 isoforms within the brain and how this compares to EP3Re. The main limitation of RT-qPCR is the resolution obtained using immunohistochemistry, detailing receptor expression within subcortical nuclei and cellular morphology, would be lost.

We also characterised the main signalling pathways activated by EP3Re. We show that EP3Re signals predominantly through Gi coupling with evidence of Gq mediated signalling. Despite the increase in CRE mediated gene transcription, there was no evidence to suggest this was mediated through Gs coupling. Second messenger assays showed no cAMP production even in the presence of pertussis toxin and CRE mediated gene transcription was maintained when cells lacking the Gαs subunit were used to perform the assays. These findings suggest the increase in CRE activity observed is not as a result of EP3Re-Gs coupling. It has been observed the Gq coupling and subsequent increase in Ca^{2+} can lead to CREB phosphorylation and CRE mediated gene transcription (Rosethorne et al., 2008; Yang et al., 2013). In our study, EP3Re-Gq coupling could have been responsible for the increase observed in CRE gene transcription. There are several limitations to using reporter gene assays to explore G-protein signalling. Although they are highly sensitive assays, the output measured is significantly downstream of the original event. Furthermore there is extensive cross talk

between the various signalling pathways contributing to a final readout that may have originated from a different G-protein than the one being assessed, as occurred in this study. This was overcome through the use of second messenger assays by measuring cAMP production for Gs coupling and IP1 for Gq and Gi. Measuring calcium mobilisation could also have been used to assess Gq and Gi coupling (with the use of the chimeric G-protein), however, IP1, a breakdown product of IP3, is upstream of calcium mobilisation. Several other factors can influence calcium levels independent of Gq coupling reducing the specificity of the assay making IP1 measurement a better choice for assessing Gq and Gi activity.

Sulprostone, PGE2 and PGD2 were all found to bind to the EP3Re receptor with sulprostone having the strongest affinity, followed by PGE2 then PGD2, with the latter two being endogenous prostaglandins. PGD2 is one of the most abundant prostaglandins within the central nervous system and has been implicated in regulation of sleep, thermoregulation and nociception (Joo and Sadikot, 2012). PGD2 has also been shown to be neurotoxic or neuroprotective depending on the receptor through which it signals. DP1 in animal models of disease has been found to be protective while DP2 is neurotoxic (Liang et al., 2005b). Further work will need to be done to determine if PGD2-EP3Re signalling occurs under normal physiology and if this has any implications for neurological disease.

These results have demonstrated that EP3Re is widely expressed in neurons throughout the brain and signals through Gi, with some evidence of Gq, mediated signalling cascades. The varying expression levels of EP3Re in specific neuronal populations could explain regional specific responses of PGE2 signalling. Further work will be required

to understand if and how this changes under inflammatory pathological conditions in a way that can be exploited as a potential therapeutic strategy.

Chapter 4

THE RELATIONSHIP BETWEEN EP3RE AND TAU

4.1 Summary

Whilst establishing the distribution of the EP3e receptor in the human brain, it was noted that the EP3Re antibody identified neurons containing what appeared to be tau neurofibrillary tangles. Inflammatory prostaglandin signalling has been implicated in the pathology of Alzheimer's dementia but a direct association between PGE2 signalling and tau pathology is yet to be identified. The possibility that the PGE2 EP3e receptor co-localises with tau tangles could provide a mechanism through which PGE2 signalling could be contributing to tau pathology. In this chapter I explore the physical relationship between EP3Re and tau in order to determine if this incidental finding could be significant. Using immunohistochemistry, we map the distribution of EP3Re in a primary tauopathy (Progressive supranuclear palsy (PSP)) and in Alzheimer's dementia, having first established the antibody was not cross reacting with tau. I then went on to extract insoluble filamentous tau from Alzheimer's and PSP brains with sarkosyl, and probed for the presence EP3Re using western immunoblotting. To investigate and determine if there is an interaction between tau and EP3Re in control as well as disease brains, proximity ligation assays on fixed sections and immunoprecipitation on brain lysates was performed. Finally we directly visualise the fibrils using electron microscopy labelling the EP3e receptor using immunogold. We show a close interaction between EP3Re and filamentous tau.

4.2 Introduction

In chapter 3 we characterised the distribution and signalling pathway of the human specific E isoform of the prostaglandin E2 (PE2) EP3 receptor (EP3Re). In the course of establishing the distribution within the human brain, it was noted that the antibody directed against EP3Re appeared to identify neurons containing neurofibrillary tangles (NFTs). Neurofibrillary tangles are a pathological hallmark of a group of neurodegenerative disorders known as tauopathies (Wang et al., 2015). These include Alzheimer's dementia, Progressive Supranuclear Palsy (PSP), Pick's disease, Corticobasal degeneration (CBD) and Frontotemporal dementia with parkinsonism-17 (FTDP-17) (Lee et al., 2001).

In the 80s several studies identified tau as the principle component of neurofibrillary tangles (Kosik et al., 1986; Wood et al., 1986). Enrichment of insoluble tau aggregates using N-lauryl-sarcosine (sarkosyl) fractionates tau into soluble and insoluble components enabling the structural study of different tau species present in aggregates across different tauopathies (Crowther, 1991; Falcon et al., 2018; Fitzpatrick et al., 2017; Goedert et al., 1989; Zhang et al., 2020). Ultrastructural analysis of fibrils isolated from Alzheimer's dementia show these to be organised as paired helical filaments (PHFs), a twisted double helicon of subunits giving rise to a structure that alternates between 8 – 20nm in width (Crowther, 1991; Goedert et al., 1989). A minor species referred to as straight filaments (SF), a variant of PHFs appearing as single stranded filaments of 15nm, is also noted (Crowther, 1991). Electron micrographs of tau filaments within inclusions in other tauopathies show some variability in structure (Arima, 2006; Falcon et al., 2018; Ksiezak-Reding et al., 1994; Melo et al., 2014). PSP consists of straight filaments of 13-14nm, CBD a combination of straight filaments

(15nm) and wide twisted filaments (20nm), whilst in Pick bodies, the principal aggregates in Pick's disease, consist of 15-18nm straight filaments of varying lengths (Arima, 2006; Falcon et al., 2018). They are structured into β strands that form a pronase resistant core with unstructured C and N tau terminal domains protruding to form a fuzzy coat resembling a two layer polyelectrolyte brush (Wegmann et al., 2013). The fibrils also differ by the tau isoform composition, with AD showing both 3R and 4R tau, Picks disease 3R tau, and, PSP and CBD 4R tau (Arima, 2006).

In addition to tau, immunohistochemical and sequencing studies have identified other cytoskeletal proteins, such as microtubule associated protein 2 (MAP2) (Alonso et al., 1994, 1997, 1996) and neurofilament (Didonna and Opal, 2019) as being components of neurofibrillary tangles in disease. NFTs have also been shown to display positive immunoreactivity to a number of different antibodies ranging from structural proteins to kinases and stress related molecules (Lennox et al., 1988; Richey et al., 1995; Takahashi et al., 2006; Yen et al., 1995). Immunoreactivity does not necessarily reflect that the protein is a structural component of NFTs, indeed most of these proteins, are either found in extracellular tau, also known as ghost tangles (thought to occur following neuronal death and disintegration of the cell membrane), or only some tangles (Takahashi et al., 2006; Yamaguchi et al., 1994). This suggests that they are unlikely to be structural or essential components of neurofibrillary tangles and could simply be trapped in the filamentous aggregates. Despite this, they may still play a role in promoting NFT formation or disease pathology. For example, the G-protein coupled receptor kinase 2 (GRK2) has been found to co-localise with 40-50% of tau tangles in Alzheimer's dementia (Takahashi et al., 2006). Studies have also shown that dysfunctional GRK2 signalling is associated with amyloid induced neuronal toxicity (Suo et al., 2004) suggesting, despite not being a structural component of NFTs, its

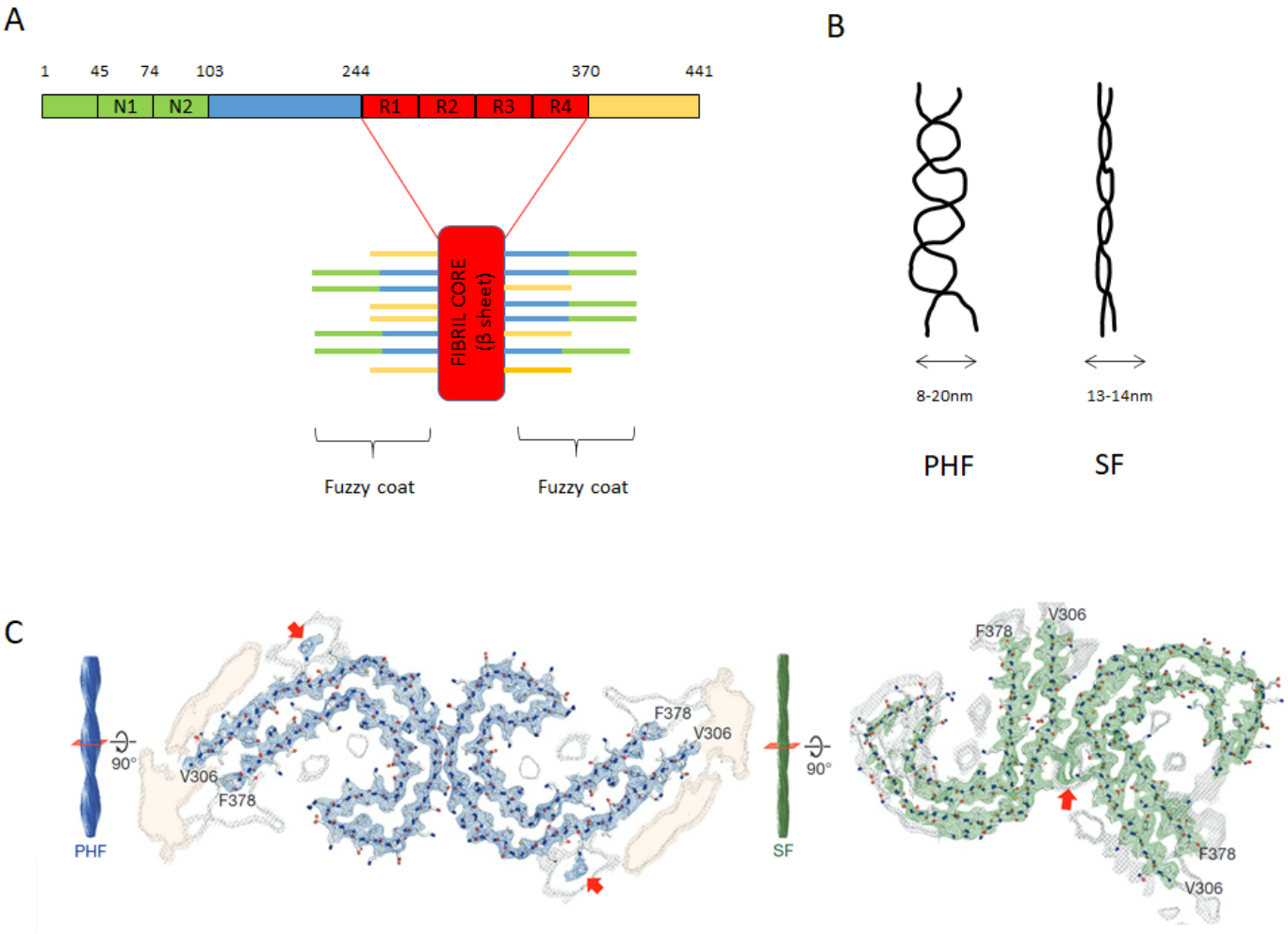


Fig. 4.1 Tau filaments. A) The longest tau isoform consists of 441 amino acids (AA) and contains 4 repeat domains (AA 244-370). In fibrillar tau aggregates the repeat domains are structured into βstrands forming the fibril core whilst the N and C termini protrude forming the 'fuzzy coat'. B) Hyperphosphorylated tau organised as paired helical filaments (PHF) and straight filaments (SF). C) Cryo-EM structure and fitted protein sequence of PHFs and SFs as seen in cross-section taken from Fitzpatrick et al. (2017).

presence within tau tangles has functional implications for disease pathology. Ubiquitin is one of the first non-cytosolic proteins to have been identified as being a structural component of tau tangles in a number of tauopathies (Lennox et al., 1988; Perry et al., 1987). Ubiquitin, so called due to its ubiquitous expression, serves multiple functions including as a cofactor for cytoplasmic proteolysis of abnormal or short lived proteins (Ristic et al., 2014) and its presence in disease could reflect its inability to degrade abnormally folded tau. The dysfunction of the ubiquitin proteasome degradation system is thought to contribute to development of tau pathology (Oddo, 2008). It is important to note that immunohistochemical analysis of tau tangles in PSP show no immunoreactivity for ubiquitin (Lennox et al., 1988) suggesting that although it may be contributing to the mechanism of disease, it is not a key component of all tau tangles. Ubiquitin is also present in other neurodegenerative conditions likely to reflect a common mechanism across several disorders rather than one unique to tauopathies as it is a target for proteasome or trafficking which are common processes (Lennox et al., 1988; Neumann et al., 2006; Spillantini et al., 1998). Other proteins that have been identified as associating with tau in tangles include the oestrogen receptor (Wang et al., 2016), and 14-3-3ζ (Qureshi et al., 2013). Loss of oestrogen signalling has been shown to negatively impact cognition with loss of neuroprotection against glutamate and amyloid (Daniel, 2006; Simpkins et al., 2012; Wang et al., 2016) whilst 14-3-3ζ has been shown to actively promote tau phosphorylation and aggregation (Hashiguchi et al., 2000; Sluchanko and Gusev, 2011). These studies all support an active role for components of NFTs in the pathogenesis of disease, either through loss or gain of a toxic function. An understanding of the constituents of tau tangles is important if we are to understand how tau pathology develops.

The EP3 receptor mediates several functions of PGE2 within the central nervous

system, and has been shown to play a critical role in the acute inflammatory response (Goulet et al., 2004; Morimoto et al., 2014). Shi et al showed in a mouse model of familial Alzheimer's, the APPSwe-PS1δE9 (APPS) transgenic mouse, not only that EP3 is upregulated correlating with disease progression, but that it is also critical for transducing amyloidβ induced inflammatory responses (Shi et al., 2012a). EP3 deficient mice displayed a marked reduction in amyloid and inflammatory proteins, with deletion of only one allele being sufficient to reverse pathology (Shi et al., 2012a). Other studies have shown that PGE2-EP3 signalling in mice impairs hippocampal synaptic plasticity. In the same transgenic APPS mouse model, mentioned above, Maingret et al show that EP3 signalling is responsible for the impaired hippocampal long term potentiation (LTP) noted in these mice (Maingret et al., 2017). Administration of an EP3R agonist in young mice is also able to reproduce impaired LTP, something that is fully rescued on blockade of EP3 receptor signalling (Maingret et al., 2017). Although one must be careful when translating animal studies to humans, particularly involving the EP3 receptor as these are species specific isoforms (Schmid et al., 1995), the involvement of EP3 in the human condition has been shown based on its upregulation in post mortem brains of patients with mild cognitive impairment and Alzheimer's dementia (Shi et al., 2012a). If EP3Re is found to be a constituent of tau tangles, this might support a role for PGE2-EP3Re signalling as contributing to disease pathology in humans.

In this chapter we focus on a possible physical interaction between tau and EP3Re. Based on the incidental finding of the EP3Re antibody identifying neurofibrillary tangles in a control brain, we hypothesised that EP3Re is present in tau tangles in disease. To establish this, we first mapped the distribution of the receptor in a primary tauopathy, PSP, and in Alzheimer's disease. We then explored the interaction between EP3Re and tau through proximity ligation assays, immunoprecipitation and

electron microscopy and demonstrate that EP3Re interacts with tau under pathological conditions.

4.3 Materials and Methods

4.3.1 Antibodies

A full list of primary and secondary antibodies can be found in **Table 2.1** and **2.2**.

4.3.2 Human brain samples

Paraffin embedded and frozen sections from 5 AD and 5 PSP post mortem human brains were obtained by ethical-committee approved procedures from the Cambridge Brain Bank. Three serial sections from 11 different brain regions (midbrain, pons, medulla, basal ganglia, hypothalamus, hippocampus, cerebellum, frontal, temporal, occipital and parietal cortices) from each subject were used for this study. See **Table 2.3** for details on clinical characteristics on each subject. Sarkosyl insoluble extract from Pick's disease, Multi-system atrophy (MSA) and Dementia with Lewy Bodies (DLB), were kindly provided by the Goedert Lab (MRC-LMB, Cambridge). Frozen tissue from a Parkinson's disease (PD) brain was obtained from the Oxford brain bank by ethical committee approved procedures.

4.3.3 Mouse sections

Paraffin embedded sections from the brainstem and cerebellum of 2 P301S transgenic mice were kindly provided by Jack Brelstaff (Spillantini lab).

4.3.4 Establishing distribution of EP3Re in tauopathies

Immunohistochemistry was performed as described in **2.2.1** on 40 µm paraffin embedded sections from 3 AD and 3 PSP subjects using antibody directed against EP3Re diluted 1:250. The results were compared with those obtained in **Section 3.4.2**. Adjacent sections from regions associated with tau pathology in both PSP (midbrain, pons, medulla, and basal ganglia nuclei) and AD (neocortex and hippocampus) were taken and stained with EP3Re or AT8 antibodies to observe EP3Re distribution in relation to tau tangles. Sections from these regions from each subject were then double stained using primary antibodies AT100 and EP3Re, followed by HRP conjugated secondary antibodies and developed in the peroxidase substrates 3-3' diaminobenzidine (DAB) or Vector VIP (V-VIP) providing brown and purple staining respectively. Sections were also double stained with AT100 and EP3Re using fluorescent-conjugated secondary antibodies following treatment by auto-fluorescence eliminator as described in **2.2.11** to determine co-localisation. In sections taken from AD, in addition to double-staining with AT100 and EP3Re, double immunohistochemistry was also performed using anti-amyloid and anti-EP3Re antibodies. Western blotting was performed to quantify EP3Re expression in the neocortex and brainstem in 3 AD, 3 PSP and 3 control subjects. This was compared with AT8 expression in each of these regions.

4.3.5 Determining if EP3Re antibody identifies tau

To determine if EP3Re antibody recognises tau tangles, further immunohistochemistry was performed on sections obtained from the hindbrain of transgenic P301S mice (see **2.1.3**). P301S mice do not express EP3Re but do develop tau tangles. Adjacent brainstem and cerebellar sections from 2 P301S mice were stained with anti-EP3Re and AT8 antibodies as per the protocol described in **2.2.1**. Recombinant monomeric tau

was run on a western blot and probed using EP3Re and tau antibodies to determine if an epitope from monomeric tau is recognised by the EP3Re antibody. Finally, human fibrillar tau was extracted using sarkosyl detergent as described in **2.2.3** from frozen brain tissue from 3x Alzheimer's case. This was run on western blot and probed using EP3Re antibody with and without EP3Re peptide preadsorbtion, as described in **2.2.2**.

4.3.6 Determining the presence of EP3Re in fibrillary tau extract

Tau fibrils were extracted from 3 AD and 2 PSP brains using sarkosyl detergent as described in **2.2.3**. Some extracts from each subject underwent further treatment with guanidinium hydrochloride and alkaline phosphatase to unravel and dephosphorylate the tau contained in the filaments as described in **2.2.4**. Western blotting was performed on all lysates which were probed for the presence of EP3Re.

4.3.7 Proximity ligation assay between EP3Re and tau

Proximity ligation assays were performed as described in **2.2.9** on paraffin embedded sections from 3 AD, 3 PSP and 3 age matched control subjects.

4.3.8 Immunoprecipitation of tau in Alzheimer's

Fresh frozen tissue from the frontal cortex and hippocampus of 3 AD patients and age matched controls was homogenised in RIPA and tau immunoprecipitated from the lysate (see section **2.2.6** for detailed protocol). This was then run on a western blot and probed for the presence of EP3Re. Sarkosyl detergent as described in **2.2.3** was then used to extract tau fibrils from the frontal cortex and hippocampus of 3 AD subjects and age matched controls. EP3Re was immunoprecipitated from the sarkosyl

insoluble extract, as well as the supernatant, which is enriched with soluble tau in AD subjects. The immunoprecipitate was then run on a western blot and probed for the presence of tau.

4.3.9 Immunogold negative stain electron microscopy of tau fibrils

Tau fibrils were extracted from 3 AD, 2 PSP and 1 Picks disease patient using sarkosyl as described in **2.2.3**. Extracts were then used for immunogold electron microscopy and stained with primary antibody directed against EP3Re and secondary antibody conjugated to gold particles. Some of the extracts from AD subjects were treated with pronase, to eliminate the external fuzzy coat from the filaments, prior to application of the primary antibody. These were then visualised using an electron microscope. To determine the specificity of any interaction seen between EP3Re and tau fibrils, extracts containing alpha-synuclein inclusions from subjects with PD, MSA and DLB were also immunogold labelled with EP3Re antibody and visualised under the electron microscope. For full details of immunogold electron microscopy protocol please see **2.2.7**.

Immunolabelling of extracted tau fibrils from 2 AD subjects was performed with the assistance of Ben Falcon and Manual Schweighauser (Goedert Lab, LMB, University of Cambridge). Immunogold labelling and images of extracted tau fibrils from 1 AD, 2 PSP, 1 Pick's disease and all alpha-synuclein fibrils were performed by Mehtap Bacioglu, (Spillantini lab).

4.3.10 Statistics

Analyses of differences in EP3Re and phospho-tau (AT8) expression across control subjects, Alzheimer's and PSP was performed using two way ANOVA with multiple comparisons. Statistical significance was considered at $p<0.05$. Correlation between EP3Re protein expression and phospho-tau (AT8) was calculated using Pearson's correlation. Statistical significance was considered at $p<0.05$.

4.4 Results

4.4.1 The EP3Re associates with neurons containing structures similar to tau aggregates in control brain

In examining the distribution of EP3Re in control brain it was noted that in some hippocampal sections the antibody identified neurons containing structures similar in morphology to neurofibrillary tangles (NFTs) (Fig. 4.2). With this unexpected finding, we needed to ensure the antibody was not cross reacting with a tau epitope causing non-specific binding.

4.4.2 The EP3Re antibody does not recognise tau

In order to establish that the staining results observed in Fig. 4.2 were not an artefact due to non-specific binding of the EP3Re antibody to tau, paraffin embedded sections from 5 month-old P301S mice were stained with EP3Re and adjacent sections stained with the anti-tau phosphorylation dependent antibody AT8. P301S mice express human tau with the P301S mutation and develop extensive pathology in the brainstem and deep nuclei of the cerebellum by 5 months of age. As EP3Re is specific to higher primates, these provide a background expressing tau NFTs and no EP3Re. Results are

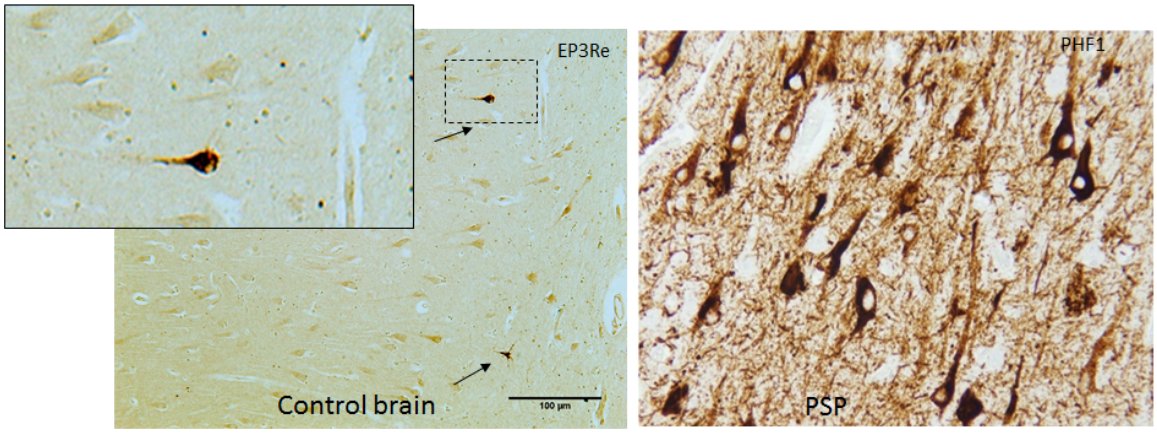


Fig. 4.2 The EP3Re antibody identifies neurons with neurofibrillary tangle-like morphology in control brain. Control brain hippocampal sections stained with the EP3Re antibody and a Progressive Supranuclear Palsy (PSP) brain section stained with the phosphorylation dependent anti-tau antibody, PHF1. The EP3Re antibody identifies neurons containing aggregates similar in appearance to tau tangles.

shown in figure 4.3A. No specific staining is seen with the EP3Re antibody while AT8 clearly identified the presence of NFTs in the P301S mouse sections. Based on these results and the staining conditions used the EP3Re antibody did not cross react with hyperphosphorylated tau.

To exclude non specific binding to monomeric tau I performed a western blot using human recombinant tau probed with the EP3Re antibody (Fig 4.3B). The EP3Re antibody failed to identify any of the tau isoforms. All 6 isoforms are seen when the same blot is probed using the total tau antibody (Dako tau). Therefore anti-EP3Re antibody does not recognise recombinant monomeric tau. Finally, fibrillar tau was extracted from an Alzheimer's brain using sarkosyl and run on a western blot. This was then probed with the EP3Re antibody preadsorbed with EP3Re peptide so as to saturate all EP3Re binding sites and determine whether the antibody could still see neurofibrillary tangles. No bands were seen. It seems therefore that following

preadsorption of EP3Re, no other independent epitopes in fibrillar tau are recognised by the antibody.

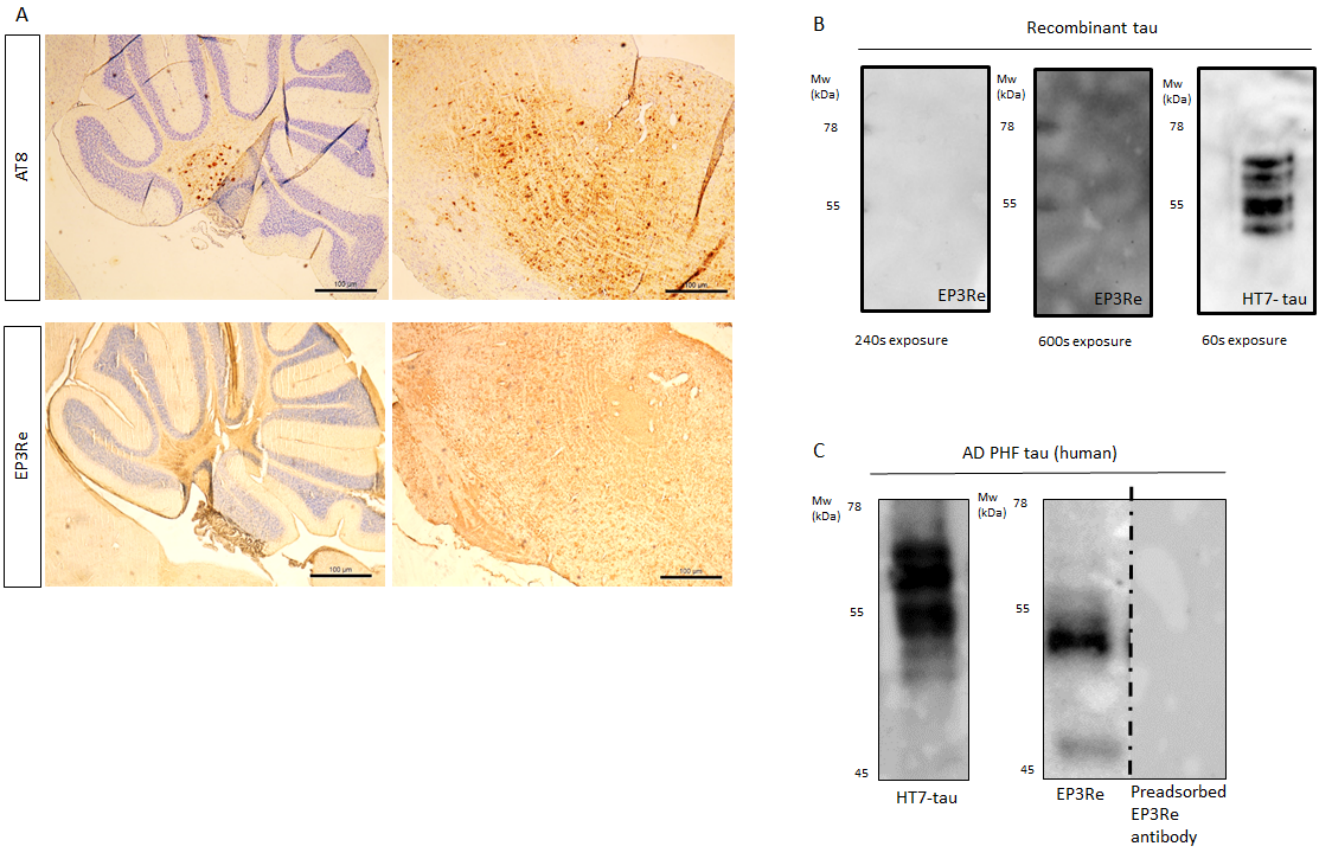


Fig. 4.3 EP3Re antibody does not recognise tau A) Sections taken from the cerebellum and brainstem of 5 month old transgenic P301S mice. Adjacent sections have been taken from each mouse. In the upper panel, sections are stained with the phosphorylation dependent tau antibody AT8. The presence of tau aggregates is clearly visible. These tau aggregates are not stained by the EP3Re antibody as shown in the lower panel indicating that EP3Re antibody does not recognise tau tangles in the absence of the receptor. n=2 B) Recombinant human tau (Sigma-Aldrich 6 human isoforms expressed in E.Coli) probed with EP3Re antibody then stripped and re-probed with tau antibody (Dako). Despite the long exposure no bands can be seen when the blot is probed with the EP3Re antibody. All 6 tau isoforms are clearly seen when probed with anti-tau antibody (HT7). The EP3Re antibody does not recognise recombinant human tau C) Western blot of sarkosyl extracts from Alzheimer's disease. The left panel shows the sarkosyl extract probed for the presence of tau (HT7). Panel on the right shows the extract probed by the EP3Re antibody before (middle panel) and after (far right panel) preadsorption with the EP3Re peptide. No bands are seen following preadsorption of the antibody. A band corresponding to the EP3Re receptor is present around 45kDa. Some protein bands are stained by the EP3Re antibody below 55kDa and one just above. These bands do not appear to colocalise with the 60, 64 and 68 kDa bands characteristic of tau from AD brain.

4.4.3 Distribution of EP3Re in Tauopathies

To investigate the apparent association between EP3Re and NFTs, I proceeded to establish the distribution of the receptor in tauopathies. Paraffin embedded sections from 11 different regions were obtained from 3 patients with a primary tauopathy, progressive supranuclear palsy (PSP) and 3 patients with a secondary tauopathy, Alzheimer's disease (AD) and stained with the EP3Re antibody. Pathologically, PSP is characterised by the presence of neuronal inclusions consisting of predominantly 4-repeat (4R) tau whilst AD has a mixture of 3-repeat (3R) and 4R tau. In both PSP and AD brains, the antibody identified neurons containing dense intracellular aggregates similar to NFTs suggesting an association between EP3Re and NFTs that is independent of tau isoform composition.

4.4.3.1 EP3Re in PSP

Staining of sections from PSP cases showed a normal pattern of EP3Re expression as seen in control brains as described in Chapter 3. In addition to the characteristic punctate cytoplasmic staining in neurons, dense neuronal aggregates are also identified by EP3Re antibody. Within the neuropil, there also appears to be stained structures consistent with neuropil threads. Given the similarity in appearance to NFTs, I decided to focus further analysis on regions associated with tau pathology in PSP, mainly the basal ganglia and brainstem nuclei. These were stained with EP3Re or the phospho-tau specific antibody, AT8. This can be seen in Fig 4.4. Aggregates identified by AT8 are very similar in morphology to those identified by EP3Re antibody supporting the initial observation that the structures being identified by EP3Re antibody are likely to be tau aggregates. To confirm possible co-localisation of EP3Re and tau within the neurons, I proceeded to perform double immunohistochemical staining using the phospho-tau antibody AT100 (DAB) and EP3Re (V-VIP). Initial results proved difficult

to interpret unequivocally. The density of the aggregates makes it difficult to visualise the different chromogens (Fig 4.5A).To address this I performed immunofluorescence staining. Double immunofluorescence staining is a common method used to determine colocalisation, however, due to the amount of autofluorescence in human tissue, it can be difficult to visualise the protein of interest. To reduce autofluorescence, sections were pre-treated using autofluorescence eliminator reagent (Millipore) and then stained with AT100 and EP3Re antibodies. Figure 4.5B shows double immunofluorescence staining confirms these are tau aggregates.

4.4.3.2 EP3Re in Alzheimer's dementia

Similarly to PSP, also in AD patients EP3Re has a distribution similar to control subjects (Fig 4.6). Neurons containing dense aggregates similar to neurofibrillary tangles were again stained by the anti-EP3Re antibody. As with PSP, sections from regions strongly associated with tau pathology in AD, the cortex and the hippocampus, were stained with the phospho-dependent anti-tau specific antibody AT8 (Fig 4.7). Neurons identified by AT8 were similar in appearance to some of those identified by EP3Re antibody further supporting that EP3Re could be associated with tau aggregates. Double immunofluorescence staining with antibodies directed against EP3Re and phosphorylated tau (AT100) suggest that also in AD, like PSP, the two proteins colocalise (Fig 4.8). Alongside intracellular NFTs, AD is also characterised by the presence of extracellular amyloid plaques. Sections from each brain region were then double stained with antibodies directed against beta-amyloid and EP3Re (Fig 4.8). Unlike the intracellular tau aggregates, there was no colocalisation of beta-amyloid and EP3Re in amyloid plaques.

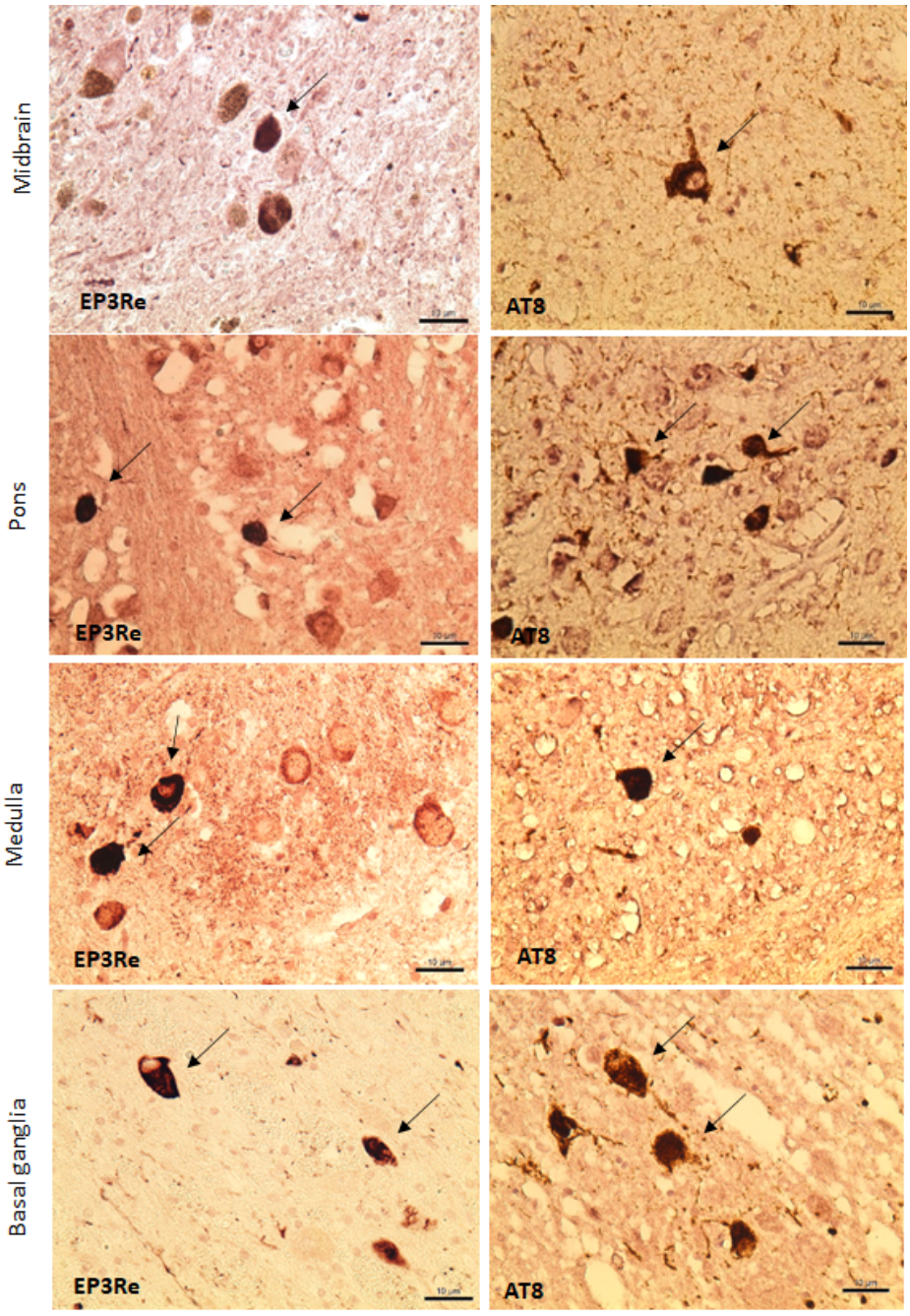


Fig. 4.4 EP3Re and Tau in Progressive Supranuclear Palsy. Sections shown have been taken from the brainstem, basal ganglia and cerebellum where extensive tau pathology can be found in PSP. From each region a section was stained with either EP3Re antibody (right panel) or AT8 (left panel) to identify tau pathology. The distribution of the receptor remains the same as control cases but in addition to the normal cytoplasmic staining observed, neurons containing dense aggregates (black arrows) are also identified by EP3Re antibody. These aggregates are similar in appearance to tau aggregates identified by AT8. n=3. Images shown are representative of the staining observed across all 3 patients.

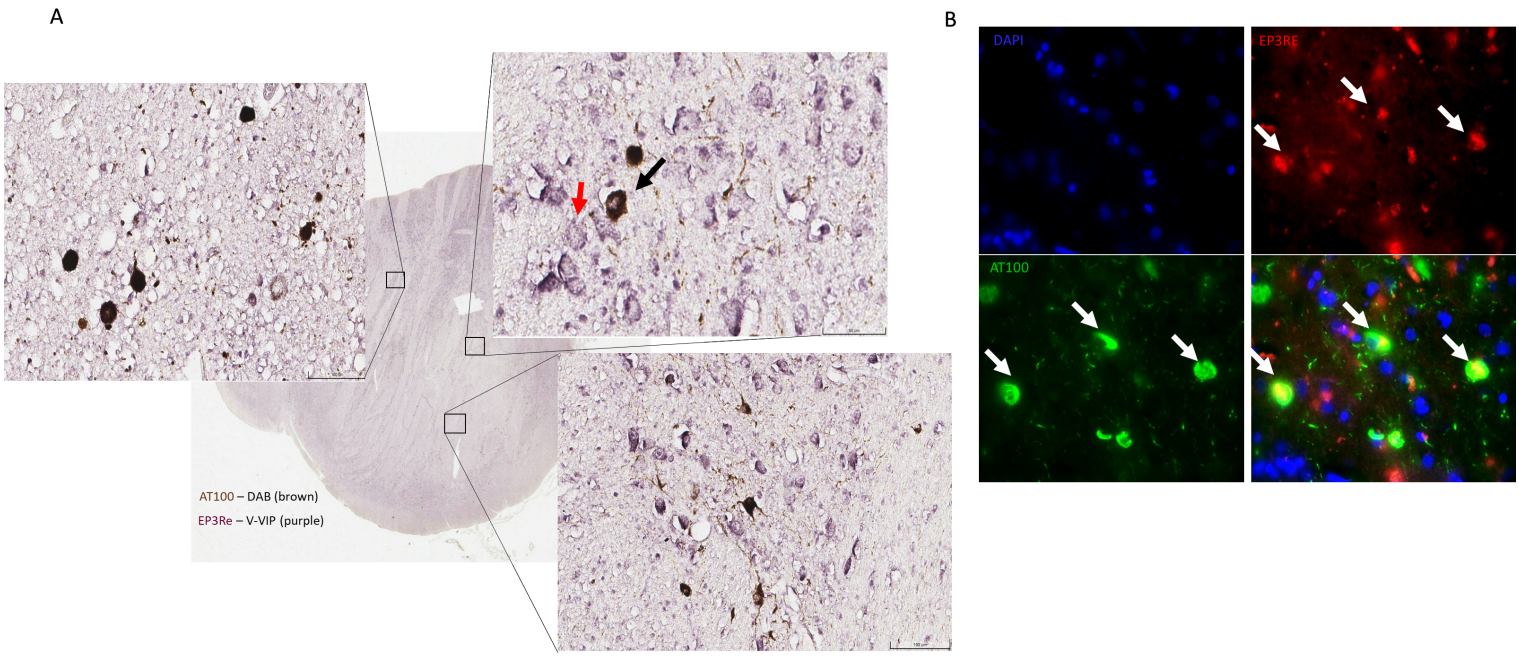


Fig. 4.5 EP3Re associates with tau aggregates in Progressive Supranuclear Palsy. A) Medulla section from a PSP patient double stained with the phospho-tau antibody AT100 and anti-EP3Re. EP3Re positive AT00 negative cells can be seen (red arrow). The density of AT100 positive inclusions (black arrow) make it is difficult to determine if the aggregate is also EP3Re positive. There is also significant background staining. B) Double immunofluorescence staining of PSP sections show EP3Re positive aggregates are tau tangles as identified by AT100.

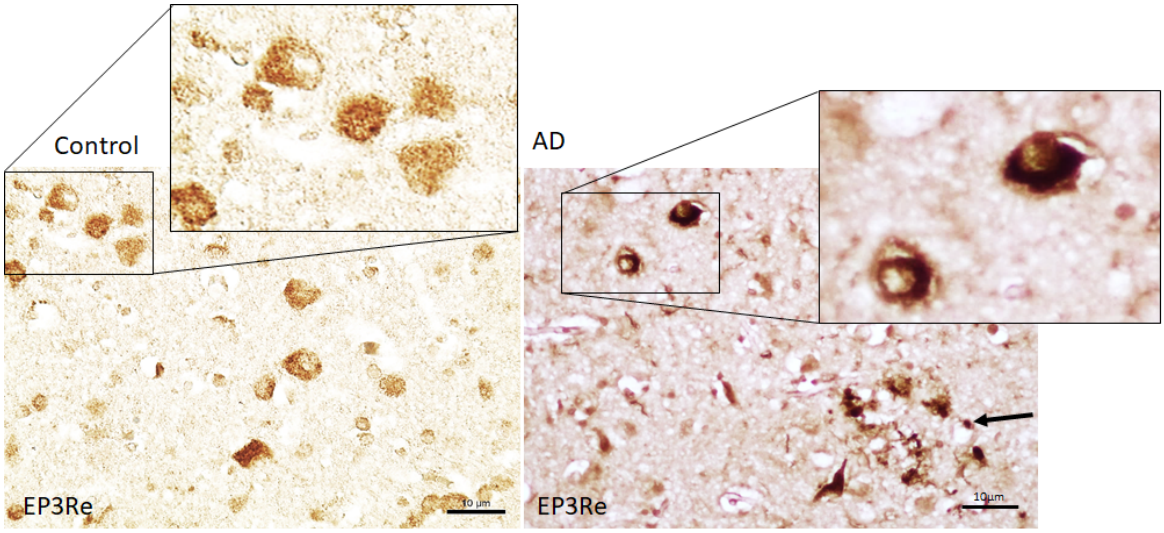


Fig. 4.6 EP3Re in Alzheimer's dementia. EP3Re staining of sections taken from the parietal cortex of an AD patient and age matched control. EP3Re distribution is the same in control and AD cases but in AD sections the antibody also identifies neurofibrillary tangles. EP3Re positive staining is noted in a neuritic plaque (black arrow). n=3. Images shown are representative of staining seen across all 3 subjects.

4.4.3.3 EP3Re protein expression is increased in regions associated with tau pathology

In order to contrast EP3Re expression levels between control and disease brains, brain lysate from the brainstem and frontal cortex, were obtained from AD, PSP and age matched controls, samples were run on a gel and immunoblotted with the anti-EP3Re antibody. This study was performed with tissue from brain regions associated with tau pathology in AD and PSP. The blot was also probed with the phospho-tau antibody AT8. There appears to be a correlation between AT8 and EP3Re (R^2=0.6, $p<0.005$) suggesting that in regions associated with high levels of phosphorylated tau there is an increase in EP3Re protein expression (Fig. 4.9).

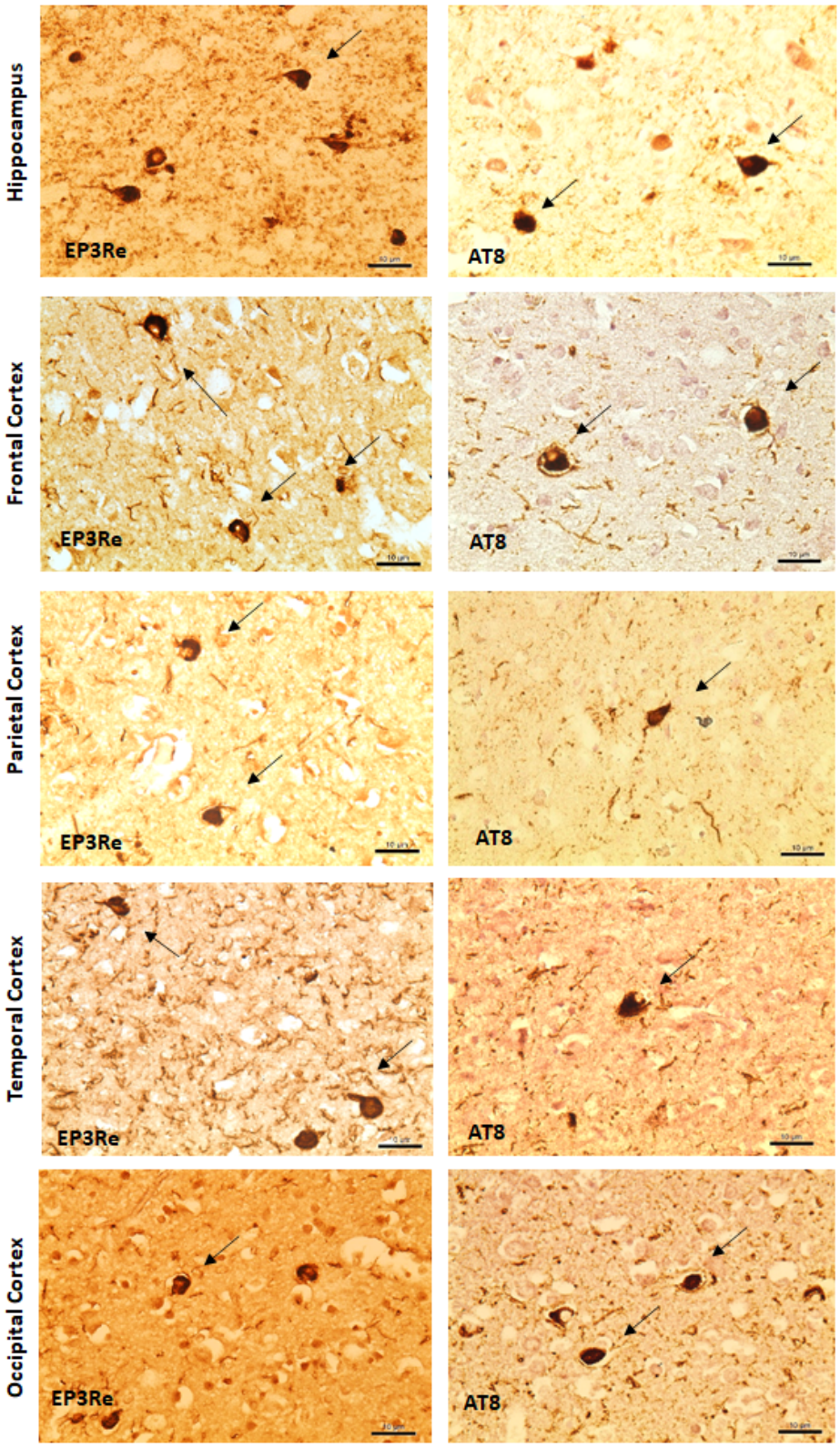


Fig. 4.7 EP3Re and Tau in Alzheimer's disease Sections taken from the neocortex and hippocampus from AD brains. Sections from each region have been stained with either EP3Re (left panel) or AT8 (right panel). Neurofibrillary tangles (black arrows) identified by EP3Re antibody are similar in appearance to AT8 positive tangles. n=3. Images representative of staining seen across all 3 subjects.

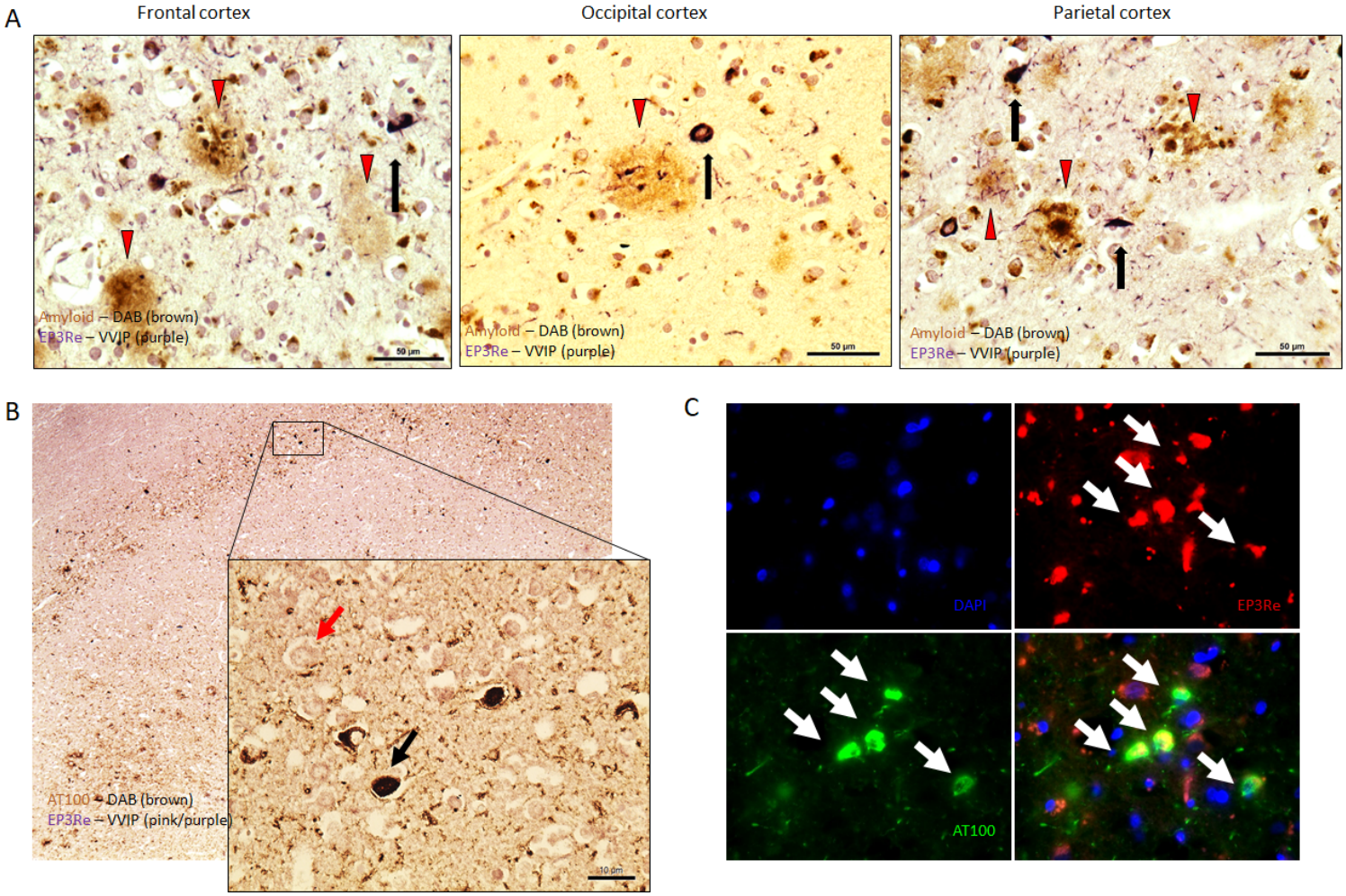


Fig. 4.8 EP3Re co-localises with tau tangles but not amyloid in Alzheimer's. A) Neocortical sections from AD subjects stained with EP3Re (VVIP) and βamyloid (DAB). Extracellular amyloid plaques (red arrowheads) do not show any EP3Re immunoreactivity. EP3Re positive neurofibrillary tangles can also be seen (black arrows). B) AD sections stained with AT100 and EP3Re antibodies. Double immunohistochemistry is difficult to interpret due to density of AT100 positive aggregates (black arrow). EP3Re positive AT100 negative cells are also seen (red arrows). C) Double immunofluorescence staining of AD sections with AT100 and EP3Re antibodies. AT00 positive neurons co-localise with EP3Re (white arrows).

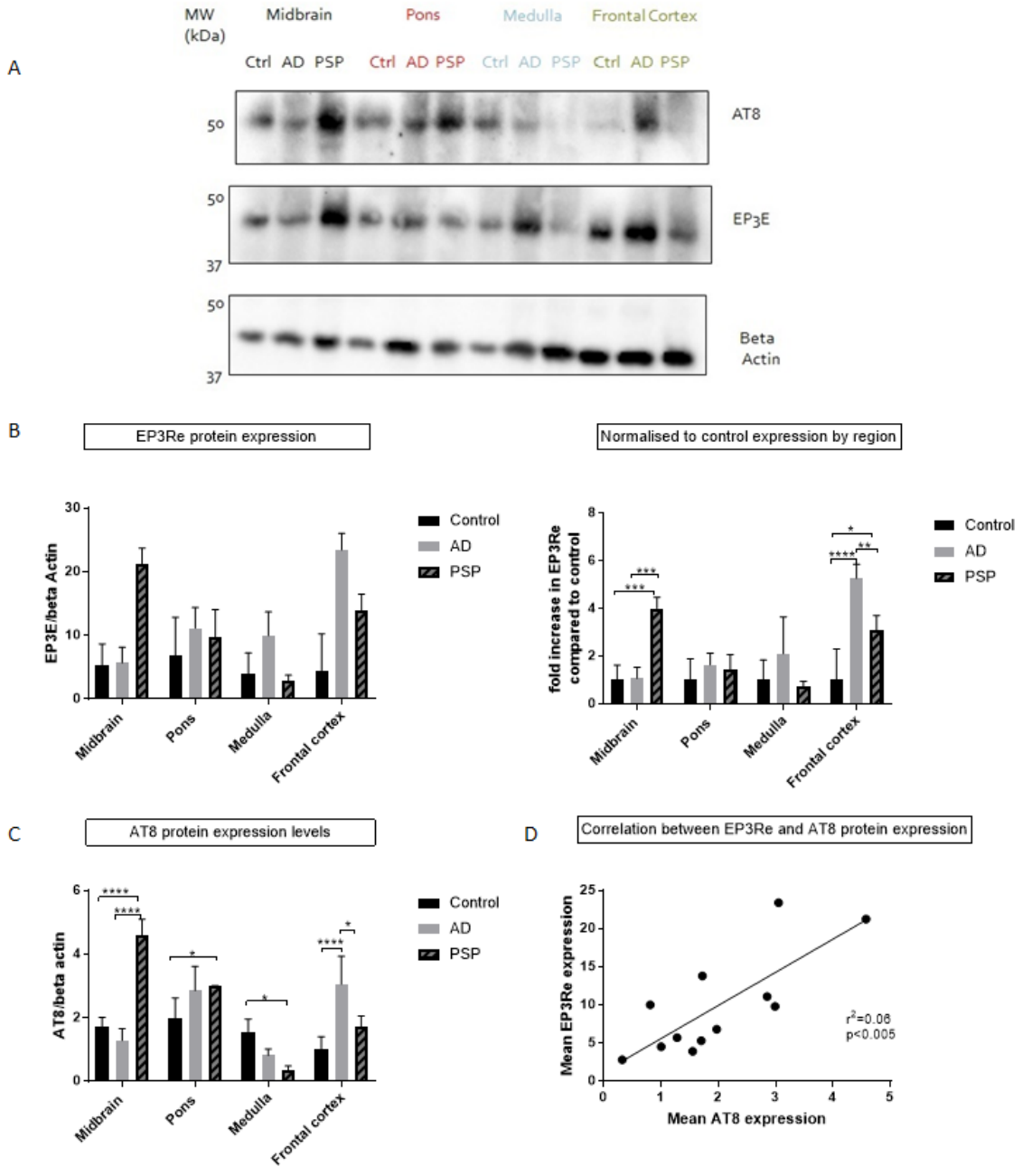

A
MW (kDa)
Midbrain
Pons
Medulla
Frontal Cortex
Ctrl AD PSP Ctrl AD PSP Ctrl AD PSP Ctrl AD PSP
50
AT8
50
37
EP3E
50
37
Beta Actin
B
EP3Re protein expression
EP3E/beta Actin
Control
AD
PSP
Midbrain
Pons
Medulla
Frontal cortex
Normalised to control expression by region
fold increase in EP3Re compared to control
C
AT8 protein expression levels
AT8/beta actin
D
Correlation between EP3Re and AT8 protein expression
Mean EP3Re expression
Mean AT8 expression
r²=0.06
p<0.005

Fig. 4.9 EP3Re expression correlates with levels of tau phosphorylation. A) Immunoblot showing EP3Re and AT8 levels in the brainstem and frontal cortex of a control, AD and PSP patient. B) EP3Re expression across AD, PSP and control brains in the brainstem and frontal cortex. The graph on the right shows EP3Re levels expressed as a fold increase in comparison to control EP3Re expression by region. Two way ANOVA performing multiple comparisons by region demonstrates significant differences in EP3Re levels in the midbrain and frontal cortex between groups. EP3Re is significantly elevated in the midbrain in PSP and frontal cortex in AD. These are both regions known to exhibit tau pathology in disease. C) AT8 (phosphorylated tau) levels across AD, PSP and control brain in the brainstem and frontal cortex. Analysis demonstrates differences in AT8 expression across regions within the different groups with higher levels of tau phosphorylation in brainstem regions in PSP when compared to control and AD, and higher levels of tau phosphorylation in the frontal cortex of AD patients. D) There is a strong correlation between EP3Re expression and levels of phospho-tau as identified by AT8, R^2=0.6, p<0.005. Results shown representative of 3 technical replicates of 1 AD, 1 PSP and 1 Control subject. AD - Alzheimer's disease, PSP - Progressive supranuclear palsy, Ctrl - control.

4.4.4 EP3Re is present in sarkosyl extract from AD and PSP brains

To further investigate the extent to which the EP3Re receptor associates with hyperphosphorylated tau, I proceeded to establish if the receptor was present in brain lysate containing insoluble filamentous-tau from diseased brains. I used sarkosyl extraction to obtain aggregates from 2 AD and 1 PSP subject. The preparations were fractionated into soluble and insoluble tau extracts, with the insoluble extracts containing filamentous-tau. The insoluble tau, alongside the supernatant containing sarkosyl soluble proteins, was then run on a gel and a western blot performed using the anti-EP3Re antibody (Fig. 4.10A). The results show that EP3Re is present in fibrillar tau extracted from both PSP and AD brains.

The next question I needed to answer was whether the EP3Re receptor was an intrinsic part of the tau fibril or if the association was only present when tau had undergone a conformational change secondary to hyperphosphorylation. To answer

this, I used guanidine isothiocyanate to disaggregate the filamentous-tau extracted using sarkosyl from frontal cortex of AD subjects and treated the samples with alkaline phosphatase to remove the phosphate groups. The samples were then probed for the presence of EP3Re. As figure 4.10B shows, the receptor is present in both the sarkosyl extract (as previously demonstrated) and in the extract treated with guanidinium and alkaline phosphatase, suggesting that in AD brains, the EP3Re receptor is intrinsically associated with the fibril. The EP3Re receptor bands appear distinct from the tau bands reducing the possibility of antibody cross-reactivity.

When probing the extracts containing fibrillar tau with the EP3Re antibody, it was noted that the antibody recognises a band of around 55kDa, which is a higher molecular weight than that expected for the receptor of 46kDa. It was important to investigate whether the band on the blot was due to non-specific binding of the antibody or if this corresponded to an oligomer of the receptor. Adding diothiothrietol (DTT) to the extract prior to running it on the gel and blotting is a method described to dissociate oligomers (Copani et al. 2000). By adding 20mM of DTT, I was able to eliminate the 55kDa band suggesting that it was an oligomer of the receptor due to disulphide bond formation (Figure 4.10C).

4.4.5 EP3Re interacts with tau in Tauopathies but not control brains

Having established the presence of the EP3Re receptor in filamentous tau, I wanted to explore the extent of this interaction. Paraffin embedded sections were taken from AD, PSP and age matched controls, probed for the presence of EP3Re and tau, and proximity ligation assays performed to determine the proximity of the two proteins. A strong signal can be seen in disease brains which is absent in sections from control suggesting

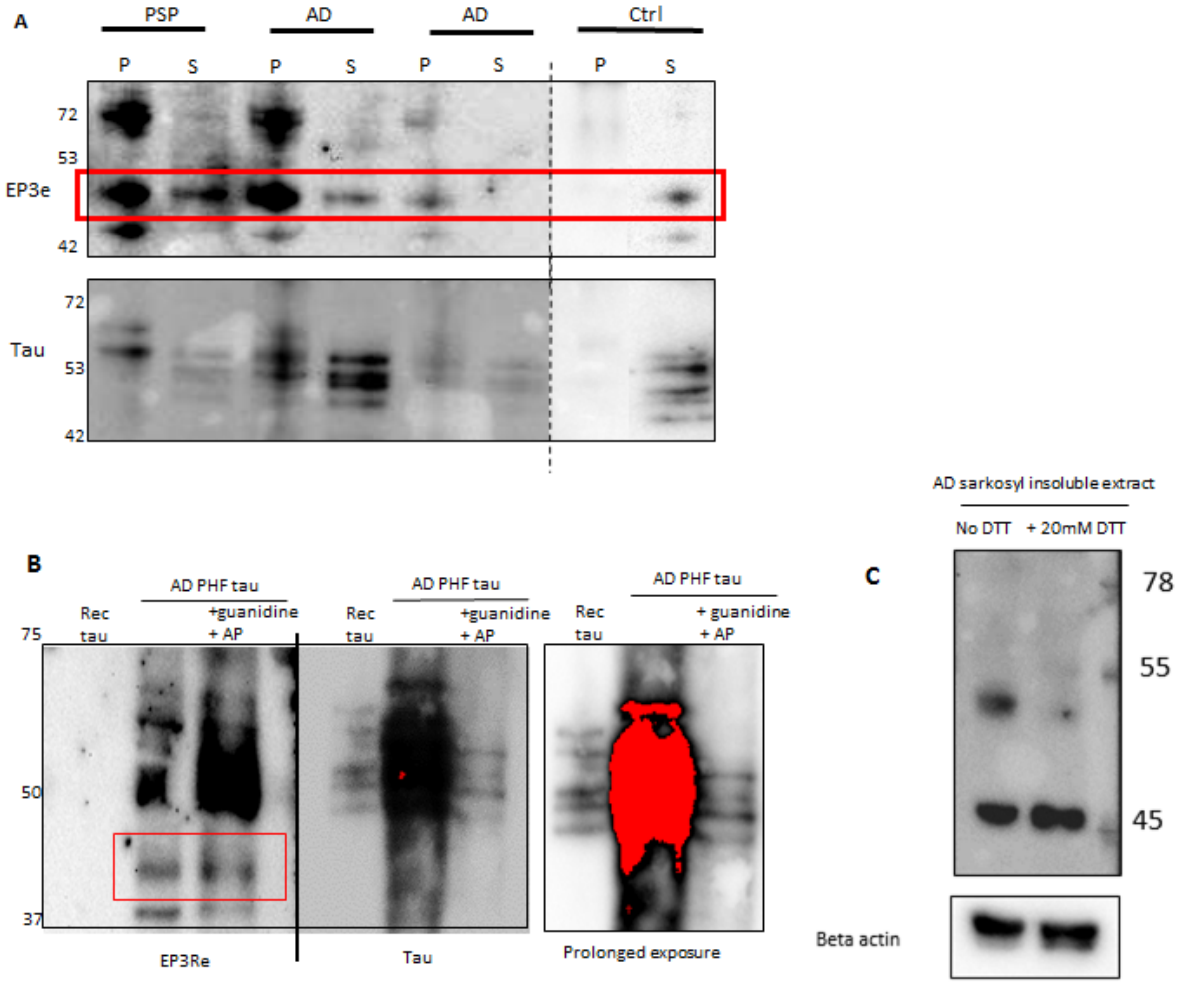


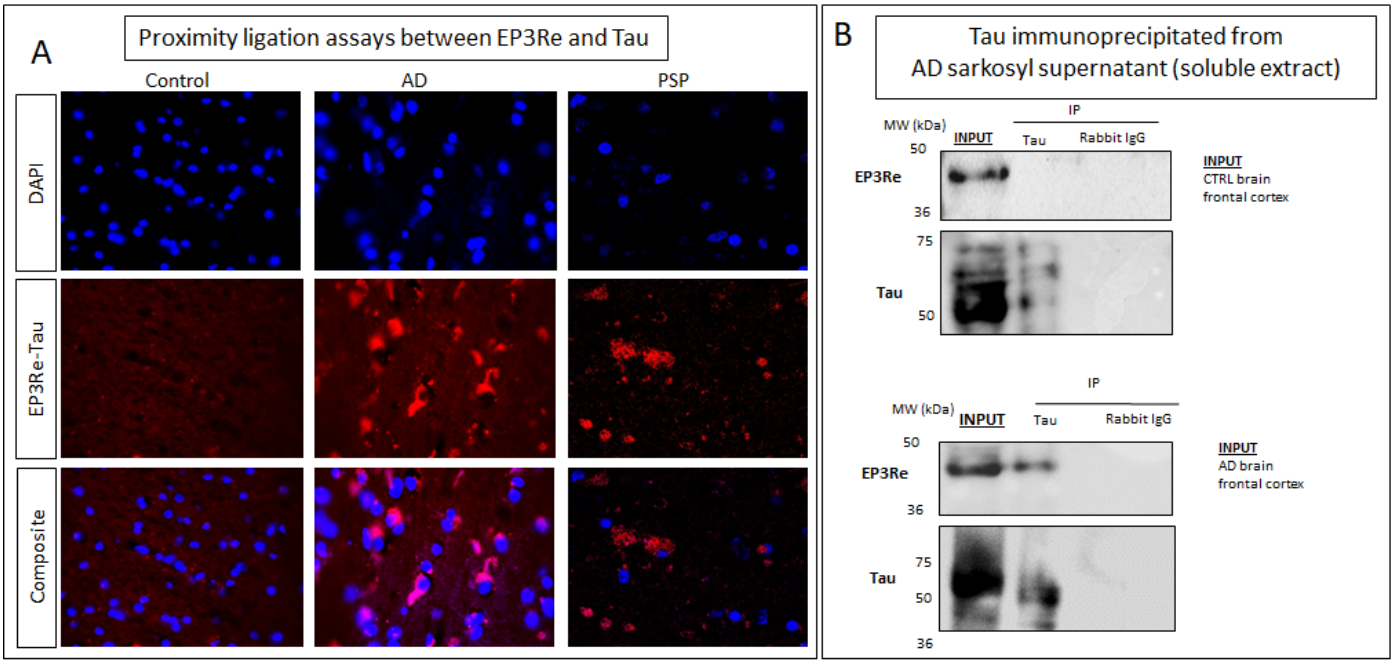
Fig. 4.10 EP3Re is present in tau fibrillar extract from AD and PSP A) Immunoblot of AD and PSP brain sarkosyl extract probed with antibodies directed against human tau (HT7) and EP3Re. With sarkosyl extraction the protein is separated into soluble and insoluble components, with the latter containing PHF-tau. Both soluble (S) and insoluble (P) components were run on the blot and probed for the presence of EP3Re. The receptor is present in both extracts (expected weight of 46kDa highlighted by red box). Representative blot of 5 independent experiments. B) The sarkosyl extract from AD brain (PHF tau) is treated with guanidinium to unravel the filaments and dephosphorylated with alkaline phosphatase. The samples probed with the anti- tau antibody (Dako) show that the extract has been successfully dephosphorylated given the downward shift in the bands when compared to the sarkosyl extract with no treatment. The immunoblot shows that EP3Re remains present in both the sarkosyl extract and the untangled and dephosphorylated extract (boxed bands). C) A heavier molecular weight band just below 55kDa is present when sarkosyl insoluble extract from AD and PSP subjects is probed for EP3Re, as shown in A and B. This band disappears following the addition of 20mM DTT to the sarkosyl extract suggesting that it corresponds to an oligomeric form of the EP3Re receptor. AD - Alzheimer's dementia, PSP - Progressive supranuclear palsy, AP - Alkaline phosphatase, DTT - dithiothreitol, PHF - Paired helical filaments, Rec tau - Recombinant tau.

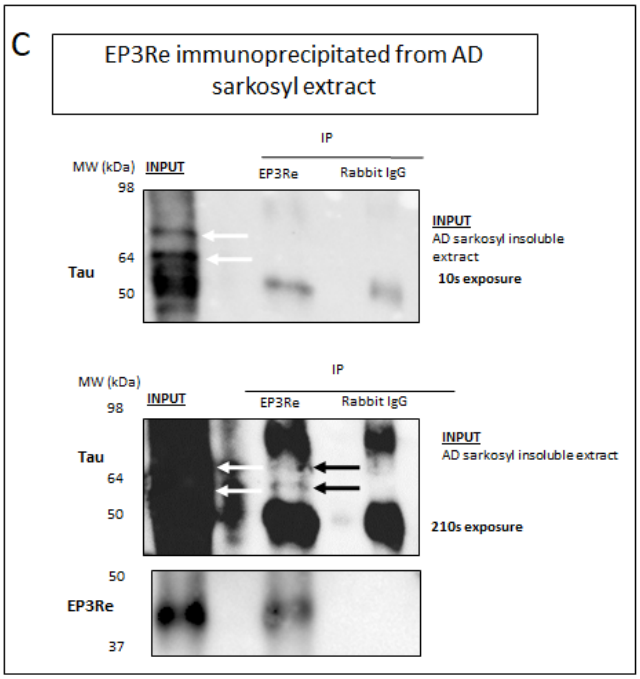
a close interaction between EP3Re and tau but only in tauopathies (Fig 4.11A).

I then went on to immunoprecipitate EP3Re from whole brain lysate from the frontal cortex extracted from Alzheimer's brains and age matched controls. The precipitated material was run on a western blot and probed for the presence of EP3Re as shown in figure 4.11B. The EP3Re receptor is immunoprecipitated alongside tau in Alzheimer's brain but not in extract from control brain suggesting a close interaction between EP3Re and tau present only in disease, further supporting the findings from the proximity ligation assay. The lysate was then separated into sarkosyl insoluble (filamentous tau) and the supernatant, containing hyperphosphorylated but not aggregated tau. In the immunoprecipitate from the sarkosyl insoluble extract bands can be seen around 64kDa which are absent from the control lane suggesting tau co-immunoprecipitates alongside EP3Re in the sarkosyl insoluble extract. The presence of heavy and light chain IgG bands make it difficult to determine if any further bands are present (Fig 4.11C). In the supernatant of the sarkosyl soluble extract no clear bands can be seen to suggest tau has been immunoprecipitated alongside EP3Re. (Fig 4.11D).

4.4.6 EP3Re is present in tau fibrils

So far I have shown that EP3Re associates with neurofibrillary tangles and closely interacts with tau in disease but to determine true co-localisation, I need to directly visualise tau fibrils. Tau filaments were extracted using sarkozyl from the frontal cortex of 3 individuals with sporadic AD, 2 with PSP and 1 with Pick's disease, a tauopathy characterised by accumulation of 3 repeat (3R) tau inclusions. Fibrils were labelled with EP3Re antibody and gold nanoparticle conjugated secondary antibody. The resulting staining on tau filaments was visualised by electron microscopy. Figure 4.12 shows tau filaments are clearly decorated with EP3Re in all analysed tauopathy cases


A
Proximity ligation assays between EP3Re and Tau
Control
AD
PSP
DAPI
EP3Re-Tau
Composite
B
Tau immunoprecipitated from
AD sarkosyl supernatant (soluble extract)
MW (kDa)
INPUT
IP
Tau
Rabbit IgG
EP3Re
Tau
INPUT
CTRL brain
frontal cortex
INPUT
AD brain
frontal cortex


C
EP3Re immunoprecipitated from AD
sarkosyl extract
MW (kDa)
INPUT
IP
EP3Re
Rabbit IgG
Tau
EP3Re
INPUT
AD sarkosyl insoluble
extract
10s exposure
INPUT
AD sarkosyl insoluble extract
210s exposure

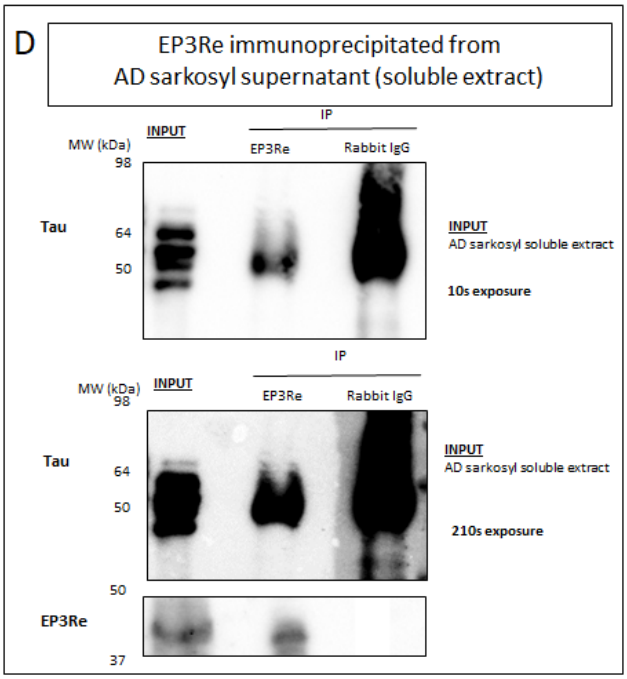

D
EP3Re immunoprecipitated from
AD sarkosyl supernatant (soluble extract)
MW (kDa)
INPUT
IP
EP3Re
Rabbit IgG
Tau
EP3Re
INPUT
AD sarkosyl soluble extract
10s exposure
INPUT
AD sarkosyl soluble extract
210s exposure

Fig. 4.11 Tau interacts with EP3Re in disease but not controls. A) PLA using EP3Re and HT7 (tau) antibodies performed on paraffin embedded sections obtained from AD, PSP and control brains. There is a strong signal in tauopathies that is absent in control brain sections suggesting a close interaction between EP3Re and tau in disease. n=3AD, 3PSP and 3 control. Representative image shown. B) Brain extract from frontal cortex of control and AD brains immunoprecipitated using anti-Tau HT7 antibody, probed for EP3Re. EP3Re is co-immunoprecipitated alongside tau in AD but not in control brain C) EP3Re immunoprecipitated from AD sarkosyl insoluble extract, containing filamentous tau, and probed for the presence of tau. Sarkosyl insoluble tau from AD brain runs as 4 bands at 62kDa, 64kDa, 68kDa and a lighter band is often seen at around 72kDa. Lower bands can also be seen in the blot which are likely to represent degradation products. Two bands are seen in the sarkosyl extract after prolonged exposure around 64kDa and 72kDa that co-immunoprecipitated alongside EP3Re (black arrows). IgG bands can be seen at around 50kDa (heavy chain) and 75kDa (combination of heavy and light chain). D) EP3Re immunoprecipitated from the supernatant fraction. No clear bands are seen, despite prolonged exposure, to suggest that tau co-immunoprecipitates with EP3Re in the supernatant.

showing that EP3Re co-localises and is a component of tau fibrils. In AD cases this can be seen with both paired helical filaments (PHFs) and straight filaments (SFs) showing the interaction is not conformation specific. The antibody directed against EP3Re fails to label tau filaments created from recombinant tau, lending further support to the specificity of the antibody. The presence of EP3Re in tau fibrils extracted from AD, PSP and Pick's disease shows this interaction is irrespective of tau isoform. Pronase treatment removing the fuzzy coat eliminates EP3Re suggesting EP3Re binds to tau in the fuzzy coat and not the pronase resistant core.

4.4.7 EP3Re associates specifically with tau and not with other protein aggregates associated with neurodegenerative disorders.

Having shown that EP3Re is associated with tau fibrils, I wanted to determine if this interaction is unique to tau or if it is an association that occurs with other aggregated protein. We know from figure 4.8 that EP3Re does not co-localise with amyloid

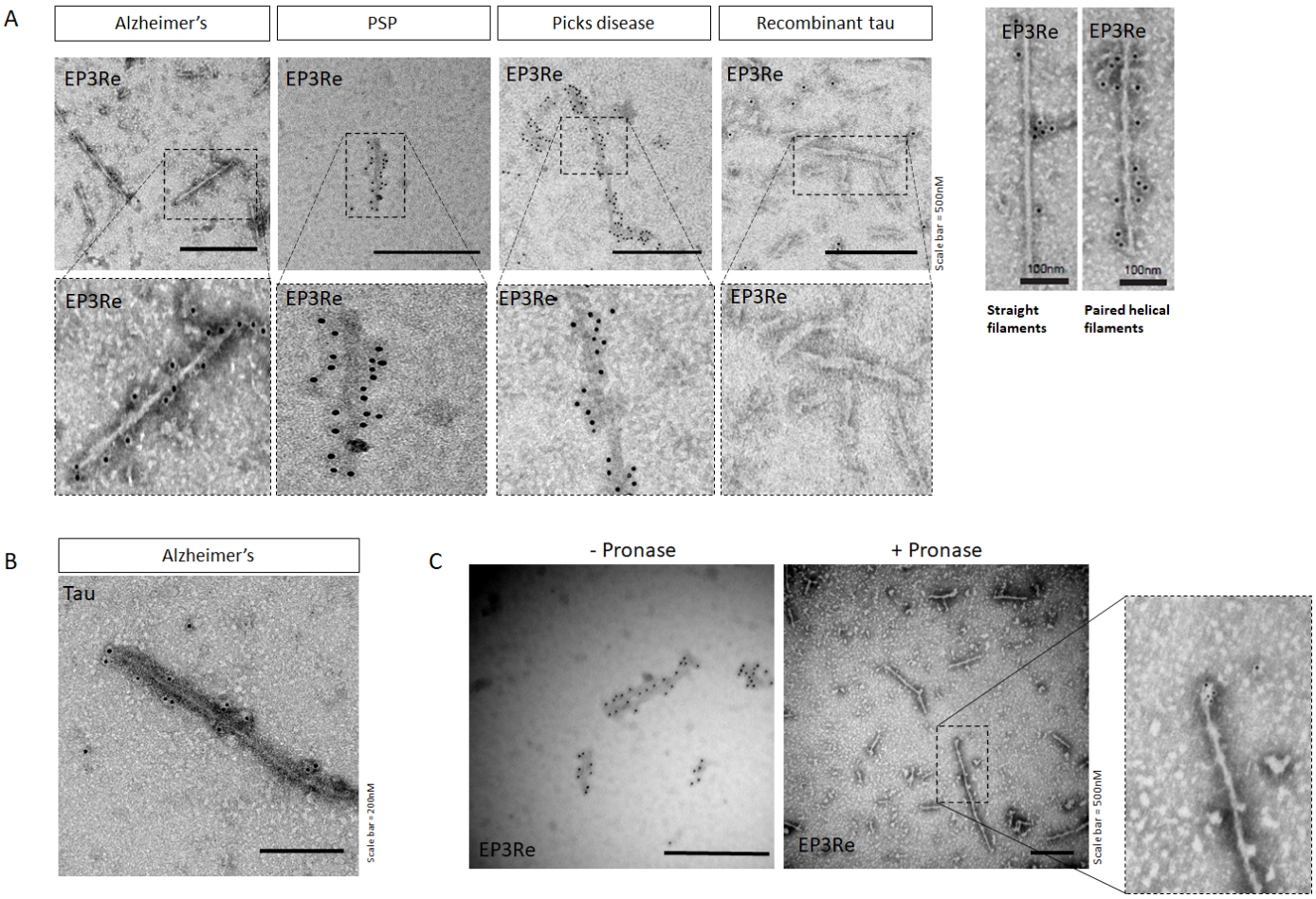


Fig. 4.12 EP3Re is present in tau fibrils. A) PHFs extracted from Alzheimer's dementia, PSP and Pick's disease negatively stained and labelled with primary EP3Re antibody and secondary gold particles. EP3Re can be seen decorating the fibrils in each disease. EP3Re is present in both straight filaments and paired helical filaments extracted from Alzheimer's brains. EP3Re is not present in fibrils made from recombinant tau. B) Fibrils extracted from Alzheimer's brain labelled with tau antibody. C) PHFs were treated with pronase stripping fibrils of the fuzzy coat leaving only the pronase resistant core. Pronase treatment removes EP3Re labelling suggesting that EP3Re is bound to tau within the fuzzy coat. n= 3 AD, 2 PSP and 1 Pick's disease

plaques. We also analysed disorders characterised by the accumulation of aggregates consisting of TAR-DNA-binding protein-43 (TDP-43) and alpha-synuclein. TDP-43 is a major component of inclusions found in sporadic and familial frontotemporal lobe degeneration with ubiquitin positive inclusions (FTLD-U) as well as most forms of amyotrophic lateral sclerosis. Sections from a patient with FTLDU were stained with both the TDP-43 antibody and EP3Re (Fig 4.13A). EP3Re fails to identify TDP-43 inclusions. Alpha-synuclein is the primary structural component of insoluble aggregates found in Parkinson's disease, Multiple system atrophy (MSA) and dementia with Lewy bodies (DLB). Paraffin embedded sections from a Parkinson's disease patient were stained with anti-alpha synuclein antibody, LB509, to identify alpha-synuclein aggregates, with adjacent sections stained with EP3Re antibody. Comparing the pattern of staining, it appears that there is no association between EP3Re and alpha-synuclein aggregates. Sections from a patient with MSA were then double stained with EP3Re and LB509. MSA presents with glial cytoplasmic inclusions consisting of alpha-synuclein. The results of immunohistochemistry are difficult to interpret due to the density of the alpha-synuclein inclusions and the identification of monomeric alpha-synuclein in EP3Re positive cells. Co-localistion between EP3Re and alpha-synuclein aggregates can not be excluded based on this immunohistochemistry study. To address this, we directly visualised alpha-synuclein fibrils extracted from a PD patient following labelling with EP3Re primary and gold labelled secondary antibody, using electron microscopy. EP3Re is not present in alpha-synuclein fibrils. (Fig 4.13D). This was repeated on alpha-synuclein fibrils extracted from MSA and DLB patients which showed no labelling of alpha-synuclein with EP3Re (not shown).

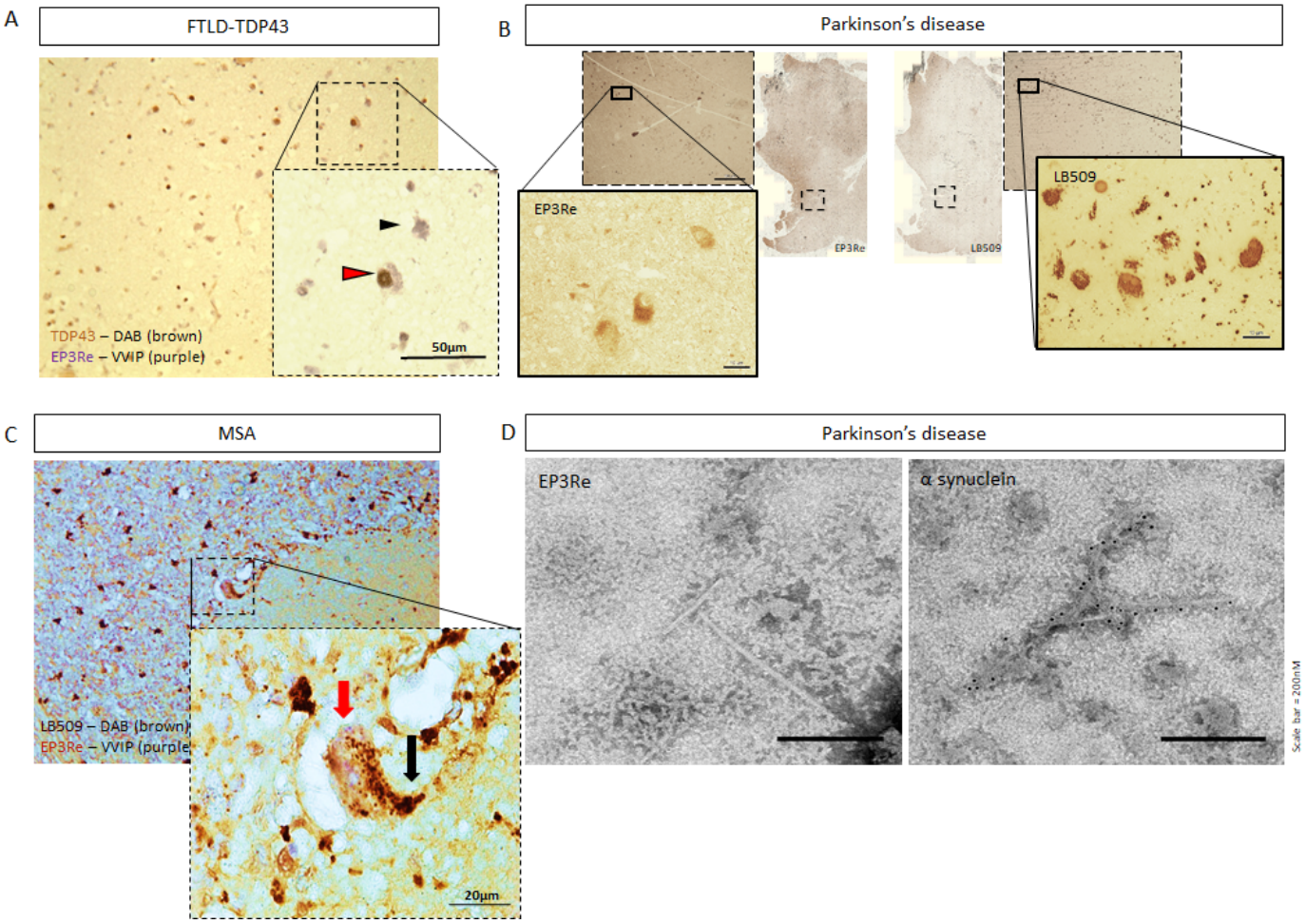


Fig. 4.13 EP3Re does not associate with other protein aggregates associated with neurodegeneration. A) FTLD-TDP43 section stained with TDP43 and EP3Re antibodies. There is no obvious co-localisation between TDP43 inclusions (red arrowhead) and EP3Re (black arrowhead). B) Adjacent sections from the basal ganglia of a PD patient stained with EP3Re antibody and LB509 (alpha-synuclein). Two different patterns of staining are seen in the same region between the two proteins. EP3Re antibody fails to identify Lewy bodies in Parkinson's sections. C) MSA section double stained with LB509 (alpha-synuclein) and EP3Re. The double staining is difficult to interpret. As shown, the EP3Re positive staining (red arrow) can be seen below dense alpha-synuclein inclusions (black arrow) in some neurons. The density of surrounding aggregates means co-localisation with EP3Re can not be determined. D) Electron microscopy of alpha synuclein fibrils stained with EP3Re antibody rules out EP3Re and alpha-synuclein co-localisation in Parkinson's disease. EM images of alpha-synuclein fibrils taken by Mehtap Baciolugu.

4.5 Discussion

This study investigates a possible link between EP3Re and tau in tauopathies and shows that EP3Re is increased in regions associated with tau pathology. EP3Re appears to be a previously unidentified component of tau tangles.

The distribution of EP3Re protein in tauopathies is similar to that of control brains with the exception that it identifies with tau aggregates demonstrating a strong correlation between EP3Re and phospho-tau protein expression. We know from previous studies, using an antibody that recognises all EP3R isoforms, that EP3R is upregulated in post mortem brains of patients with mild cognitive impairment and Alzheimer's disease (Shi et al., 2012a) and therefore this is not unexpected. What has previously not been described is a specific interaction between EP3Re and tau. Immunohistochemistry alone proved insufficient to show co-localisation due to the density of aggregates. This was overcome through the use of immunofluorescent staining as well as proximity ligation assays, immunoprecipitation and electron microscopy. In paraffin embedded sections we show through proximity ligation assays an interaction between EP3Re and tau in Alzheimer's disease and PSP but not in sections from control subjects. We obtained paired helical filaments (PHFs) from a number of Alzheimer's patients and in each case we found that EP3Re co-immunoprecipitated with tau indicating EP3Re is closely associated to tau in PHFs. This association was not present in control brain extract, containing monomeric tau. EP3Re fails to identify with other protein aggregates associated with neurodegenerative disorders examined as part of this study, such as amyloid, alpha-synuclein or TDP-43 as demonstrated by immunohistochemistry and immunogold electron microscopy. The specificity of this interaction suggests a possible role of EP3Re in tau pathology.

We also show, through electron microscopy studies, that EP3Re is present in tau tangles irrespective of the type of tau isoforms present in the filaments. EP3Re receptor was present in fibrils extracted from Pick's disease (3R tau), PSP (4R tau) and Alzheimer's dementia (combination of both 3R and 4R tau). Tau fibrils consist of a core, formed from short β strands, with unstructured tau terminal domains protruding from the core, known as the fuzzy coat (Wegmann et al., 2013). This coat can be stripped off by proteolytic digestion as demonstrated here with pronase. In this study we show removing the fuzzy coat with pronase, also eradicates EP3Re labelling of the fibrils suggesting the interaction between EP3Re and tau is within the fuzzy coat and not the pronase resistant core. We also show, through western blotting, that the interaction between EP3Re and tau remains present following unravelling and dephosphorylation suggesting EP3Re is not simply being sequestered by the fuzzy coat but there is an intrinsic interaction between the tau terminal domains and EP3Re. Prior to this study, ubiquitin is the only other non-cytosolic protein to have been identified as being bound to tau in NFTs. Although we have performed many experiments to exclude that our results represent a cross reactivity between the EP3Re antibody and tau, showing that the antibody fails to recognise tau in filaments made of recombinant tau or in mouse brain, only mass spectrometry of sarkosyl tau extract could definitely confirm the absence of cross reactivity and presence of EP3Re in the filaments.

Studies have shown the propensity of tau aggregates to form clusters is influenced by the charge of the N-terminal projection within the fuzzy coat (Wegmann et al., 2013). A more acidic environment providing a positive charge results in a filament more prone to forming clusters. Arachidonic acid, the precursor to PGE2 is known to rapidly promote tau fibrillisation *in vitro*, a finding that has been demonstrated for a

number of free fatty acids (Lim et al., 2014; Wilson and Binder, 1997). The presence of a PGE2 receptor within the N-terminal projections could reflect a mechanism by which PGE2 contributes to formation of tau fibrils by creating a more pro-aggregation environment. This raises the question of whether or not EP3Re remains functional if bound to tau within the cytoplasm.

As previously described EP3Re is a G-protein coupled receptor (GPCR). Tau has been known to sequester other GPCRs, such as the oestrogen receptor, resulting in their loss of function with subsequent clinical implications (Wang et al., 2016). EP3R receptors in the median preoptic area have been shown to be necessary for the febrile response with EP3R receptors in the hypothalamus playing an inhibitory role (Lazarus et al., 2007). Although the specific role of the EP3Re isoform has not been explored, if the receptor in tauopathies is bound to tau fibrils with subsequent loss of function, this could lead to deficits in thermoregulation. Thermoregulatory deficits have been shown to correlate with a rise in the incidence of Alzheimer's (Tournissac et al., 2017) with both *in vitro* and *in vivo* studies showing an increase in tau phosphorylation in relation to hypothermia (Bretteville et al., 2012; Whittington et al., 2010). Impaired thermoregulation due to dysfunctional EP3 signalling, could potentially be contributing or exacerbating tau pathology.

EP3Re, as a GPCR, is normally bound to the cell membrane. Signal transduction by GPCRs has traditionally thought to be uniquely confined to the plasma membrane but recent studies contradict this showing that internalised GPCRs can continue to signal (Godbole et al., 2017; Irannejad et al., 2013; Jensen et al., 2017; Pavlos and Friedman, 2017). GPCRs are able to initiate signalling from various subcellular locations with location specific responses (Irannejad et al., 2017). This opens the possibility that

instead of a loss of EP3Re function, the translocation of EP3Re from the membrane to within the cytoplasm results in normal receptor function or an altered function. This is something that will need to be further explored to determine the functional significance of this finding. Beyond the functional implications of this finding, the other question this raises is how the interaction occurs. How does a membrane bound receptor interact with tau within the cytoplasm? There is accumulating evidence to suggest that tau interacts with the plasma membrane (Maas et al., 2000; Pooler and Hanger, 2010; Pooler et al., 2012). The projection domain, located in the N-terminal, is thought to mediate this process, localising tau to the plasma membrane (Brandt et al., 1995). It has been suggested that the targeting of tau to the plasma membrane plays a role in cell signalling pathways and neurodevelopment (Pooler and Hanger, 2010). Once at the plasma membrane tau has been shown to interact with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor under ischaemic conditions (Cardona-Gomez et al., 2006) suggesting other membrane bound receptors could be targeted by tau at the plasma membrane, including EP3Re, when the neuron is under stress. Alternatively, tau could be interacting with EP3Re on the extracellular surface. Tau secretion has been observed in several models of tauopathies (Chai and et Al., 2012; Katsinelos and et Al., 2018; Sayas et al., 2019). However 90% of extracellular free tau is monomeric (Dujardin et al., 2014; Wang et al., 2017) and this study finds no interaction between monomeric tau and EP3Re. EP3Re could still interact with extracellular aggregated tau. Several *in vivo* and *in vitro* studies support the hypothesis that tau pathology propagates in a prion-like manner (Clavaguera et al., 2009, 2015; Sonawane and Chinnathambi, 2018). Mechanisms that have been proposed to explain intra-neural transfer of tau aggregates include being transported into the extracellular space in exosomes or through ectosomes (Asai et al., 2015; Dujardin et al., 2014; Wang et al., 2017), through endocytosis and macropinocytosis (Zeineddine and

Yerbury, 2015), through diffusion and receptor mediated uptake (Holmes et al., 2013; Takahashi et al., 2015). If, as we have shown, EP3Re interacts with tau aggregates, then this observed interaction could play a potential role in tau uptake and spreading through receptor mediated endocytosis. How and when the interaction occurs goes beyond the scope of this study but will need to be explored further to determine if and to what extent EP3Re could be contributing to disease pathology.

The main aim of this study was to explore if the incidental finding observed was a genuine co-localisation between EP3Re and tau tangles or an artefact. One of the biggest problems I encountered was the ability to accurately interpret immunohistochemistry. Double-immunohistochemistry is challenging to interpret. The density of aggregates make it difficult to distinguish the presence of more than one chromogen. Immunofluorescence was initially avoided as human brain tissue exhibits significant auto-fluorescence. This was eventually overcome through the use of commercially available auto-fluorescence eliminator.

These experiments, using proximity ligation assays, immunohistochemistry and electron microscopy, have shown that EP3Re interacts with tau fibrils. One of the limitations to the study is that we have not examined other isoforms of the receptor and therefore it is unclear if this interaction is unique to this isoform. The EP3 isoforms differ by their C-terminus and if the interaction between EP3Re and tau occurs within the cytoplasm, then it is possible, that only EP3Re binds to tau. However, if this interaction occurs with extra-cellular tau, and the interaction occurs at the extracellular N-terminus of EP3Re, then this sequence is shared by all EP3 receptors and I would not expect there to be a difference across all isoforms. The time limitations and lack of commercially available antibodies for each isoform have meant this is not something

that was fully explored but may have significant implications should the receptor prove to be contributing to tau pathology. Further mass spectrometry studies of sarkosyl insoluble preparations, and the detection in them of the EP3Re receptor, are needed to definitely rule out the possibility of a cross reaction between the anti-EP3Re antibody and aggregated tau.

Chapter 5

INVESTIGATING THE INTERACTION BETWEEN EP3RE AND TAU

5.1 Summary

In this chapter, I explore the functional relationship between EP3Re and tau. We hypothesised, based on the colocalisation of EP3Re and tau neurofibrillary tangles in tauopathies, that EP3Re is interacting with tau and contributing to tau pathology in disease. As tau hyperphosphorylation is seen as a key contributing factor to disease, I initially hypothesised that signalling through the receptor could be contributing to tau phosphorylation. To test this hypothesis I first stimulated EP3Re in SHSY5Y cells which express both endogenous EP3Re and tau and measured tau phosphorylation and kinase activation using western blotting in an attempt to outline a potential pathway and mechanism. I then went on to explore the function of the EP3Re receptor in hypothermia, a physiological condition that is known to increase tau phosphorylation and in which EP3 has been shown to play a role in, to understand how the receptor behaves under conditions known to drive tau phosphorylation. To better model pathological conditions, I used HEK293 cells transfected with EP3Re and wild type tau or tau with the P301S mutation (associated with fronto-temporal dementia) and examined changes in tau phosphorylation using western blotting in response to EP3Re activation. To better understand what role the receptor could be playing in pathological conditions, we explore the functional relationship between tau and EP3Re in human iPSC derived neurons from a patient with tau mutation as a better model of disease. Finally, we examine how the presence of the P301S mutation in tau affects the activity of EP3Re. Using inositol monophosphate (IP1) accumulation as a measure of EP3Re activity, having previously established the receptor as signalling via Gαi, we performed assays in the presence of P301S mutation and wild type tau as well as using microscopy and cell surface ELISA to examine how this tau mutation impacts receptor cell surface expression and internalisation. We show that activating EP3Re, but only

in the presence of P301S and P301L tau mutations increases tau phosphorylation and EP3Re intrinsic activity is necessary for hypothermia mediated tau phosphorylation. In turn, the presence of the P301S and P301L tau mutations impairs EP3Re activity as measured by IP1 accumulation and appears to increase EP3Re expression on the cell surface in both transfected HEK293 cells and human iPSC derived neurons. Our findings are consistent with a functional relationship between EP3Re and tau. Further work is required to fully establish if EP3Re is exacerbating or driving tau pathology in disease and whether this could be a potential therapeutic target.

5.2 Introduction

We have shown in previous chapters the distribution of the receptor under normal physiological conditions and in tauopthies and that the receptor is a component of neurofibrillary tangles, co-localising with tau filaments in disease. In this chapter, we aim to understand the function of EP3Re in relation to tau and if this could be contributing to tau pathology. Here, we discuss the known functions of EP3 in the context of neurodegenerative disorders before exploring the proposed mechanisms in tauopathies in which EP3Re could be playing a role.

The EP3 receptor mediates several functions within the central nervous system (CNS) with the most well characterised being thermoregulation. EP3 expressing neurons in the preoptic area are critical for the febrile response and stimulation of cutaneous vasoconstriction and brown adipose tissue thermogenesis (Ivanov and Romanovsky, 2004; Morrison, 2016). In models of CNS disorders, the receptor has been strongly implicated in mediating inflammatory responses leading to neuronal damage and impaired neuronal function. EP3 has been shown to mediate PGE2 effects on vascular permeability, oedema, blood-brain barrier damage, neutrophil infiltration and microglial infiltration (Han et al., 2016; Iadecola et al., 2001; Ikeda-Matsuo et al., 2011, 2010; Lan et al., 2017). EP3 accumulate at sites of ischaemic injury (Iadecola et al., 2001) and is implicated in secondary neuronal damage following an initial vascular insult (Han et al., 2016; Ikeda-Matsuo et al., 2011, 2010; Saleem et al., 2009). Oxygen glucose deprivation of mouse hippocampal slice cultures from EP3 -/- deficient mice show cell death is significantly reduced in the absence of EP3 (Saleem et al., 2009). Further supporting a toxic role for EP3 in neurological disorders, treatment of mice and rats with an EP3 agonist increases infarct volumes and exacerbates NMDA induced toxicity, findings absent on

inhibition or genetic deletion of the EP3 receptor (Ahmad et al., 2007; Ikeda-Matsuo et al., 2010). In models of intracerebral haemorrhage, both EP3-/- deficient mice and those treated with an EP3 antagonist show a significant reduction in lesion volume with reduced astrogliosis, microglial activation, blood brain barrier breakdown, and neutrophil infiltration with better survival rates (Han et al., 2016; Leclerc et al., 2015, 2016).

Alongside vascular disorders, EP3 activity has also been described in the context of neurodegenerative diseases. In models of Amyotrophic Lateral Sclerosis (ALS), PGE2 has been shown to be elevated promoting inflammation and motor neuron death (Almer et al., 2002; Drachman et al., 2002). In contrast, administration of PGE2 at physiological levels to organotypic spinal cord slices in a model of ALS paradoxically rescues motor neurons (Bilak et al., 2004). Bilak et al show this to be mediated by the EP3 receptor. Treating cells with pertussis toxin, which inhibits Gi signalling, prevents this effect showing that this is mediated by EP3-Gi coupling signal transduction pathway (Bilak et al., 2004). This suggests that EP3 can mediate both neurotoxic and neuroprotective effects depending on levels of inflammation and PGE2 production. In mouse models of Alzheimer's dementia EP3 has been shown to mediate amyloid induced toxicity (Maingret et al., 2017; Shi et al., 2012a) and very recently Cao et al show that tau phosphorylation in the transgenic APP/PS1 mouse is dependent on EP3 activity, alongside EP1 and EP2 (Cao et al., 2019). They show that EP3 promotes tau phosphorylation through a p25-dependent Cyclin dependent kinase 5 (CDK5) mechanism (Cao et al., 2019). Although these mice do not develop tau aggregates, the cardinal pathological finding in human tauopathies, the rise in phospho-tau is associated with cognitive deficits (Cao et al., 2019).

The mechanism of development of tau aggregates, is poorly understood. The identifica-

tion of hyper-phosphorylated tau as the main component of tangles (Lee et al., 1991) has meant that tau phosphorylation is one of the most studied aspects of disease development. Tau is a microtubule associated protein and serves to promote microtubule stability. The microtubule binding domain is situated at the carboxyl terminus where there are three or four repeat domains (regulated by alternative splicing of exon 10) (Xie et al., 2016). This region is flanked by serine and threonine residues that when phosphorylated regulate tau's interaction with microtubules (Wang et al., 2013). This is a highly dynamic and necessary process to regulate axonal transport (Rodríguez-Martín et al., 2013). In tauopathies however, tau becomes hyperphosphorylated and detaches from microtubules resulting in progressive destabilisation and loss of microtubules, synapses and neuronal disintegration (Mietelska-Porowska et al., 2014). There is also evidence to suggest phosphorylation of tau inhibits degradation of tau by proteolysis (Poppek et al., 2006). Assessment by a combination of monoclonal phospho-tau antibodies and mass spectrometry reveals several site of tau phosphorylation, some of which are unique to pathology (Laurent et al., 2018; Noble et al., 2013). There are considered to be 85 potential sites of phosphorylation in tau (Laurent et al., 2018) and it has become apparent that some epitopes are phosphorylated in a sequential manner. For the monoclonal antibody AT100, to recognise phospho-tau, tau is required to have undergone a conformational change as a result of sequential phosphorylation at Thr212 and Ser214 (Zheng-Fischhofer et al., 1998) and allows AT100 to recognise filamentous aggregates of tau more specifically than other anti-tau antibodies (Allen et al., 2002). This has not been shown to occur under normal physiological conditions (Zheng-Fischhofer et al., 1998). Figure 5.1 shows known tau phosphorylation sites accompanied by the epitopes recognised by major tau antibodies (Šimić et al., 2016).

Elucidating the signalling pathways that influence tau phosphorylation has been an

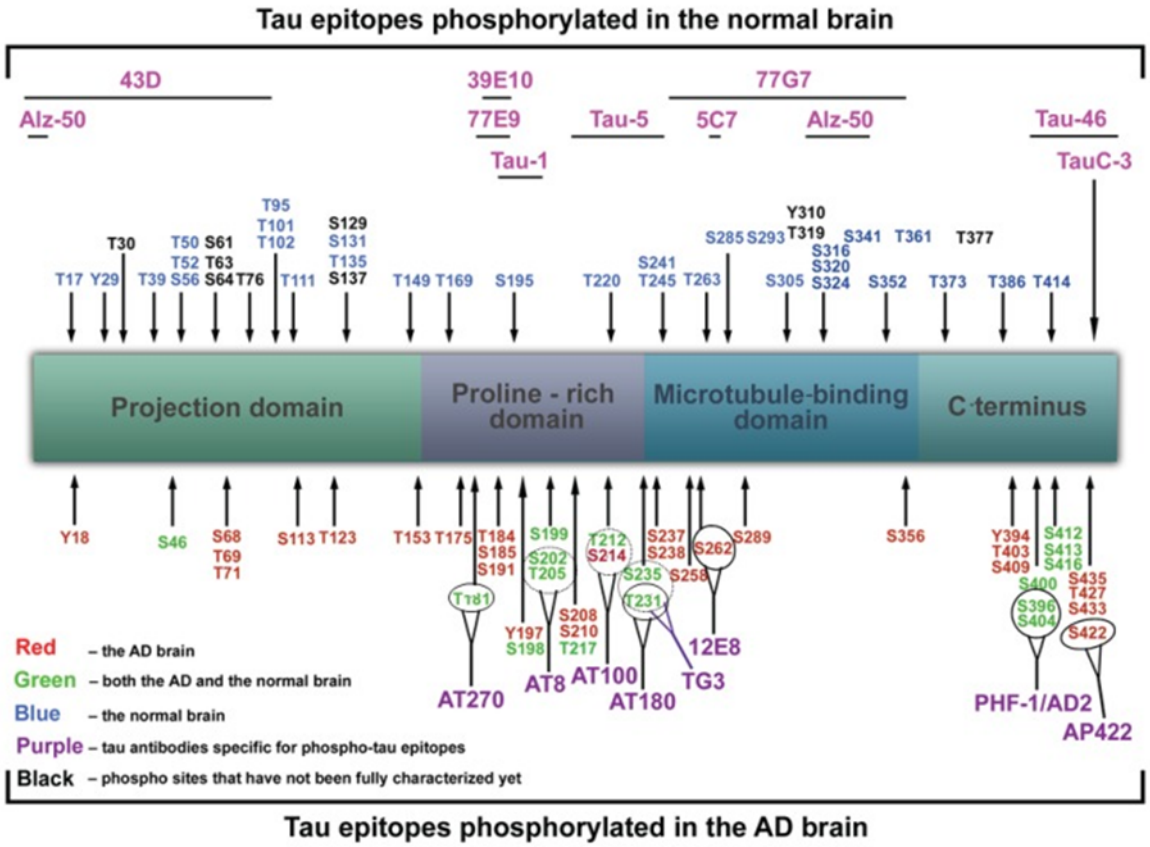


Fig. 5.1 Sites of tau phosphorylation and epitopes recognised by major tau antibodies. Epitopes phosphorylated only in the normal brain, only in Alzheimer's dementia and sites the can be associated with both normal and Alzheimer's dementia are shown. Figure adapted from Simic et al., 2016

important focus for understanding disease pathogenesis. Excessive tau phosphorylation is thought to occur when there is a disruption in the balance between tau kinases and phosphatases. Several kinases have been implicated in disease pathology including glycogen synthase kinase 3 (GSK3), cyclin dependent kinase 5 (cdk5), and p38 mitogen activated protein kinases (MAPK) (Ferrer et al., 2005; Hanger et al., 1992; Lee and Kim, 2017). Although the association between tau phosphorylation and aggregation is not fully understood, it is thought that progressive phosphorylation leads to misfolding of tau and its subsequent oligomerization into insoluble aggregates (Augustinack et al., 2002; Su et al., 2008). There are both *in vitro* and *in vivo* studies that support a direct association between tau phosphorylation and subsequent aggregation. Despres et al. show using kinase assays and nuclear magnetic resonance (NMR) spectroscopy that the combined phosphorylation of serine 202, threonine 205 and serine 208, in the absence of serine 262 phosphorylation yields a tau sample that readily forms aggregates as identified by thioflavin T and electron microscopy, directly linking tau phosphorylation and aggregate formation *in vitro* (Despres et al., 2017). In animal studies, inhibition of kinases such as GSK3 and p38 MAPK, reduce levels of tau phosphorylation and tau pathology with significant improvements in memory and cognitive deficits (Lee and Kim, 2017; Onishi et al., 2011). Targeting tau phosphorylation thus remains an important therapeutic strategy. Other post-translation modifications that have been implicated in disease include tau truncation acetylation, oxidation, ubiquitination, and methylation (Delobel et al., 2008; Ercan-Herbst et al., 2019; Martin et al., 2011).

There is growing evidence that changes in tau occur years before the onset of clinical disease by which point significant neuronal loss has already occurred (Bateman et al., 2012; Khan, 2018). Intervening at this preclinical stage is thus the most likely therapeutic strategy to yield positive results. Inflammatory prostaglandin signalling

is thought to play an important role in this preclinical stage of disease, in particular PGE2 (Andreasson, 2010; Breitner et al., 2011). Studies have shown non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of developing Alzheimer's dementia, but this beneficial effect is most pronounced at the preclinical stage of disease ((ADAPT Research Group et al., 2009; Breitner et al., 2011). Identifying which PGE2 E-prostanoid (EP) receptors are involved at this stage in disease will be critical in providing a more targeted therapeutic strategy. Although the association between EP receptor mediated signalling and tau pathology is poorly understood, several studies have shown EP receptors to contribute to amyloid pathology in Alzheimer's dementia (Andreasson, 2010; Bazan et al., 2002; Figueiredo-Pereira et al., 2016; Johansson et al., 2015; Liang et al., 2005a; Shi et al., 2012a; Wei et al., 2010; Wood, 2012; Woodling and Andreasson, 2016). Liang et al show that deletion of EP2 in the APPSwe-PS1δE9 mouse model of familial Alzheimer's disease results in a marked reduction in oxidative damage and reduced amyloid burden (Liang et al., 2005a). They suggest this to be through increased clearance of Aβ42 and/or reduced generation. In the presence of EP2, they note an increase in β-CTF, a product of APP cleavage by BACE1, suggesting EP2 modulates BACE1 cleavage increasing generation of Aβ42. EP2 has also been shown to impair microglial function resulting in reduced Aβ42 clearance (Johansson et al., 2015). Inhibiting microglial EP2 signalling not only decreases inflammation but in mouse models of disease also rescues memory (Johansson et al., 2015). The EP3 receptor has also been implicated in Alzheimer's disease pathology. Using the APPSwe-PS1δE9 mouse model of familial Alzheimer's disease, Shi et al showed that EP3 contributes to synaptic dysfunction as well as amyloid plaque burden, both of which are rescued on EP3 deletion. They show that deletion of just one allele is sufficient to reduce amyloid plaque burden and rescue pre-synaptic proteins. EP3 signalling is thought to suppress amyloid clearance whilst increasing inflammatory oxidative stress and

βsecretase activity (Shi et al., 2012a). EP3 activity also negatively correlates with the pro-survival kinase, protein kinase B (AKT) (Shi et al., 2012a; Wang et al., 2010).

There are fewer studies exploring EP receptors and tau pathology. In tauopathies it has been proposed that an initial stimulus, such as generation of amyloid plaques in Alzheimer's disease, activates glial cells releasing inflammatory cytokines and prostaglandins which induce neurotoxicity (McGeer and McGeer, 2013). This then leads to impairment of the ubiquitin/proteasome pathway and activation of caspase which in turn cleaves tau creating a tau species prone to aggregation generating paired helical filaments and tangles (Gamblin et al., 2003; Metcalfe et al., 2012). Arnaud et al. (2009) demonstrated in cell culture that prostaglandins are able to induce caspase mediated cleavage of tau in keeping with the proposed hypothesis linking inflammatory prostaglandin signalling to tau pathology. More recently PGE2 has also been implicated in tau phosphorylation. Alzheimer's dementia is associated with dysregulated calcium signalling (Wang et al., 2017) and Cao et al. show this to cause an increase in PGE2 leading to tau phosphorylation via EP1, EP2 and EP3 (Cao et al., 2019) providing a direct correlation between EP3 activity and tau phosphorylation in murine cells.

An understanding of the role of EP3Re in tauopathies relies on experimental conditions in which the behaviour of human tau can be studied. The species-specific isoforms of EP3 make animal models of tauopathy unsuitable for the purpose of the project. A suitable model that recapitulates all the features of human tauopathies is yet to be established. Cellular models using human cell lines provide an accessible way of studying complex pathogenic events at a single cell level. Both neuronal and non-neuronal cell lines are currently used. Models based on non-neuronal cell lines lack endogenous tau making them a simple and inexpensive model to evaluate the effects of overexpressing

exogenous tau with mutations associated with familial tauopathies in comparison to exogenous wild type tau. Most of these cells are also very well characterised making them suitable for a wide variety of molecular and biochemical techniques. Cell lines, such as the human neuroblastoma cell line, SHSY5Y, have the added advantage of a neuronal phenotype (Agholme et al., 2010). They express tau when undifferentiated, and when differentiated express levels of mature tau comparable to the human adult brain (Agholme et al., 2010). Most tau models are based on the hypothesis that the key pathogenic event is tau aggregation. *In vitro* this can be replicated by adding co-factors to recombinant tau, such as sulphated glycosaminoglycans, heparin, or arachidonic acid to promote tau polymerisation (Goedert et al., 1996; Pérez et al., 2002), seeding cells with tau fibrils (Karikari et al., 2019; Xu et al., 2016), inducing tau phosphorylation by inhibiting phosphatase activity or activating tau kinases (Shea and Fischer, 1996), or, overexpressing tau with fronto-temporal dementia (FTD) associated mutations. Xu et al. show that exposing HEK293 cells to extracellular tau fibrils converts cellular transfected soluble tau to insoluble thioflavin-S positive tau resembling neurofibrillary tangles (Xu et al., 2016) and propose this as a mechanism of being able to study disease pathogenesis *in vitro*. This has been replicated by several groups and is a well-established method of studying the spread and propagation of tau pathology (DeVos et al., 2018; Falcon et al., 2015; Frost et al., 2009; Woerman et al., 2016). In vitro models of tauopathy can also be generated by altering the balance between tau phosphorylation and dephosphorylation. Loss of tau dephosphorylation through inhibition of tau phosphatases produces a tau species that is prone to aggregation, a pathological hallmark of tauopathies. Treating cells with okadaic acid, an inhibitor of protein phosphatases PP1 and PP2A, results in tau hyperphosphorylation, including phosphorylation of Alzheimer's dementia associated epitopes (Caillet-Boudin and Delacourte, 1997; Seidel et al., 2012; Shea and Fischer, 1996; Yadikar et al., 2020). Okadaic

acid treated cells also show impaired microtubule assembly and stability (Shea and Fischer, 1996). PP2A inhibition and rapid and extensive tau phosphorylation has also been noted as a result of anaesthesia (Planel et al., 2007). Planel et al show this to be due to anaesthesia induced hypothermia, as the phenotype is rescued on return to normothermia (Planel et al., 2007). They then went on to propose a cell-based model of tauopathy based on hypothermia induced tau phosphorylation (Bretteville et al., 2012). Exposing primary cell lines and *ex vivo* brain slices of wild type mice to hypothermia results in phosphorylation of Alzheimer's dementia related epitopes without affecting total tau levels, proposing a model compatible with endogenous levels of tau expression (Bretteville et al., 2012). They show this to be mediated by GSK3β and CDK5 as treatment with lithium chloride and roscovitine, GSK3β and CDK5 respective inhibitors, prevent this (Bretteville et al., 2012). Cell based models of tauopathy can also be created by overexpressing tau (Houck et al., 2016), or expressing tau with fronto-temporal dementia (FTD) associated mutations (Ritter et al., 2018). Tau mutations, such as P301L, promote β sheet structure formation enhancing aggregation and creation of paired helical filaments (Lewis et al., 2000; Rizzu, 2000; Rodríguez-Martín et al., 2016). Ritter et al. (2018) show in COS7 cells that the presence of the FTD N279K mutation is sufficient to increase levels of tau phosphorylation in comparison to wild type tau (Ritter et al., 2018). Although tauopathy models using primary cell lines do have certain limitations, due to their viability and ease of use, they provide a simple and effective way of modelling disease.

A more recent approach to modelling tauopathies has been the use of induced pluripotent stem cells (iPSCs). The discovery that terminally differentiated cells, such as fibroblasts, can be reprogrammed into stem cells has allowed the creation of a system that captures the patient's exact genetic make-up to study the underlying disease

mechanisms. Expressing the transcription factors Oct3/4, Sox2, Klf4 and c-Myc (Takahashi et al., 2006) returns cells to pluripotency allowing for subsequent differentiation into neurons. Neurons derived from iPSCs can be generated from individuals bearing genetic mutations in *MAPT* associated with familial frontotemporal dementia (Iovino et al., 2015; Strang et al., 2019). Several models of tauopathy rely on tau overexpression but the ability to grow neurons from these individuals with familial disease allow us to study these mechanisms under normal physiological expression levels. Neurons derived from iPSCs from patients with familial tauopathies as well as sporadic disease have been shown to recapitulate several of the pathological features of the disease (Iovino et al., 2015; Muratore et al., 2014; Shi et al., 2012c). Neurons derived from iPSCs of patients harbouring mutations in the amyloid precursor protein (APP), associated with familial Alzheimer's dementia, and those obtained from sporadic disease, display increase Aβ production as well as tau phosphorylation (Muratore et al., 2014; Ochalek et al., 2017; Ovchinnikov et al., 2018). Neurons derived from iPSCs harbouring mutations in the *MAPT* gene , alongside increases in levels of tau phosphorylation, display a unique neuronal phenotype with changes in 3R:4R tau isoform ratios, deficiencies in neurite outgrowth, and, activation of stress response pathways (García-León et al., 2018; Iovino et al., 2015; Silva et al., 2016; Verheyen et al., 2018). These neurons display an upregulation of stress pathway genes such as superoxide dismutase 1 (*SOD1*), sulfiredoxin 1 (*SRXN1*) and, nuclear factor erythroid-2-related factor 2 (*NRF2*) a phenotype that can be rescued on downregulation of *MAPT* expression using CRISPR/Cas9 (Silva et al., 2016; Verheyen et al., 2018) showing that these neurons are able to capture several aspects of tauopathies. These make them an ideal model to study the behaviour of EP3Re in tauopathies and validate any findings observed in primary cell lines.

In this present chapter, we hypothesise, based on what we know on PGE2 signalling in

tauopathies and our previous findings of EP3Re and tau tangle co-localisation, that the receptor is contributing to tau pathology. We have shown through electron microscopy that EP3Re interacts with tau in the fuzzy coat, where most epitopes identified by tau phospho-dependent antibodies also localise (Friedhoff et al., 2000; Wegmann et al., 2013). This raises the possibility that EP3Re physically interacts with tau tangles and could play a role in tau phosphorylation. As part of our investigation to understand this, our aims in this study are to:

- Understand changes in tau in response to EP3Re signalling under normal physiological conditions
- Understand changes in tau in response to EP3Re signalling in models of tauopathy
- Determine if EP3Re signalling is altered in tauopathies

To this end we will be using primary cell lines as well as neurons derived from induced pluripotent stem cells to model disease.

5.3 Materials and Methods

5.3.1 Immunocytochemistry

Localisation of the EP3Re receptor was examined in the human neuroblastoma cell line, SHSY5Y cells (See **2.2.7.1** for further details). In view of their neuronal phenotype it was possible that they would express the EP3Re receptor. SHSY5Y cells were stained for the presence of the receptor using the human EP3Re specific antibody. The presence of the receptor was assessed by confocal microscopy on the Leica SP8.

5.3.2 Silencing EP3Re expression

Predesigned siRNA duplexes were obtained from Qiagen directed against PTGER3. Standard forward transfection was initially performed using lipofectamine RNAiMAX (Invitrogen) according to manufacturers instructions. SHSY5Y cells were plated into 6 well plates and grown till they were 60-80% confluent. SiRNA lipid complexes were created using 5-20μl/ml of lipofectamine RNAiMAX and siRNA varying in concentration from 30-150pmol to determine optimal transfection conditions. The lipid complex was left to incubate for 5min before being applied to cells. The media was then changed after 72hrs before being used for experiments. Green fluorescent protein (GFP) cDNA was used as a positive control for transfection whilst non-targeting siRNA obtained from Qiagen was used as a negative control. For reverse transfection, lipid complexes were prepared inside the wells prior to adding SHSY5Y cells.

5.3.3 Detecting changes in tau phosphorylation in response to EP3Re signalling in SHSY5Y and HEK293 cells

Tau phosphorylation in response to EP3Re activation was assessed in SHSY5Y cell and HEK293 cells.

SHSY5Y cells were plated into 12 well plates at a density of 0.1 x 10^6 per well. Prior to experiments serum was changes to serum free media. The EP3 agonist sulprostone was then added for 30min at concentrations varying from 0 – 10^{-6}M and changes in tau phosphorylation measured by immunoblotting using the phospho-tau specific antibodies PHF1 or AT8. Western immunoblots were also probed for the presence of active kinases AKT and GSK3 and normalised to total kinase present and GAPDH. Changes observed were verified by silencing the receptor using siRNA directed against

EP3Re as described in **2.2.10**. All experiments were done in duplicate and repeated 3 times.

HEK293 cells were plated into 12 well plates at a density of 0.1 x 10^6 per well. The following day cells were transfected with DNA constructs encoding EP3Re alongside wild type tau or tau with the P301S mutation as detailed in **2.2.9**. On termination of transfection, cells were maintained in serum free media till the following day when they were exposed to varying concentrations of the EP3 agonist sulprostone for 30min. Tau phosphorylation in response to EP3Re activity was determined by immunoblotting using the phospho-tau antibody AT8 and normalised to total tau.

5.3.4 Modelling tauopathies in SHSY5Y cells

5.3.4.1 Tau seeding as a model of tauopathy

The protocol was performed as described by Falcon et al. (2015). SHSY5Y cells were plated onto poly-l-lysine coated coverslips in 12 well plates at a density of 0.1 x 10^6. Cells were then seeded with sarkozyl insoluble extract from Alzheimer's dementia subjects (see **2.2.4**) diluted in OptiMEM (Gibco) at a concentration of 100ng/ml. After 3hr the media was replaced with normal growth media (RPMI supplemented with 10% FBS). Cells were then incubated for 3 days before being washed in PBS and fixed in methanol for immunohistochemistry or protein lysate extracted for immunoblotting. The presence of phosphorylated tau was assessed by immunohistochemistry using the phospho-tau antibody AT8 whilst aggregated tau was assessed using pFTAA. This was validated on western immunoblotting using the phospho-tau antibody AT100, which has been shown to be specific for pathological filamentous aggregated tau.

5.3.4.2 Hypothermia as a model of tauopathy

Hypothermia was used as a model of tauopathy following the protocol described by Bretteville et al. (2012). SHSY5Y cells were plated into 2x 12 well plates at a density of 0.1 x 10^6 per well and kept in serum free media for 24hr prior to performing experiments. One plate was then transferred to a 32°C incubator whilst the other was kept at 37°C for 2hr. If cells were treated with sulprostone, this was applied after 1hr30min at which point cells were then returned to their respective incubators for a further 30min. After 2hr cells were lysed in RIPA buffer and immunoblotting performed to measure levels of tau phosphorylation. To determine the role of EP3Re in hypothermia induced tau phosphorylation, experiments were performed following transfection with siRNA targeting EP3Re 72hrs prior to performing experiments as described in **2.2.10**. All experiments were repeated 3 times.

5.3.5 EP3Re signalling in the presence of Tau

To determine if tau affects EP3Re function, using Gi coupling as a measure of EP3Re function, we performed IP accumulation assays. HEK293 cells were plated into 96 well plates and transfected with EP3Re and either wild type tau or tau with the P301S mutation alongside the chimeric G-protein G$\alpha\delta$6qi4myr. Cells were then stimulated with the EP3 ligand sulprostone and IP1 accumulation measured. For details of protocol see **2.2.16**. All assays were run in quadruplicate and repeated 3 times.

5.3.6 Molecular interaction between EP3Re and Tau

To understand if there is a dynamic interaction between tau and EP3Re in a cell model of disease, we used a NanoLuc® Binary Technology (NanoBiT®) protein:protein interaction assays. EP3Re and MAPT constructs were ligated into NanoBiT® vectors

containing a Large BiT (LgBiT) subunit, an 18kDa peptide, or its complementary Small BiT (SmBiT) 11kDa peptide as described in **2.2.18**. The SmBiT and LgBiT peptides were expressed at either the C or N terminus of each protein to allow us to identify the terminus at which the interaction occurs. HEK293 cells were seeded into a 96 well plate and transfected with the following combinations of gene constructs;

- EP3E_NSmBiT:MAPT_NLgBiT
- EP3E_NLgBiT:MAPT_NSmBiT
- EP3E_NSmBiT:MAPT_CLgBiT
- EP3E_NLgBiT:MAPT_CSmBiT
- EP3E_CSmBiT:MAPT_CLgBiT
- EP3E_CLgBiT:MAPT_CSmBiT
- EP3E_CSmBiT:MAPT_NLgBiT
- EP3E_CLgBiT:MAPT_NSmBiT

Luminescence was measured over a period of 20min. Assays were run in quadruplicates and repeated 3 times for wild type tau and once for P301S tau. The vectors used were created with the assistance of Edson Mendes de Oliveira (Institute of Metabolic Science, Cambridge). Assays for wild type tau were performed by Kieran Ryan and for P301S tau by Edson Mendes de Oliveira.

5.3.7 iPSC derived neurons

Neural embryonic stem cells derived from female dermal fibroblasts using the Shi protocol (Shi et al., 2012b) were obtained from Axol. Neurons derived from induced pluripotent stem cells with wild type tau and tau with the P301L mutation were grown

as described in **2.2.8** and stained for the presence of the EP3Re at D0 and D30. After 35 days of differentiation, beta tubulin positive neurons were detected denoting mature neurons. The interaction between EP3Re and tau in these neurons was assessed using proximity ligation assays as described in **2.2.12**. Neurons were treated at D35 with the EP3 agonist sulprostone for 30min, fixed and stained for the presence of phosphorylated tau, using the phospho-tau antibody AT8. The presence of tau aggregates was assessed by staining cells with the fluorescent pentameric oligothiophene, pFTAA.
All results were assessed using confocal microscopy on the Leica SP8.
Two rounds of differentiation were done by myself and the third round was performed by Mehtap Baciologu.

5.3.8 Statistics

Statistical analysis was carried out using GraphPad prism 7.0. For signalling assays, half maximal effective concentration (LogEC50) was calculated from data normalised and fitted to a non-linear regression curve in GraphPad prism 7.0. Two way ANOVA with multiple comparisons were performed to determine effect of tau on EP3Re signalling assays as well as differences in EP3Re expression and tau phosphorylation in iPSC derived neurons. EP3Re expression in cells was calculated by measuring integrated density and subtracting the area of the cell x mean background intensity to give the corrected total cell fluorescence. This was obtained from 10 fields of view from each round of cell differentiation. Results of NanoBiT protein:protein interaction assays were plotted in GraphPad prism 7.0. Significance for all tests was assumed at $p<0.05$.

All materials and methods are described in detail in Chapter 2.

5.4 Results

5.4.1 EP3Re signalling and wild type tau pathology

We first wanted to understand how EP3Re interacts with wild type tau. As most tauopathies are sporadic, it was important to investigate if EP3Re activity alone is sufficient to drive disease pathology. Focusing predominantly on abnormal tau phosphorylation, we used SHSY5Y cells, that express endogenous tau, as well as HEK293 cells transfected with wild type tau to determine if signalling through EP3Re could be contributing to disease pathology.

5.4.1.1 SHSY5Y cells express endogenous EP3Re

SHSY5Y cells are a human neuroblastoma cell line and express a number of human-specific proteins and protein isoforms, although EP3Re expression has never previously been investigated. SHSY5Y cells were stained with antibody directed against EP3Re to determine their suitability for further experiments. SHSY5Y cells were positive for EP3Re, demonstrating the characteristic punctate cytoplasmic staining identified previously in human tissue (Fig. 5.2).

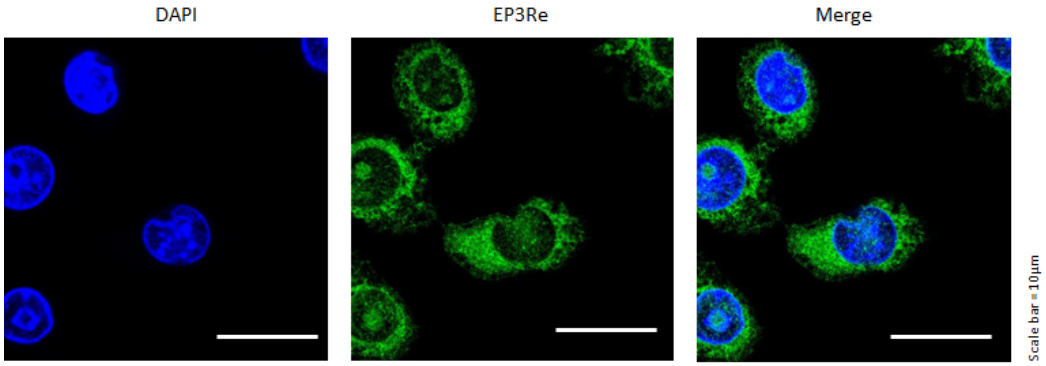


Fig. 5.2 SHSY5Y cells stained with EP3Re antibody. SHSY5Y cells are positive for endogenous EP3Re, which appear localised to the cytoplasm.

5.4.1.2 Silencing EP3Re expression

Prior to understanding if EP3Re mediated signalling affects tau phosphorylation, I needed to establish a system by which EP3Re expression could be regulated. Small interfering RNA (siRNA) is an established method to silence gene expression by degrading mRNA after transcription, preventing translation. Predesigned siRNA directed against EP3Re were obtained from Qiagen and delivered using lipofectamine. Successful silencing was verified using western immunoblotting. Initial attempts using 10nM siRNA and standard forward transfection, where cells are attached prior to transfection, showed minimal transfection efficiency with no statistical significant reduction in EP3Re protein expression (Fig. 5.3D). Transfection efficiency was significantly improved using higher concentrations of siRNA (Fig. 5.3F). A maximum effect was seen at a concentration of 50nM siRNA using reverse transfection, where cells are plated into wells containing siRNA-lipid complexes, with a resulting reduction of over 60% in EP3Re protein expression. Although both forward and reverse transfection at concentrations of 20nM and 50nM siRNA yielded statistically significant reductions in EP3Re expression, subsequent experiments were performed using reverse transfection at 50nM siRNA due to the high efficiency and shorter experiment times.

5.4.1.3 EP3Re and Tau phosphorylation

Having established endogenous EP3Re expression could be silenced in SHSY5Y cells, I wanted to explore the effect of EP3Re mediated signalling on tau phosphorylation. This was examined in SHSY5Y cells, expressing endogenous wild type tau, and in HEK293 cells, transfected with both EP3Re and wild type tau.

SHSY5Y cells were treated with the EP3 selective agonist sulprostone and changes in tau phosphorylation identified by the phosphorylation specific antibody PHF1

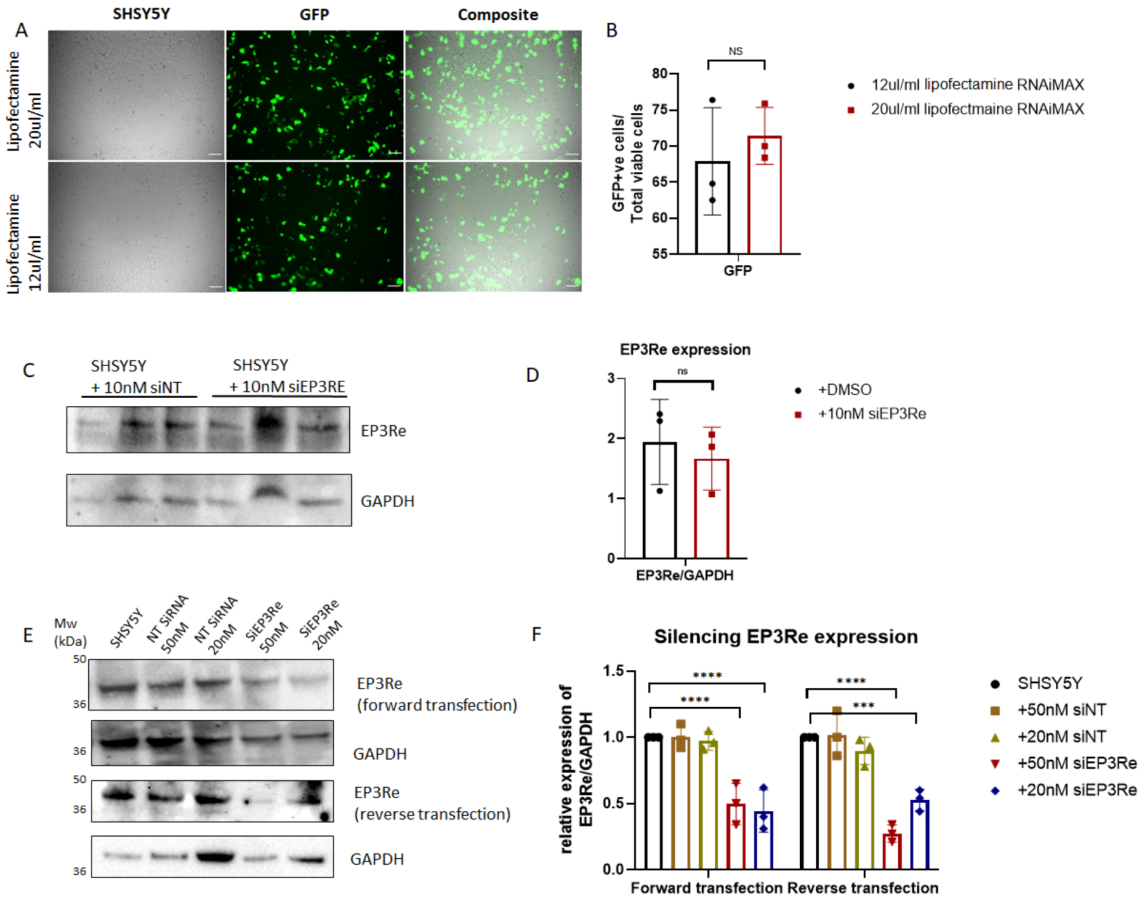
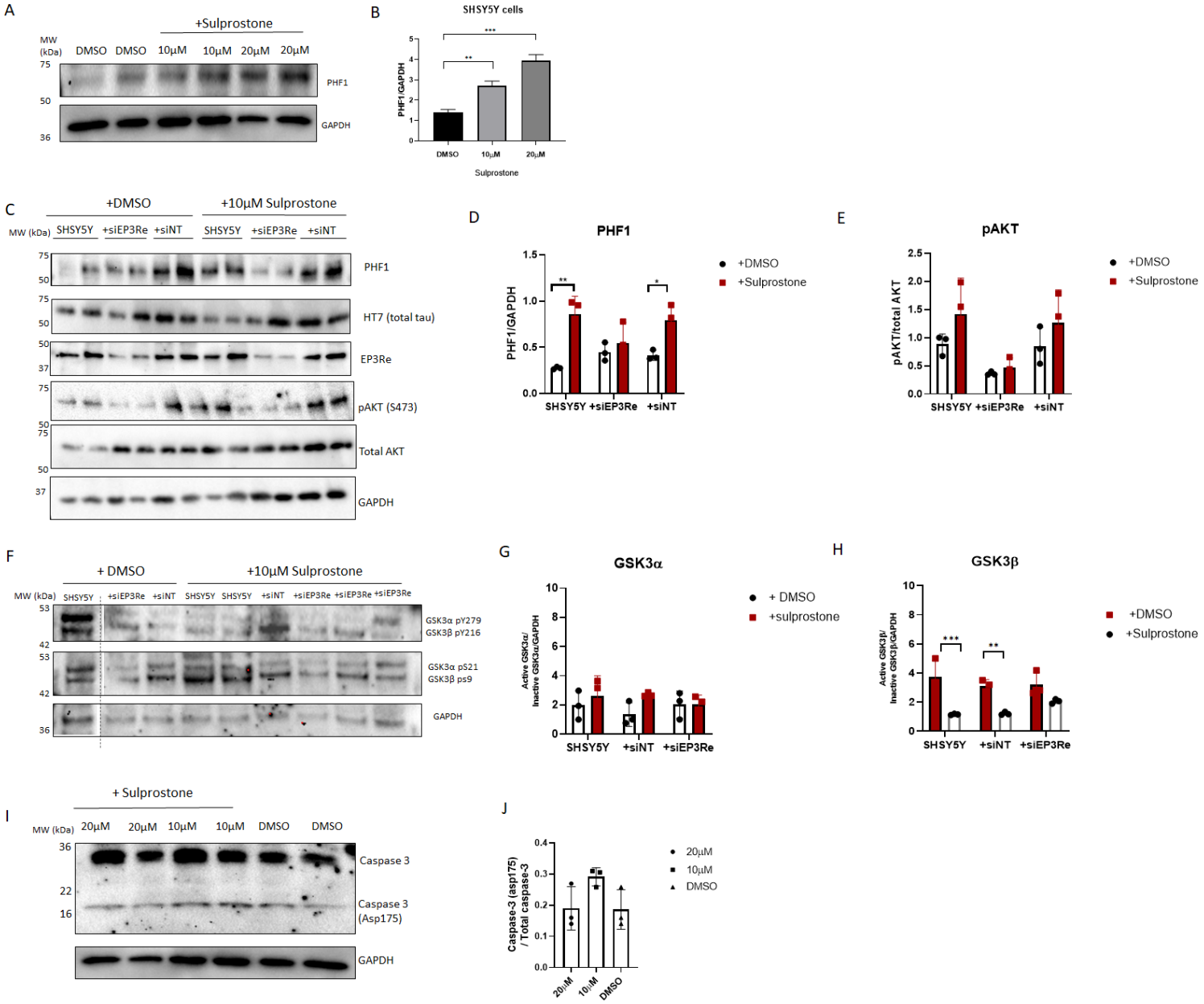


Fig. 5.3 Silencing EP3Re expression using siRNA. A) GFP was used as a positive control and to optimise transfection conditions. siRNA-lipid complexes were formed using recommended lipofectamine concentrations of 5 µl/ml, 12 µl/ml and 20 µl/ml. Significant uptake was not seen at 5 µl/ml (not shown) but at 12 µl/ml and 20 µl/ml up to 75% of viable cells were GFP+ve following the media change at 72hrs. B) Data shown from 3 independent experiments. No statistical significant difference in transfection efficiency so 12 µl/ml used for subsequent experiments. Analysed using two tailed T-test with mean+/- standard deviation shown. Statistical significance determined at $p<0.05$. C) Initial experiments performed using 10nM siRNA showed no reduction in EP3Re expression as quantified by western blotting. D) Graphical representation of (C). Individual data points with mean +/- standard deviation shown. Two tailed t-test showed no statistical significance taken at $p<0.05$. E) Cells were transfected with 20nM or 50nM siRNA using standard forward and reverse transfection. Representative western blot shown. Graphical representation of 3 independent experiments can be seen in (F). Maximum reduction in EP3Re expression is noted using 50nM siRNA reverse transfecting cells. Data analysed using two-way ANOVA with multiple comparisons. Statistical significance determined at $p<0.05$.

(Ser202/Thr205). Cells exposed to sulprostone show an increase in tau phosphorylation in a dose dependent manner (Fig. 5.4A). As sulprostone targets all EP3 isoforms, to verify the contribution of EP3Re, EP3Re expression was silenced and experiments repeated. In the absence of EP3Re expression, the increase in phosphorylated tau was no longer statistically significant (Fig. 5.4D). In SHSY5Y cells, EP3Re is the main isoform mediating tau phosphorylation. To understand the mechanism behind this increase, I then examined the activity of tau kinases AKT, GSK3α and GSK3β. EP3 has previously been described as negatively correlated with the pro survival kinase AKT whilst some studies show EP3 to increase AKT activity. In this present study, although a trend is noted suggesting an increase in AKT in response to EP3Re activation, this failed to reach statistical significance (Fig. 5.4E). GSK3α did not shown any change in activity in response to EP3Re stimulation (Fig. 5.4G), however GSK3β showed a statistically significant reduction in activity. In the absence of EP3Re this reduction is no longer significant (Fig. 5.4H) suggesting the noted reduction was mediated by EP3Re. Phosphorylation is not the only post translational modification associated with disease. Tau truncation at Asp-421 by caspase 3 is thought to be an early event in the oligomerisation and formation of aggregates in Alzheimer's (Means et al 2015; Flores-Rodriguez et al 2015). Activated caspase 3 has been shown to co-localise with NFTs in tauopathies, alongside truncated tau. It has previously been proposed that prostaglandin signalling increases caspase activity (Arnaud, Myeku and Figueiredo-Pereira, 2009). To explore if EP3Re could be contributing to disease pathology via this mechanism, SHSY5Y cells were exposed to the EP3 agonist sulprostone and the presence of active caspase 3 measured by western blotting. No difference is noted in caspase 3 activity before and after EP3Re stimulation by sulprostone. EP3Re mediated activity does not appear to contribute to tau truncation by caspase 3 (Fig. 5.4I).


A
+Sulprostone
MW (kDa)
DMSO DMSO 10μM 10μM 20μM 20μM
75
50
36
PHF1
GAPDH
B
SHSY5Y cells
PHF1/GAPDH
DMSO
10μM
20μM
Sulprostone
C
+DMSO
+10μM Sulprostone
MW (kDa)
SHSY5Y +siEP3Re +siNT SHSY5Y +siEP3Re +siNT
PHF1
HT7 (total tau)
EP3Re
pAKT (S473)
Total AKT
GAPDH
D
PHF1
PHF1/GAPDH
SHSY5Y +siEP3Re +siNT
+DMSO
+Sulprostone
E
pAKT
pAKT/total AKT
SHSY5Y +siEP3Re +siNT
+DMSO
+Sulprostone
F
+ DMSO
+10μM Sulprostone
MW (kDa)
SHSY5Y +siEP3Re +siNT SHSY5Y SHSY5Y +siNT +siEP3Re +siEP3Re +siEP3Re
GSK3α pY279
GSK3β pY216
GSK3α pS21
GSK3β ps9
GAPDH
G
GSK3α
Active GSK3α/
Inactive GSK3α/GAPDH
SHSY5Y +siNT +siEP3Re
+ DMSO
+sulprostone
H
GSK3β
Active GSK3β/
Inactive GSK3β/GAPDH
SHSY5Y +siNT +siEP3Re
+DMSO
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20μM 20μM 10μM 10μM DMSO DMSO
Caspase 3
Caspase 3
(Asp175)
GAPDH
J
Caspase-3 (asp175)
/ Total caspase-3
20μM
10μM
DMSO

Fig. 5.4 EP3Re signalling and tau phosphorylation in SHSY5Y cells. A) SHSY5Y cells exposed to sulprostone showed an increase in tau phosphorylation in a dose dependent manner. B) Graph showing results of 3 independent experiments. All experiments run in duplicate. C) Western blot showing SHSY5Y cells treated with the EP3 agonist sulprostone. Levels of tau phosphorylation (ser396/ser404) and AKT were assessed. There was an increase in tau phosphorylation in response to sulprostone that does not occur when EP3Re is silenced. Although a trend is noted suggesting an increase in AKT, this fails to reach statistical significance. D) Graphical representation of PHF1 levels in response to EP3R activity following 3 independent experiments. All experiments performed in duplicate. E) Graphical representation of active levels of AKT following sulprostone treatment following 3 independent experiments. All experiments performed in duplicate. F) Western blot showing levels of active GSK3α and β, as identified by phosphorylation on tyrosine279 and tyrosine 216 respectively and inactive GSK3α and β as identified by phosphorylation on serine21 and serine9 respectively. There was a reduction in the ratio of active:inactive GSK3β which was not seen in GSK3α in response to sulprostone treatment. G) Graphical representation of data obtained from 3 independent experiments measuring active:inactive GSK3α. H) Graphical representation of data obtained from 3 independent experiments measuring active:inactive GSK3β. The reduction in active GSK3β failed to reach statistical significance when EP3Re expression was silenced suggesting EP3Re activity was necessary to inactivate GSK3β. I) SHSY5Y cells exposed to sulprostone show no differences in caspase 3 activation. J) Graphical representation of 3 independent experiments. All experiments done in duplicate. All graphs show individual data points+/- standard deviation. Data analysis was done using 2 way ANOVA with multiple comparisons. Statistical significance determined at $p<0.05$.

In Chapter 3, using HEK293 cells, we established that EP3Re signals through Gi coupling. To correlate tau phosphorylation with EP3Re activity, HEK293 cells were exposed to varying concentrations of sulprostone, selected to correspond with different points of EP3Re activity as measured by IP1 accumulation. Figure 5.5 shows an increase in tau phosphorylation (AT8 ser202/thr205) in response to sulprostone in a dose dependent manner ($p<0.0001$). An increase is however also noted in the absence of EP3Re suggesting that other EP3 receptors are present in the cells, which is confirmed on western blotting using the pan-EP3 antibody. Any increase noted can therefore not be attributed to EP3Re activity alone.

HEK293 cells treated with the highest concentration of sulprostone, showed a higher increase in tau phosphorylation in the presence of EP3Re in comparison to cells lacking the e isoform when compared to untreated cells.

5.4.1.4 Effect of tau on EP3Re signalling

To determine if EP3Re signalling is affected by the presence of wild type tau, HEK293 cells were transfected with both the receptor and wild type tau alongside the chimeric G protein, G$\alpha\delta$6qi4myr, which couples Gi binding residues on the internal surface of the GPCR to a Gq signalling pathway. This allows for measurement of inositol monophosphate (IP1) accumulation in response to Gi coupling. On stimulation of cells with and without wild type tau by the EP3R agonist sulprostone, no differences in IP1 accumulation are noted. In the presence of wild type tau, there is no change in EP3Re receptor activity as measured by Gi coupling (Fig. 5.6).

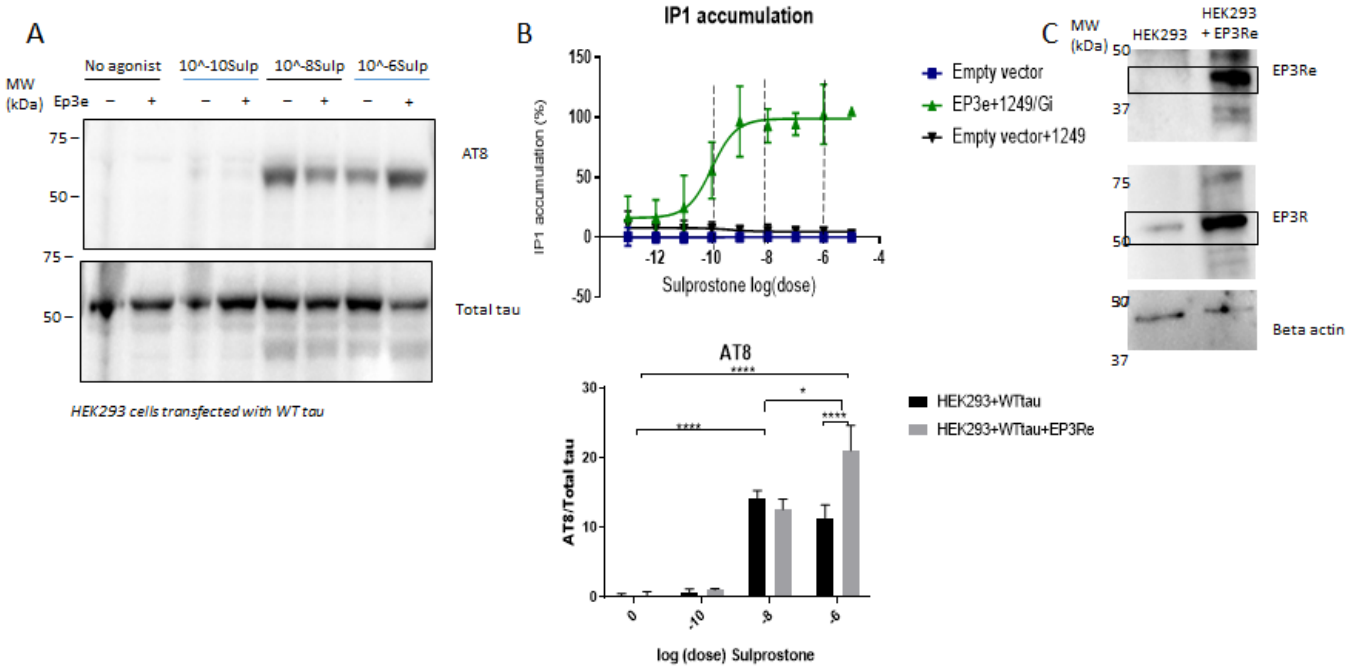


Fig. 5.5 EP3Re signalling increases phosphorylation of wild type tau in HEK293 cells. A) Immunoblot showing levels of phospho-tau (AT8) in response to treatment with sulprostone in HEK293 cells expressing wild type tau with and without EP3Re. B) Graphical representation of data of 3 independent experiments. Level of tau phosphorylation is positively correlated with level of sulprostone and EP3 activity both in the presence and absence of the e isoform ($p<0.0001$). At higher concentrations of sulprostone, the presence of the EP3Re receptor results in higher levels of tau phosphorylation ($p<0.0001$) in comparison to EP3 alone. Analysis done using 2 way ANOVA with multiple comparisons. Statistical significance determined at $p<0.05$. C) Western blotting confirms that HEK293 cells express other isoforms of EP3 as recognised by a pan-EP3 antibody. They do not express EP3Re. n=2

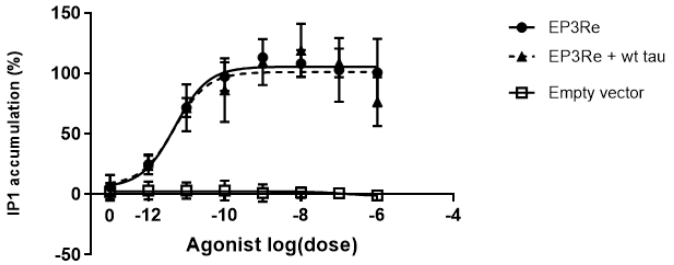


Fig. 5.6 EP3Re stimulation in HEK293 cells with and without wild type tau HEK293 were transiently transfected with EP3Re, wild type tau and the chimeric G protein G$\alpha\delta$6qi4myr. Inositol monophosphate (IP1) accumulation was measured in response to stimulation by sulprostone. A dose dependent increase in IP1 accumulation is noted in HEK293 cells with and without wild type tau. No differences are noted in receptor activity in response to the presence of wild type tau. Cumulative data are shown from 3 different experiments with the mean and standard deviation. Data was fitted to a non-linear regression curve and normalised in GraphPad prism 7.0. Differences in signalling were assessed by performing two tailed unpaired t-tests. Statistical significance was considered at $p<0.05$.

5.4.1.5 Molecular interaction between EP3Re and Tau

We next went on to explore the molecular interaction between EP3Re and wild type tau. Previous experiments using proximity ligation assays and immunoprecipitation exploring the interaction between EP3Re and tau, failed to identify an association between EP3Re and monomeric tau. All these studies, however, describe a static interaction. To investigate how they might interact in a dynamic context I used a structural complementation reporter system, NanoLuc® binary technology (NanoBiT®). MAPT and EP3Re constructs were fused to a large 18kDa peptide (LgBiT) or a complementary small 11 amino acid peptide (SmBiT) at either the N- or C- terminus. In the presence of the substrate, a luminescent signal is emitted when the two proteins are in close proximity to one another. By creating multiple constructs where the fused peptides are at either the C- or N- terminus, if a signal is seen, this allows for orientation and localisation of the site of interaction. HEK293 were transfected with EP3Re and MAPT fused to a LgBiT or SmBiT and the baseline interaction measured. Cells were then exposed to sulprostone to determine if an interaction occurs following

activation of the receptor. Figure 5.7 shows no increase in luminescence relative to the negative control. There is no detectable interaction between EP3Re and monomeric wild type tau.

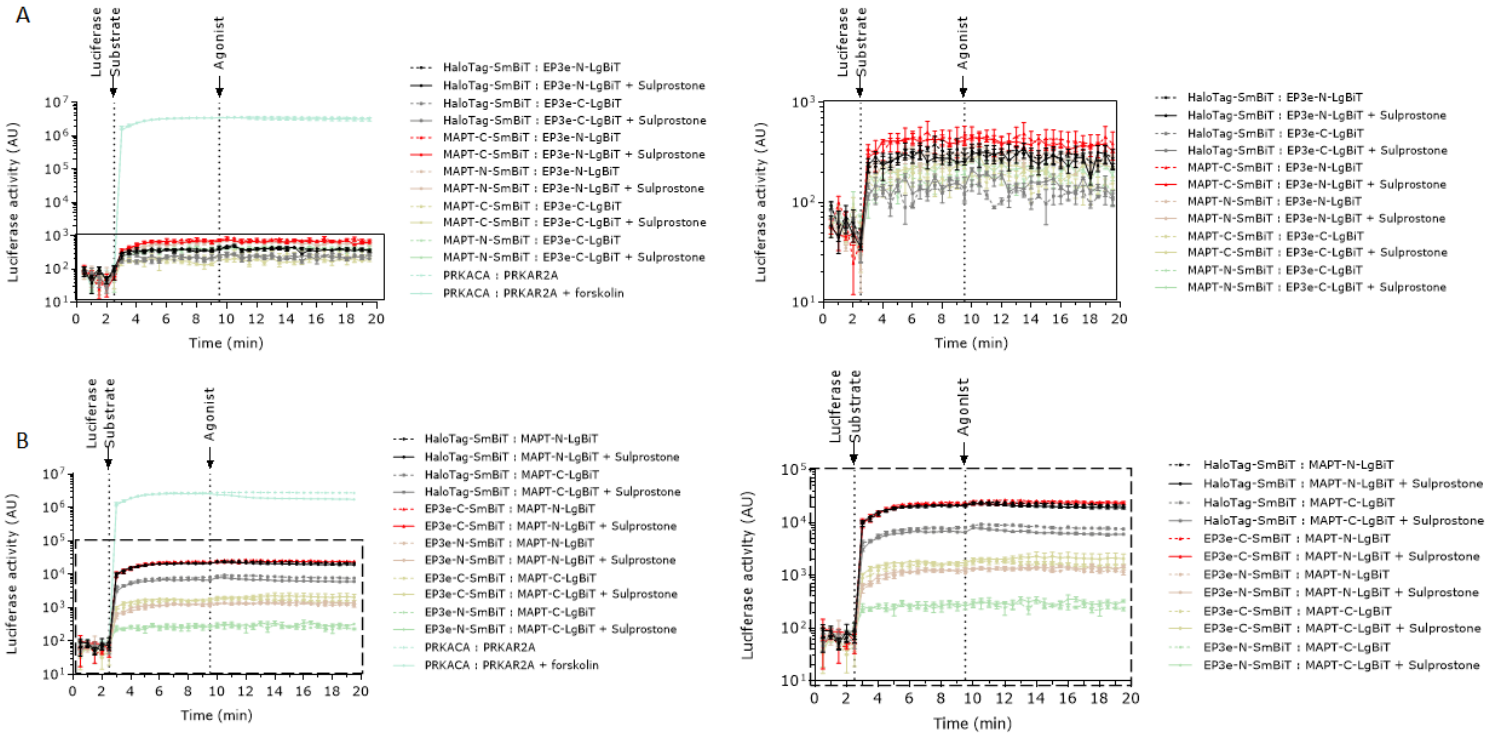


Fig. 5.7 There is no detectable molecular interaction between EP3Re and wild type tau. HEK293 cells were transfected with MAPT and EP3Re constructs fused with either a LgBiT or SmBiT peptide at the N- or C- terminus. A and B show results from all combinations of vectors used to determine if there is a dynamic molecular interaction between EP3Re and tau. No interaction is detected. A non-interacting protein (HaloTag) with the corresponding interacting half of the pair was used as a negative control. Protein Kinase C Alpha (PRKACA) and Protein Kinase CAMP-Dependent Type II Regulatory Subunit Alpha (PRKAR2A), two proteins known to interact, were used as a positive control. Experiment performed with 8 technical replicates of each interaction with 3 independent experiments. Data shown from one experiment.

5.4.2 EP3Re signalling in a model of disease

So far, I have examined the relationship between EP3Re and wild type tau in primary cell lines. To understand how the receptor behaves in tauopathies, it was important to

use an appropriate disease model, given EP3Re's specificity to higher primates. There are few *in vitro* models that allow for the study of the human nervous system. Here we explore models of disease using both human primary cells lines and iPSC derived neurons.

5.4.2.1 Modelling disease in SHSY5Y cells

SHSY5Y cells express both endogenous EP3Re and tau. They are often used as models of disease due to their neuronal phenotype. An initial SHSY5Y based model of disease was created by exposing cells to fibrillar tau extract from an AD subject. Three days after an initial 3hr incubation with tau fibrils, cells were assessed for the presence of tau aggregates using pFTAA, which was then further validated by western blots by probing for the presence of Thr212/Ser214 phosphorylated tau (AT100). Despite initial promising results by microscopy suggesting the presence of tau aggregates in the fibril treated sample and not the control, as identified by pFTAA, this could not be validated by western blot (Fig. 5.8). Both the treated and untreated cells appeared to express tau phosphorylated at AT100 and AT8 epitopes with no discernible difference in AT100 phosphorylation levels. Following three independent experiments, with the same result, this model was not used for any further experiments.

Hypothermia as a model of tauopathy in SHSY5Y cells

Hypothermia has been shown to lead to tau hyperphosphorylation and has been proposed as a mechanism behind age related tau pathology. Given the involvement of EP3 in thermoregulation, and the association with hypothermia and hyperphosphorylation, we hypothesised that EP3Re could be involved in hypothermia mediated tau phosphorylation. We initially repeated the experiment as described by Planel et al. (2012) to show that a drop in temperature does increase tau phosphorylation.

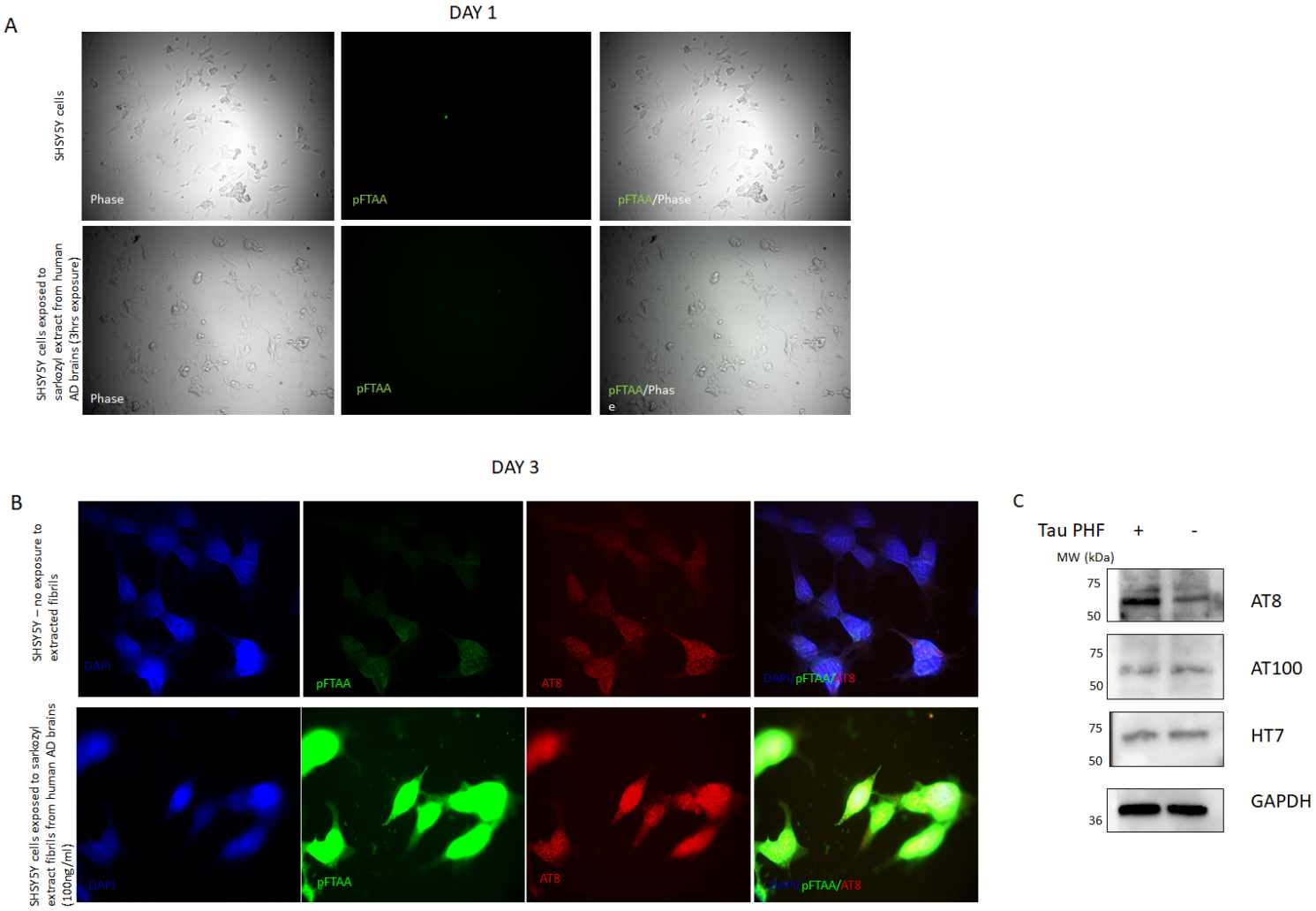


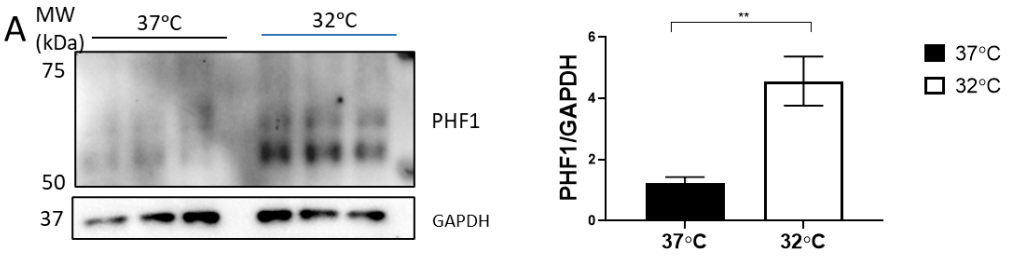
Fig. 5.8 Modelling tauopathies in SHSY5Y cells using tau fibrils. A) Live image of SHSY5Y cells on day 1 of seeding assay. No pFTAA positive cells were seen prior to seeding cells with fibrils extracted from AD brain. B) Cells were exposed to fibrillar tau extracted from AD brain for 3hrs before the media was changed and fixed 3 days later. Cells exposed to fibrillar tau show positive pFTAA staining. C) Protein extract lysed from cells and immunoblotted for phosphorylated tau. Differences in tau phosphorylation could not be validated by western blotting despite consistent pFTAA staining.

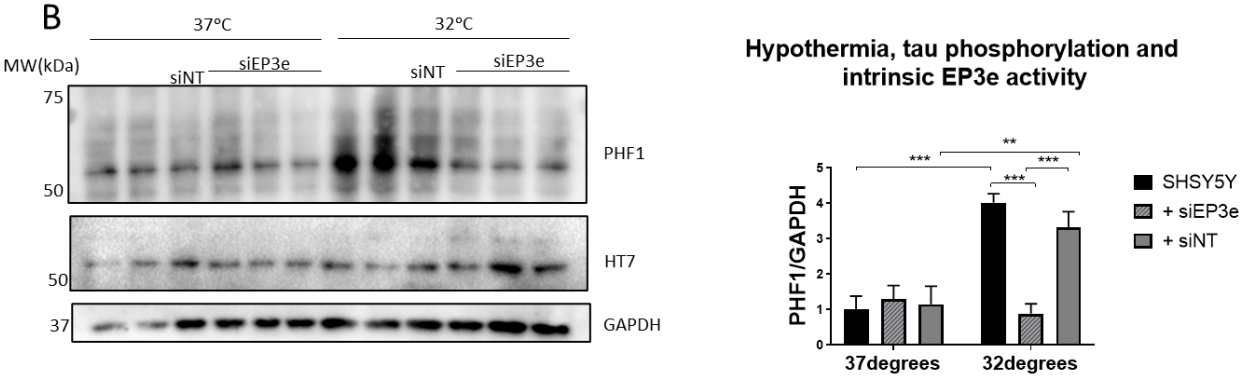
SHSY5Y cells were kept at 32°C for 2 hrs (Fig. 5.9). This resulted in an increase in tau phosphorylation by a factor of 4.5. EP3Re expression was then silenced and experiments repeated. Reduced EP3Re expression prevented hypothermia induced tau phosphorylation. Treating the cells with sulprostone at 32°C was not associated with any further changes in tau phosphorylation compared with untreated cells. To determine if this could be correlated with EP3Re signalling, IP1 accumulation assays were performed in HEK293 cells at 37°C and at 32°C. A reduction in temperature resulted in a 50% reduction in receptor activity as measured by Gi coupling. Reduced EP3Re activity as measured by Gi coupling, is thus unlikely to account for changes in tau phosphorylation associated with hypothermia.

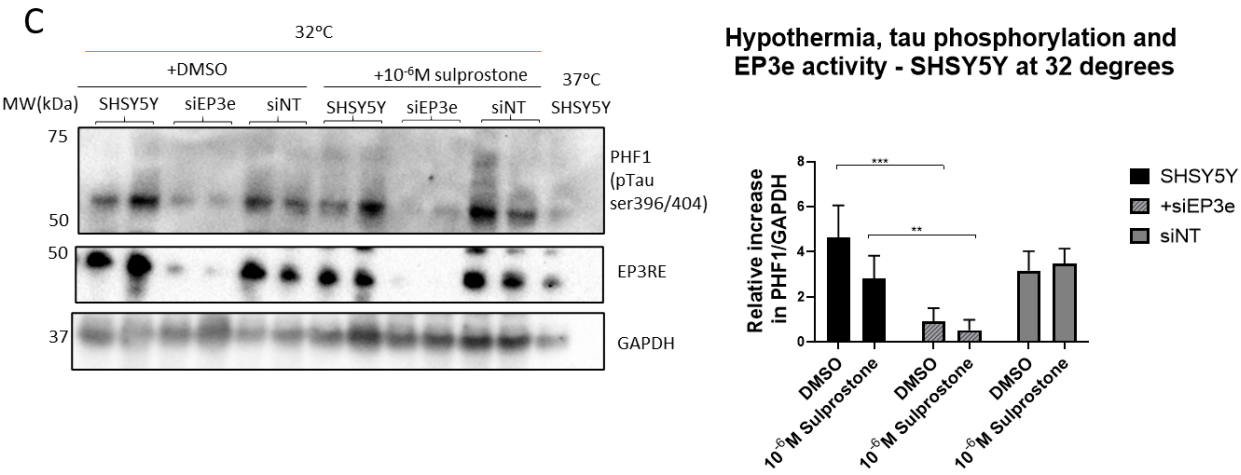
5.4.2.2 Modelling disease in HEK293 cells

To further explore EP3Re function in the context of tauopathy, we then went on to examine the relationship between EP3Re and tau in a model of familial tauopathy. The *MAPT* P301S mutation was first reported in an Italian family with early frontotemporal dementia and parkinsonism (Bugiani et al 1999) and has been reported in several families since (Sperfeld et al. 1999; Lossos et al 2003; Yasuda et al, 2000). The mutation alters microtubule binding and promotes aggregation of tau (Bugiani et al 1999). P301S tau and EP3Re were transfected into HEK293 cells and treated with sulprostone for 30min. Levels of tau phosphorylation were then assessed by immunoblotting. Similar to results seen in HEK293 cells transfected with wild type tau, an increase in tau phosphorylation was seen in response to EP3 activity. This increase in tau phosphorylation was also noted in the absence of EP3Re, suggesting other EP3 isoforms also contribute to tau phosphorylation in HEK293 cells (Fig. 5.10).

To further characterise the relationship between EP3Re and P301S tau, we next


A
MW (kDa)
37°C
32°C
75
50
37
PHF1
GAPDH
PHF1/GAPDH
37°C
32°C


B
MW(kDa)
37°C
32°C
siNT
siEP3e
PHF1
HT7
GAPDH
Hypothermia, tau phosphorylation and intrinsic EP3e activity
PHF1/GAPDH
SHSY5Y
+ siEP3e
+ siNT
37degrees
32degrees


C
32°C
+DMSO
+10-6M sulprostone
37°C
MW(kDa)
SHSY5Y
siEP3e
siNT
PHF1 (pTau ser396/404)
EP3RE
GAPDH
Hypothermia, tau phosphorylation and EP3e activity - SHSY5Y at 32 degrees
Relative increase in PHF1/GAPDH
SHSY5Y
+siEP3e
siNT
DMSO
10-6M Sulprostone

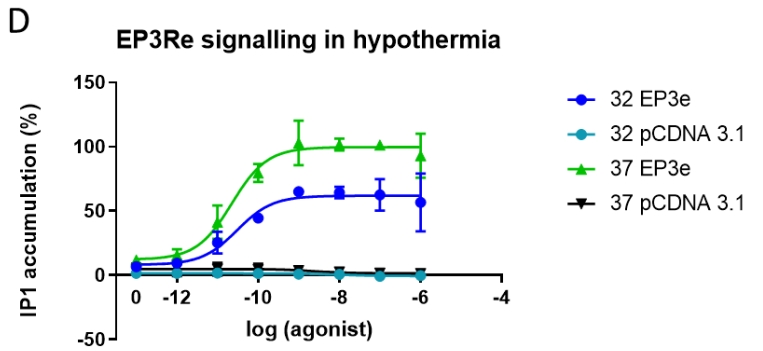

D
EP3Re signalling in hypothermia
IP1 accumulation (%)
log (agonist)
32 EP3e
32 pCDNA 3.1
37 EP3e
37 pCDNA 3.1

Fig. 5.9 Hypothermia as a model of tauopathy. A) Western immunoblot showing levels of tau phosphorylation in response to hypothermia with graphical representation of blot. A drop in 5°C is enough to increase levels of tau phosphorylation by a factor of 4.5. Experiments run in triplicate. Data shown representative of one independent experiment. Data analysed using unpaired t-test, p=0.0022. B) SHSY5Y cells were then transfected with either siRNA directed against EP3Re (siEP3e) or non targetting siRNA (siNT). Silencing of EP3Re inhibits hypothermia induced tau phosphorylation. Data shown results of 3 independent experiments. Analysed using 2 way ANOVA with multiple comparisons. Statistical significance taken at $p<0.05$. C) To determine role of active EP3Re signalling in hypothermia induced tau phosphorylation, cells were transfected with either siNT or siEP3e. SHSY5Y cells lacking EP3Re fail to show the increase in tau phosphorylation associated with the drop in temperature. There is no statistical difference between untreated and sulprostone treated cells. Experiments were run in duplicates with data generated from 3 independent experiments shown. Data analysed using 2 way ANOVA with multiple comparisons. Statistical significance accepted at $p<0.05$. D) IP1 accumulation assays performed in HEK293 cells in the presence of the chimeric G-protein, G$\alpha\delta$6qi4myr, so as to measure Gi activity. A 50% reduction is noted in EP3Re activity in response to a 5°C reduction in temperature. All experiments were run in triplicates. Cumulative data are shown from 3 different experiments with the mean and standard deviation. Data was fitted to a non-linear regression curve and normalised in GraphPad prism 7.0

examined the cellular localisation of EP3Re in this model. HEK293 cells transfected with flag tagged EP3Re alongside wild type tau or P301S tau, were treated with either sulprostone or ligand diluent (PBS), fixed and stained for the presence of the receptor using a FLAG antibody and a wheat germ agglutinin dye to identify the membrane. Results were examined by confocal microscopy. HEK293 cells expressing P301S tau appeared to show an increase in EP3Re expression on the membrane following exposure to sulprostone than the cells expressing wild type tau. To quantify this, FLAG ELISA was performed before and after sulprostone exposure. Although a trend was seen suggesting that the presence of P301S tau resulted in reduced FLAG tagged EP3Re internalisation following ligand exposure, this did not reach statistical significance (Fig. 5.10).

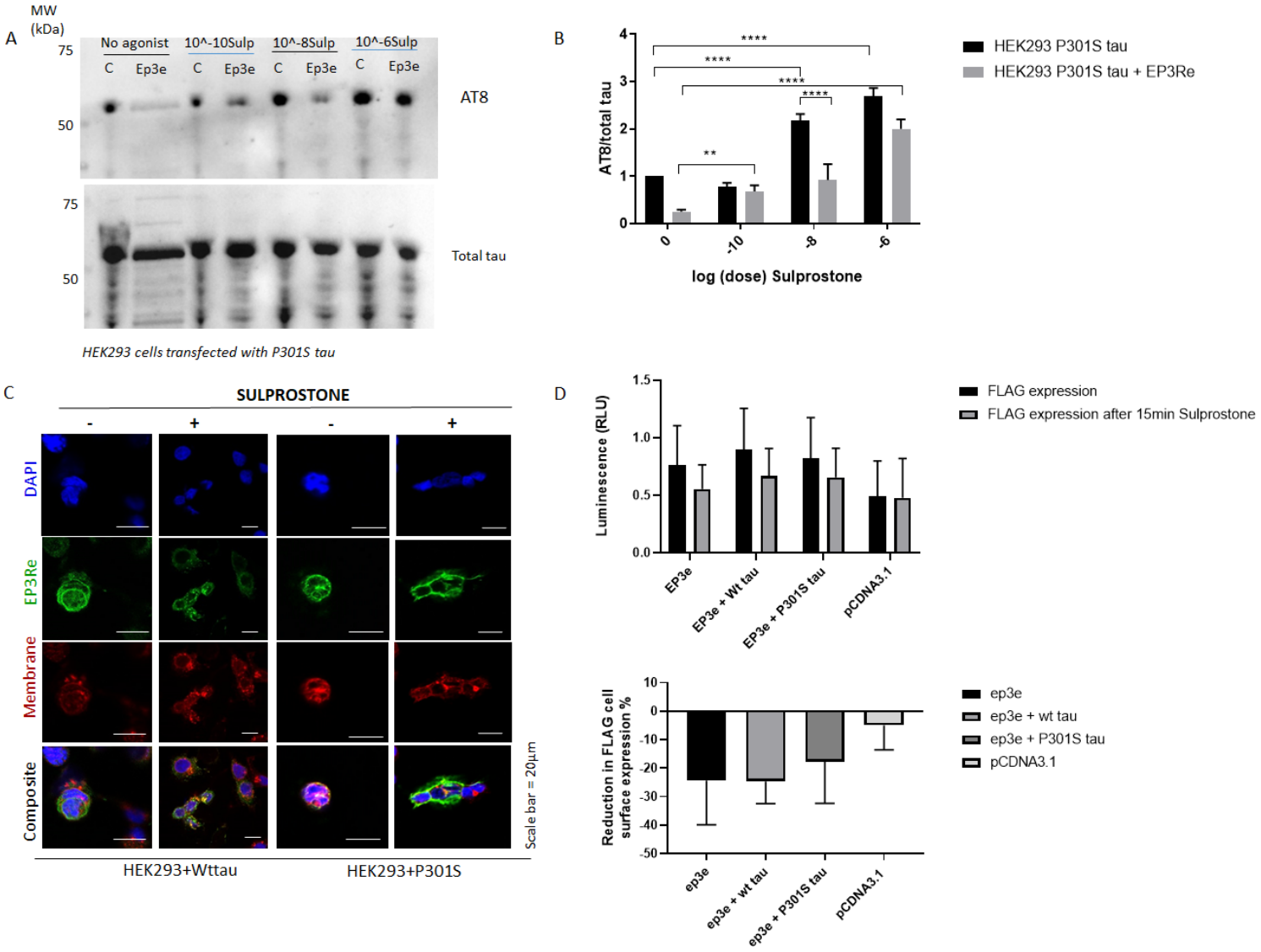


Fig. 5.10 Modelling tauopthies in HEK293 cells. A) Immunoblot showing levels of phospho-tau (AT8) in response to treatment with sulprostone in HEK293 cells expressing P301S tau with and without EP3Re. B) Graphical representation of data of 3 independent experiments. Level of tau phosphorylation increases in association with sulprostone levels ($p<0.0001$). The presence of the e isoform does not increase levels of tau phosphorylation and, in contrast to the results we show with wild type tau, at certain concentrations the presence of the e isoform results in lower levels of tau phosphorylation in response to sulprostone. Analysis done using 2 way ANOVA with multiple comparisons. Statistical significance determined at $p<0.05$. C) HEK293 cells were transfected with FLAG tagged EP3Re alongside wildtype tau or P301S tau. Cells were treated with sulprostone for 15min and stained before and after treatment for the receptor as well as the membrane. Following sulprostone treatment, EP3Re distribution in cells containing wild type tau appeared to move away from the membrane suggesting receptor internalisation, in contrast with cells containing P310S tau, where the receptor remained prominent on the membrane. D) EP3Re internalisation following 15min sulprostone exposure was assessed using a FLAG ELISA. Although a trend is seen suggesting reduced EP3Re internalisation in response to sulprostone exposure, this fails to reach statistical significance. Twelve technical repeats were performed for each condition with data shown from 3 independent experiments. Results were analysed with One way ANOVA using multiple comparisons. Statistical significance was taken at $p<0.05$.

5.4.2.3 Modelling disease in induced Pluripotent Stem Cell (iPSC) derived neurons

Human iPSC derived cortical neural stem cells were obtained from Axol from a control subject and an isogenic line edited using CRISPR-Cas9 technology to introduce the P301L mutation, the most frequent cause of familial frontotemporal dementia, into the *MAPT* gene. iPSCs with the *MAPT* N279K mutation were also differentiated, however, several attempts at differentiation were unsuccessful and therefore this iPSC line was not used for experiments.

EP3Re expression in iPSC derived neurons

EP3Re expression was initially examined in neural stem cells. Figure 5.11A shows that both lines expressed EP3Re at this stage with no significant differences in EP3Re intensity. Cells were then differentiated into neurons and by day 35, mature neurons as identified by beta III-tubulin, were noted in the cultures. Mature neurons continued to be positive for EP3Re, with predominant expression noted in cell bodies and little immunoreactivity noted in axons.

EP3Re interacts with tau in iPSC derived neurons with P301L MAPT mutation but not wild type tau

We next went on to characterise the physical interaction between tau and EP3Re in iPSC derived neurons. Proximity ligation assays between tau and EP3Re were performed on neurons derived from the control and isogenic P301L line. Figure 5.11F shows a signal was noted in the P301L line but not the control suggesting an interaction between EP3Re and tau that is present only in the P301L line.

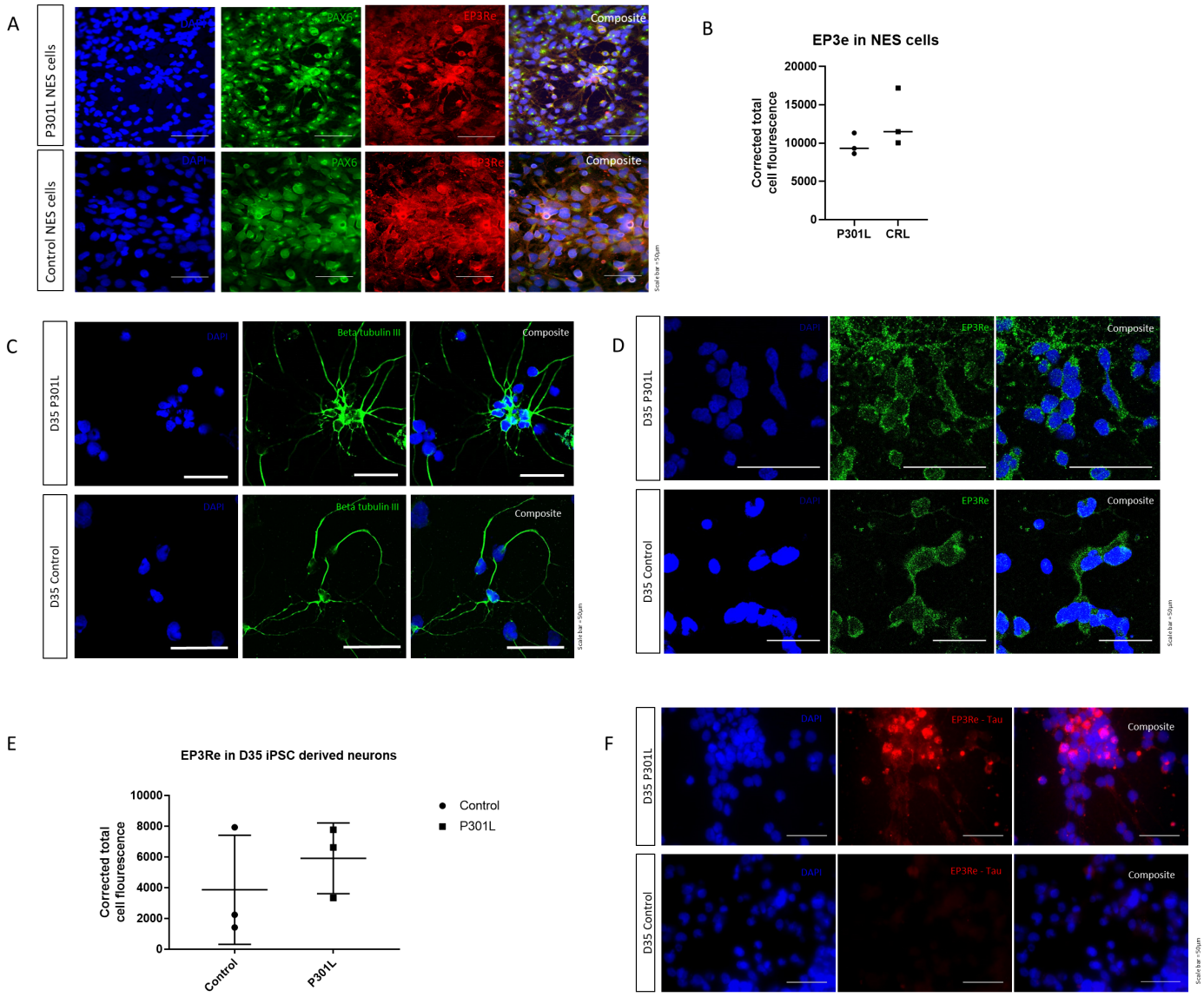


Fig. 5.11 Modelling tauopathies in iPSC derived neurons. A) Neural stem cells have been stained for the presence of EP3Re as well as PAX6, a marker for neural progenitor cells. EP3Re was expressed in both the control and P310L heterozygous line. B) Graph showing intensity of EP3Re fluorescence staining. There was no difference in EP3Re signal between control and P301L neural embryonic stem (NES) cells. Average intensity calculated from 10 fields of view from 3 rounds of differentiation. All experiments performed in triplicate. Unpaired t-test was used to determine differences in staining intensity. Statistical significance was taken at $p<0.05$. C) At D35 both control and P301L cultures showed the presence of neurons as identified by beta tubulin III. D) D35 iPSC derived neurons expressed EP3Re. E) Graph showing intensity of immunofluorescence EP3Re staining in both control and P301L iPSC derived neurons. There was no significant difference in EP3Re staining between the two lines. Data shown from 3 independent rounds of differentiation. Analysed using unpaired t-test. Statistical significance taken at $p<0.05$. F) Duolink proximity ligation assay done in fixed cells using antibodies directed against EP3Re and tau. There was an interaction between EP3Re and tau in the P301L line which was absent in the control line. Proximity ligation assays done in triplicates. Representative image of two rounds of differentiation.

EP3R stimulation increases tau phosphorylation in iPSC derived neurons

Day 35 neurons were exposed to the EP3 agonist sulprostone for 30min and levels of tau phosphorylation assessed by immunocytochemistry using the phospho-tau antibody AT8. At D35 the control line failed to show significantly increased AT8 staining in comparison to untreated neurons. In contrast, the neurons expressing the P301L mutation showed an increase in AT8 levels (Fig. 5.12). In iPSC derived neurons, EP3 signalling increased tau phosphorylation but only when the P301L mutation was present does it reach statistical significance. Neurons were also assessed for the development of tau aggregates using the fluorescent pentameric oligothiophene pFTAA, which has been shown to identify filamentous tau (Brelstaff et al 2015). No positive pFTAA staining was noted in the treated neurons. Although EP3 stimulation increases tau phosphorylation this did not lead to development of filamentous tau at day 35.

5.4.2.4 Effect of the tau P301S mutation on EP3Re signalling

We have shown that the presence of wild type tau does not affect EP3Re activity as measured by Gi coupling. To determine if the tau P301S mutation affects EP3Re activity, IP1 assays were repeated in HEK293 cells transfected with the chimeric G protein G$\alpha\delta$6qi4myr alongside EP3Re and P301S tau. There was a 40% reduction in EP3Re activity, as measured by IP1 accumulation, in the presence of P301S tau when compared to wild type tau or no tau. P301S tau impaired EP3Re/Gi coupling in HEK293 cells. The loss of the inhibitory effect of Gi should result in an increase in cAMP and any downstream effects of this. When we examined cAMP response element binding protein (CREB) using a luciferase reporter gene assay, this was noted to increase in the presence of P301S tau supporting a loss of inhibitory Gi signalling.

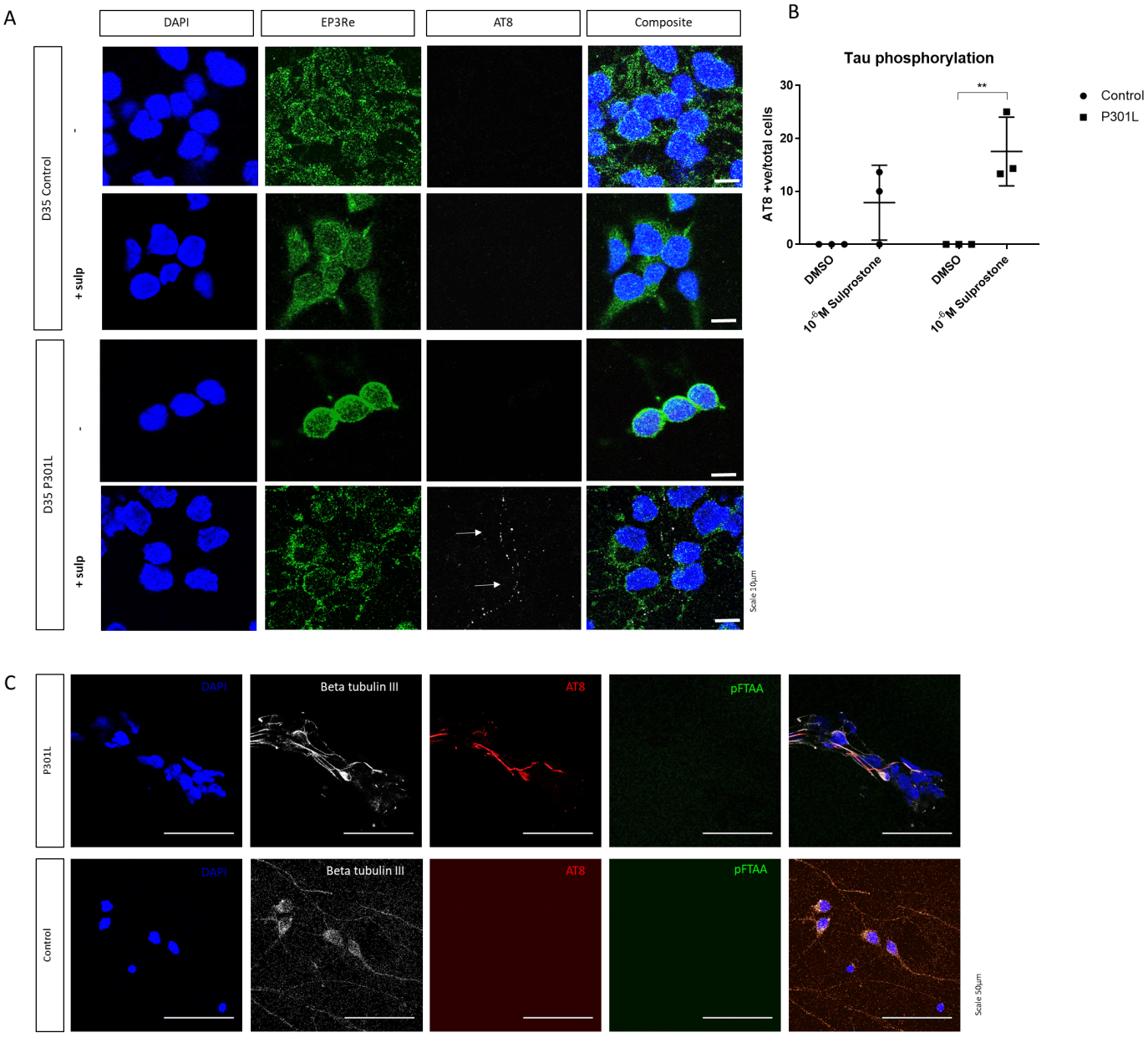


Fig. 5.12 EP3 stimulation increased tau phosphorylation in iPSC derived neurons. A) Confocal images of iPSC derived neurons exposed to sulprostone. There was an increase in tau phosphorylation but only in the MAPT P301L line did this reach statistical significance (B). Data shown from 3 independent rounds of differentiation and analysed using two way ANOVA with multiple comparisons. Statistical significance taken at $p<0.05$. C) Sulprostone treated iPSC derived neurons did not show any evidence of aggregated filamentous tau as identified by pFTAA. Experiments repeated 3 times per round of differentiation. Representative image following 2 rounds of differentiation.

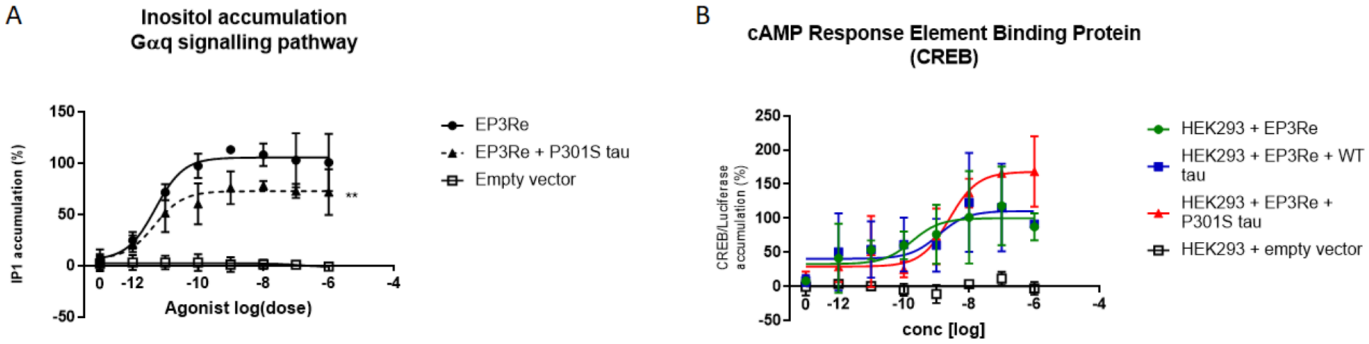


Fig. 5.13 EP3Re signalling in the presence of P301S tau. A. Inositol accumulation in HEK293 cells transfected with EP3Re and P301S tau alongside the chimeric G-protein G$\alpha\delta$6qi4myr. There was a 40% reduction in EP3Re activity in the presence of P301S tau. Data was fitted to a non-linear regression curve and normalised in GraphPad prism 7.0. Experiments were done in quadruplicates with cumulative data from 3 different experiments shown. Two-tailed unpaired t-tests were performed to determine if P301S tau affected EP3Re signalling. Statistical significance determined at $p<0.05$. B. EP3Re and CREB in the presence of P301S tau. An increase in CREB activity was noted supporting impaired EP3Re-Gi coupling with a subsequent loss in cAMP inhibition in the presence of P301S tau. Cumulative data shown from 3 independent experiments. All experiments done in triplicate. Data was fitted to a non-linear regression curve and normalised in GraphPad prism 7.0 and one-way ANOVA with multiple comparisons performed on Emax values. Statistical significance determined at $p<0.05$.

5.4.2.5 Molecular interaction between EP3Re and P301S tau

Finally we went on to examine the dynamic molecular interaction between EP3Re and P301S tau using the NanoBiT® protein:protein interaction assay. NanoBiT® vectors were fused to the C-terminal of the EP3Re receptor construct and either the C- or N-terminus of tau with the P301S mutation. HEK293 cells were transfected with both vectors and assays run for 20min to determine if EP3Re and P301S tau interact. No detectable signal was noted suggesting there was no interaction between P301S tau and the EP3Re C-terminal in HEK293 cells (Fig. 5.14). An interaction between the extracellular EP3Re N-terminus and tau was not examined.

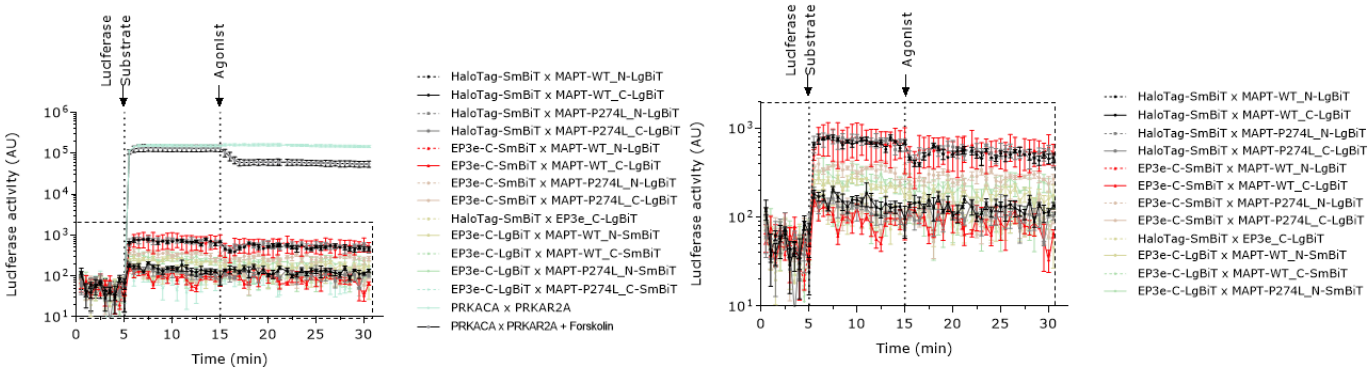


Fig. 5.14 There was no detectable molecular interaction between EP3Re and P301S tau. HEK293 cells were transfected with MAPT with the P301S mutation and EP3Re constructs fused with either a LgBiT or SmBiT peptide at the N- or C terminus. There was no detectable interaction. A noninteracting protein (HaloTag) with the corresponding interacting half of the pair was used as a negative control. Protein Kinase C Alpha (PRKACA) and Protein Kinase CAMP-Dependent Type II Regulatory Subunit Alpha (PRKAR2A), two proteins known to interact, were used as a positive control. Eight technical replicates were performed for each interaction. Data shown from one experiment, representative of three independent experiments.

5.5 Discussion

In this study we show that signalling through EP3 increases levels of tau phosphorylation in cell-based models of tauopathy. A summary of key findings regarding EP3Re and tau phosphorylation is summarised in table 5.1.

Table 5.1 Summary of results by cell-based model

CELL	WILD TYPE TAU	DISEASE MODEL
SHSY5Y	EP3Re stimulation increased tau phosphorylation and reduced GSK3β activity	In a hypothermia based model EP3Re activity mediated hypothermia induced tau phosphorylation
Transfected HEK293	EP3 stimulation increased tau phosphorylation (not specific to e isoform)	EP3 stimulation increased tau phosphorylation in models based on P301S tau (not specific to e isoform)
	Wild type tau had no effect on EP3Re signalling as measured by EP3Re-Gi coupling	P301S tau impaired EP3Re signalling as measured by EP3Re-Gi coupling
human iPSC derived neurons	No change in tau phosphorylation in response to EP3 stimulation	EP3 stimulation increased tau phosphorylation

Initial results obtained in SHSY5Y cells expressing endogenous tau, show an increase in tau phosphorylation at PHF1 tau epitopes (serine 396/404) in response to EP3 activity. In SHSY5Y cells we show this to be predominantly mediated via EP3Re. Unexpectedly, we noted an inhibition by EP3Re of GSK3β, an important kinase implicated in Alzheimer's dementia. Although unexpected, this is not surprising as PGE2, the endogenous EP3Re ligand, has been shown to inhibit GSK3 activity (Beurel et al., 2010). GSK3 is modulated via its phosphorylation by AKT at serine 9, and although the data is conflicting on the relationship between EP3 and AKT, studies in rat cere-

bellar astrocytes have shown PGE2 to promote AKT phosphorylation and subsequent activation via the EP3 receptor (Paniagua-Herranz et al., 2017). Although a trend is noted suggesting EP3Re activity promotes AKT activity in SHSY5Y cells, providing a mechanism by which GSK3 activity is inhibited, this failed to reach statistical significance in our present study. Other studies have shown PGE2 induced GSK3 inhibition to be driven via protein kinase A and cAMP through EP2 and EP4 (Fujino et al., 2002).

Study of EP3Re activity in HEK293 cells, as measured by Gi coupling, shows an increase in tau phosphorylation in association with an increase in EP3Re activity, regardless of whether the receptor is in the presence of wild type tau or tau with the P301S mutation, although these results are confounded by the presence of other EP3 isoforms. Overexpression of the e isoform when examining the signal transduction pathways, seems sufficient to differentiate EP3Re activity from other EP3 isoforms but, when examining levels of tau phosphorylation, it is difficult to determine the precise contribution of the e isoform. EP3 activity increases tau phosphorylation in a sulprostone dose dependent manner, but what is unclear is to what extent the e isoform contributes to this, given that an increase is noted in both the presence and absence of EP3Re. The absence of inositol accumulation in the HEK293 cells lacking the e isoform suggest that increases in tau phosphorylation, in cells without EP3Re following sulprostone treatment, is not mediated by Gi coupling. The finding in HEK293 cells that other EP3 isoforms contribute to tau phosphorylation suggests that in SHSY5Y cells, where no effect is seen in the absence of EP3Re, the e isoform is likely to be the predominant isoform expressed due to their neuronal phenotype. The fact that tau is endogenously present may also be relevant.

In this study we also show that intrinsic EP3Re activity is necessary for tau in-

duced hypothermia. Signalling assays previously performed in HEK293 cells showed a degree of EP3Re intrinsic activity in the absence of its ligand. Further activation of the receptor, using sulprostone, fails to result in any further changes in tau phosphorylation. The mechanism behind this is unclear. Signalling assays to correlate this with changes in EP3Re activity, show a reduction in temperature results in a depression of EP3Re activity, as measured by IP1 accumulation, which fails to support our findings. The discrepancy could be attributed to an alternative EP3Re signalling cascade independent of Gi coupling. Bretteville et al. 2012 propose that hypothermia induces tau phosphorylation by downregulating protein phosphatase 2A (PP2A) activity (Bretteville et al., 2012). EP3Re intrinsic activity may be necessary for this to occur, but the scope of this study does not examine a possible interaction between EP3Re and phosphatase activity, something that would have been interesting to explore.

We subsequently went on to examine how tau impacts EP3Re functions and show EP3Re activity, as measured by Gi coupling, is inhibited in the presence of tau with the P301S mutation. In keeping with this, when we examined EP3Re signalling by looking at the cAMP response element binding protein, this was increased in the presence of P301S tau confirming the loss of cAMP inhibition due to impaired Gi activity. A trend was also noted suggesting the presence of P301S tau disrupts receptor internalisation, although, this did not reach statistical significance. A number of pathological *MAPT* P301 mutations have been described in association with frontotemporal dementia, P301L, P301S and P301T (Bugiani et al., 1999; Erro et al., 2019; Spillantini et al., 1998). P301 is located within the microtubule binding domain in exon 10, and thus P301 mutations impair tau's ability to promote microtubule assembly (Goedert et al., 2000). Disrupted microtubule assembly can lead to defects in intracellular transport and may explain why we see an impairment in the function of the receptor. GPCRs following

activation, are targeted for internalisation into endocytic compartments (Mohan et al., 2012). It is then recycled back to the plasma membrane following re-sensitisation (Mohan et al., 2012). The classical canonical model of GPCR signalling states that receptor internalisation is a prerequisite for re-sensitisation. If this is impacted due to disrupted cellular transport and trafficking due to dysfunctional microtubules then this could result in reduced EP3Re function due to impaired re-sensitisation. Another possible theory is that the receptors physical interaction with tau impairs its ability to function, however assessing the molecular interaction between EP3Re and tau using a dynamic protein:protein interaction showed no interaction between EP3Re and wild type or P301S tau in HEK293 cells.

The NanoBit® protein:protein interaction assay has several limitations. The assay only explores the interaction between monomeric tau and not aggregated tau, and unless the assay is run till the point pathology develops, it is difficult to determine at which point EP3Re interacts with tau. The cells only express both proteins before experiments are performed for 24hrs and observed for 20mins to determine if an interaction is present in contrast to the assessments done in fixed cells or tissue, which examine a single time point of an interaction between two endogenous proteins. Although EP3Re fused to a Nanobit® vector at the extracellular facing N-terminal was used for experiments, tau would only have been expressed within the cytoplasm. Any interaction between tau and the N-terminal of EP3Re would not be detectable. The current experimental design only allows for detection of interactions that occur within the cytoplasm. The presence of a tag at each terminal may also have implications as to how the two proteins are able to interact.

We have established that in primary cell lines EP3Re activity increases tau phos-

phorylation in the presence of wild type tau as well as in models of disease, either by using hypothermia in SHSY5Y cells or expressing P301S tau in HEK293 cells. To understand if this is reflected in human disease, a better human model of tauopathy was required. To this end, we used neurons derived from iPSCs to understand if EP3Re signalling could be contributing to tau pathology by increasing tau phosphorylation.

The use of iPSC derived neurons allows us to study the disease pathogenesis using patient derived pathogenic cells endogenously expressing both proteins. This is particularly important given that we are trying to understand how a human specific receptor behaves in disease. The iPSC derived neurons recapitulated some of the results seen in studies performed on fixed tissue and primary cell lines. The iPSC derived neurons are shown to express the receptor at day 35 of differentiation, when neurons are present as shown by the presence of beta tubulin. Interestingly positive EP3Re immunoreactivity is also present in neural stem cells. Although we didn't look at EP3Re expression at multiple time points in between, it would have been interesting to see if expression varied as neurons matured. There is no discernible difference between expression of the receptor at the stages looked at between neurons derived from control lines and those derived from the P301L heterozygous line. Proximity ligation assays, similar to those done in fixed tissue sections, show an interaction between EP3Re and tau in neurons derived from the P301L line, but not control lines at day 35 of differentiation. The tau antibody used for these experiments was not conformation specific, and therefore it is difficult to say if this interaction is dependent on the tau conformation. These neurons at day 35 do not have tau tangles, as defined by pFTAA, suggesting that in the iPSC derived neurons, EP3Re interaction with tau is not dependent on tau being aggregated.

We show EP3 activation increases tau phosphorylation in P301L iPSC derived neurons but not neurons derived from isogenic controls. We know from work down by Iovino et al. characterising these neurons that those with the P301L *MAPT* mutation mature faster than those with wild type tau. Exposing neurons derived from the isogenic control to sulprostone at a later time point may have yielded a statistically significant increase in tau phosphorylation not seen at 35 days. Attempts to silence EP3Re to determine the contribution of this specific isoform to tau phosphorylation using siRNA targeting the receptor were unsuccessful. This will need to be further investigated using a more suitable tool to delete EP3Re expression. In this present study I can only state that EP3 signalling in iPSC derived neurons increases tau phosphorylation. We were unable to show this subsequently leads to development of tau aggregates in our experimental models of tauopathy. Previous characterisation of the P301L line (Iovino et al., 2015) show even if left to mature for up to 150 days they do not develop filamentous tau aggregates and therefore do not recapitulate all the features of tauopathy. Neurons derived from patients with the *MAPT* N279K mutation have also been described (Iovino et al., 2015). At 150 days, these cells have increased levels of tau phosphorylation, but importantly also develop filamentous tau aggregates (Iovino et al., 2015). Unfortunately, attempts to study EP3 signalling in this line were unsuccessful. They developed into non-neuronal cells or failed to survive. Due to time constraints, several of the experiments on iPSC derived neurons were only performed on two biological replicates and will need to be repeated a third time to ensure these findings remain valid.

In conclusion, in this present study we show that EP3 signalling increases tau phosphorylation in models of tauopathy based on P301 tau mutations but not necessarily in the presence of wild type tau under normal physiological conditions. In turn P301S tau impairs EP3Re activity as measured by Gi coupling. We also show that EP3Re

is necessary for hypothermia induced tau phosphorylation. The study is limited by the fact only one tau mutation was examined to study the behaviour of EP3Re in tauopathies. Future studies will need to explore the behaviour of the receptor in the presence of other tau mutations to determine if the effects seen are unique to P301 mutations or if this occurs in all mutations associated with tauopathies. The findings of the study also suggest the e isoform is not the only receptor mediating the effects of sulprostone on tau phosphorylation but other EP3 receptors are also playing a role. Further work will be required to study each isoform in turn to determine which of the EP3 isoforms are likely to be playing a role in tauopthies.

Chapter 6

DISCUSSION

6.1 Summary

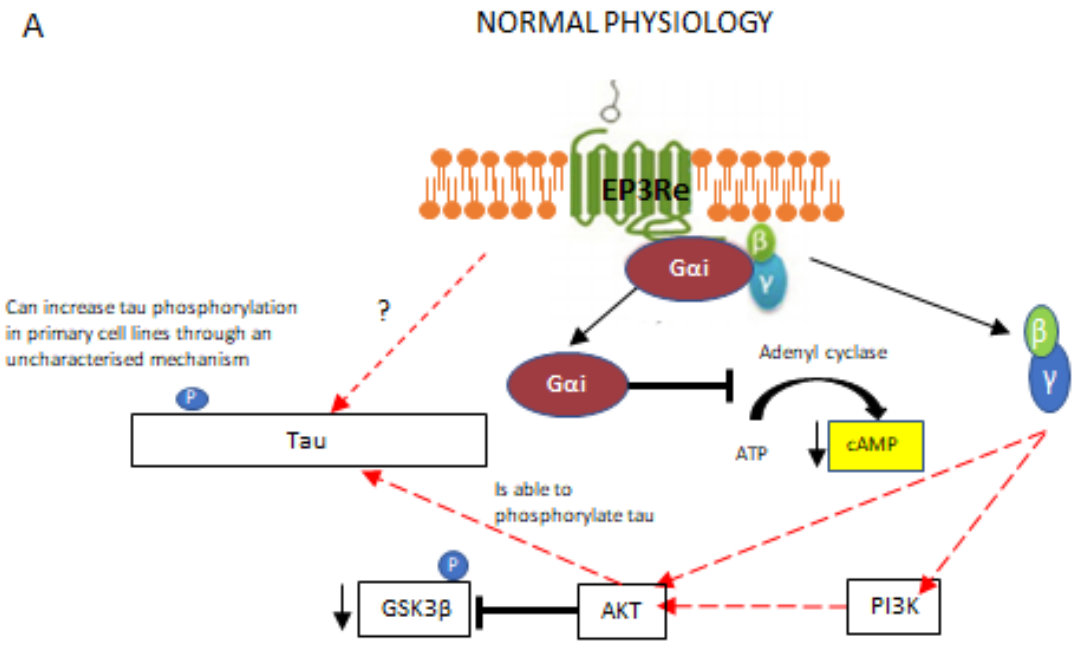
This project evolved from an incidental finding suggesting an interaction between tau and EP3Re. Our aim was to better understand EP3Re distribution and function in human brain and its relationship, if any with tau. Studies have implicated inflammatory PGE2 signalling in tauopathies but due to the broad effects of PGE2, inhibiting its production would have systemic effects and implications beyond disease pathology. A greater understanding of the tau-EP3Re interaction is required to consider taking a more targeted approach. The work performed during my PhD shows that the e isoform of the PGE2 EP3 receptor is expressed throughout the human brain and appears to localise with tau aggregates in tauopathies. This is the first study to characterise the distribution of EP3Re in the human brain, where we found neuronal expression throughout the brain, with strong expression in brainstem nuclei and the hypothalamus. We also show that EP3Re signals through Gi coupling and the presence of the tau P301S mutations appears to impede this. We also found that signalling through EP3 increases tau phosphorylation in our models of disease. Based on studies using P301S tau mutations, the EP3 receptor, and in particular the e isoform, was found to play a role in hypothermia mediated tau phosphorylation.

6.2 EP3Re signalling and tau phosphorylation - A potential role in tau pathology

The PGE2 EP receptors are all G-protein coupled receptors (GPCRs) and thus signal via Gi, Gq or Gs and associated second messenger signalling cascades. Similar to other studies looking at EP3 signalling (Hatae et al., 2002b; Hizaki et al., 1997; Morimoto et al., 2014), which suggest EP3 couples predominantly to Gi, we found the human

EP3Re receptor to signal preferentially through Gi coupling. The original linear dogma of one receptor coupling to one G-protein has however proven to be inadequate. Most G-protein coupled receptors interact with diverse G proteins eliciting multiple intracellular signals. To this end several other EP3 isoforms have been shown to interact with multiple G-proteins and in this present study our assays have shown EP3Re is also capable of coupling with Gq. It is unclear why certain GPCRs are able to couple to multiple Gα subunits whilst others are more selective. Three-dimensional modelling of GPCRs have revealed multiple latent intracellular cavities to which G-proteins interact with at varying strengths (Sandhu et al., 2019). It has been proposed that incorporation of selective residues throughout evolution within these cavities is sufficient to reshape the intracellular cavity making multiple binding partners possible (Sandhu et al., 2019). Targeting these residues, inhibiting or promoting one signalling cascade over another, creates a potential therapeutic strategy should EP3Re-Gi or EP3Re-Gq coupling have functional implications in disease. To this end, we attempted to correlate EP3Re signalling with tau pathology, mainly focusing on tau phosphorylation. In figure 6.1 we summarise the key findings and postulate possible mechanisms underlying the relationship between EP3Re and tau under normal physiological conditions and in our models of tauopathy.

Under normal physiological conditions and in the presence of wild type tau, we found EP3Re activation inhibited GSK3β but not GSK3α. GSK3, and in particular GSK3β, is an important serine/threonine kinase strongly implicated in tau phosphorylation and Alzheimer's dementia, and is a proposed therapeutic target for disease (Griebel et al., 2019; Hooper et al., 2008; Lauretti and Praticò, 2020; Llorens-Martín et al., 2014; Serenó et al., 2009). The mechanism through which EP3Re inhibits GSK3β is not yet clear, however, we found a trend suggesting an increase in active AKT. AKT, when


A
NORMAL PHYSIOLOGY
EP3Re
Gαi
β
γ
Can increase tau phosphorylation in primary cell lines through an uncharacterised mechanism
?
Adenyl cyclase
Gαi
ATP
cAMP
Tau
P
Is able to phosphorylate tau
GSK3β
AKT
PI3K

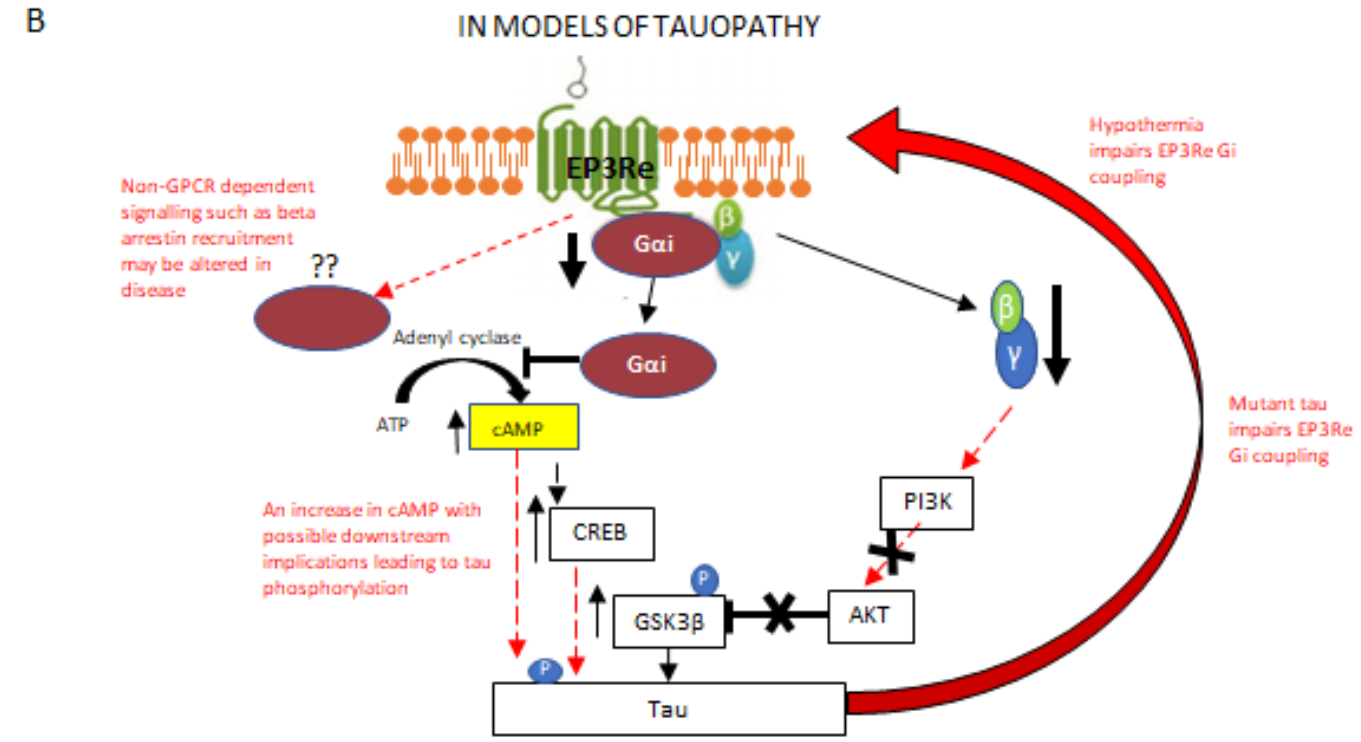

B
IN MODELS OF TAUOPATHY
EP3Re
Gαi
Hypothermia impairs EP3Re Gi coupling
Non-GPCR dependent signalling such as beta arrestin recruitment may be altered in disease
??
Adenyl cyclase
Gαi
ATP
cAMP
Mutant tau impairs EP3Re Gi coupling
An increase in cAMP with possible downstream implications leading to tau phosphorylation
CREB
PI3K
GSK3β
AKT
Tau

Fig. 6.1 A) EP3Re signalling under normal physiological conditions. EP3Re signals predominantly through Gi coupling. Upon ligand binding the receptor undergoes a conformational change resulting in dissociation of the Gαi subunit from the $\beta\gamma$ subunit. The Gαi subunit inhibits adenyl cyclase preventing cAMP production from ATP. Data obtained from experiments in SHSY5Y cells show EP3Re activation to be associated with a reduction in active GSK3β. This could be mediated by the increase observed in AKT which in turn phosphorylates and inactivates GSK3. Studies have shown that AKT can be activated through direct binding of the $\beta\gamma$ subunit or indirectly through PI3K. The increase in tau phosphorylation observed in our SHSY5Y and HEK293 cell lines should be interpreted with caution given that this is not observed in the iPSC derived neurons. It remains to be determined whether the noted increase in tau phosphorylation is mediated by AKT, or by a yet uncharacterised mechanism.
B) EP3Re signalling in models of tauopathy. In our models of tauopathy based on hypothermia and P301L tau mutations, we observed a reduction in EP3Re-Gi coupling. This would result in an increase in cAMP and downstream signalling targets such as cAMP dependent protein kinases and CREB. In keeping with this, in our disease model based on P301S tau, we also observed an increase in CREB. We also note an increase in tau phosphorylation in our models of disease in response to EP3Re activation by sulprostone. This is not mediated through EP3Re Gi-coupling, as our signalling assays in HEK293 cells show an increase in tau phosphorylation in the absence of EP3Re-Gi coupling. This is likely to occur either due to the loss of previously observed GSK3β inhibition by EP3Re, loss of cAMP inhibition, activating kinases downstream of cAMP, such as PKA, or, alterations in non-GPCR dependent mechanisms, such as changes in beta-arrestin recruitment. ATP- Adenosine triphosphate, cAMP – cyclic adenosine monophosphate, CREB – cAMP response element binding protein, AKT – RAC-alpha serine/threonine-protein kinase, PI3K – phosphoinositide 3-kinase, GSK3β - glycogen synthase kinase 3β.

phosphorylated is, in turn, able to inhibit GSK3 via phosphorylation at serine9 and EP3Re could reduce GSK3β activity via this mechanism. With this apparent reduction in GSK3β, one would expect an associated reduction in phosphorylated tau, however, data obtained from our experiments in SHSY5Y and HEK293 cells show an increase in tau phosphorylation in response to EP3Re activation by the EP3 agonist sulprostone. This does appear to be independent of Gi coupling and not specific to EP3Re as this response is seen in HEK293 cells lacking the e isoform suggesting this is being mediated by other EP3 isoforms. In figure 6.1 we suggest this increase in tau phosphorylation, could be mediated by AKT, which is known to phosphorylate tau at serine 214 (Kyoung Pyo et al., 2004), or an uncharacterised mechanism. In the control iPSC derived neurons expressing wild type tau, no increase in tau phosphorylation is seen in response to EP3Re stimulation by sulprostone. This contrasts with our findings in SHSY5Y and HEK293 cell lines where an increase in tau phosphorylation is noted. In view of the findings in iPSC derived neurons, the results obtained in our human cell lines should be interpreted with caution. HEK293 cells were transfected and would likely have expressed levels of tau above endogenous levels unlike the iPSC derived neurons, where one would expect a more physiological level of tau expression comparable to what we find *in vivo*. SHSY5Y cells also express PHF1 and AT8 phospho-tau epitopes during proliferation (Pope et al., 1994) which could have contributed to the levels of tau phosphorylation noted. The discrepancy noted between iPSC derived neurons and human cell lines creates some uncertainty as to the contribution of EP3 or EP3Re to tau phosphorylation under normal physiological conditions. Continuous treatment of control iPSC derived neurons with sulprostone, modelling a chronic inflammatory process, may have resulted in a similar increase in phosphorylated tau, as seen in human cell lines but time limitations meant we were unable to fully explore this hypothesis. The evidence that EP3Re increases tau phosphorylation under pathological conditions

is more consistent. We have found an increase in phospho-tau in SHSY5Y and HEK293 in our models of tauopathy based on expressing P301S tau, hypothermia, as well as iPSC derived neurons expressing tau with the P301L mutation. The finding that EP3Re increases tau phosphorylation in our models of disease but not consistently in the presence of wild type tau would suggest that inflammatory signalling via EP3Re on its own is not sufficient to drive disease pathology but a primary stimulus, such as hypothermia or tau mutation, is required. Our results also demonstrate that the effects on tau are not unique to the e isoform. This is supported by the recent findings that the murine EP3 receptor increases tau phosphorylation in murine neuroblastoma cells (Cao et al., 2019).

Signalling assays performed in HEK293 cells expressing P301S tau, as well as cells exposed to hypothermia, showed a reduction in EP3Re-Gi coupling as measured using our second messenger IP1 assay, not seen when wild type tau is expressed at normothermia. This supports our view that any resulting tau phosphorylation seen in the presence of EP3Re activity is independent of Gi coupling. In keeping with loss of inhibitory Gi signalling, we also note an increase in cAMP response element binding protein (CREB) in the presence of P301S tau but not wild type tau. The downstream effects observed are likely to be mediated by an alternative signalling cascade.

6.3 Signalling via G-protein or alternative pathways

G-protein independent signalling such as β-arrestin dependent mechanisms, have been shown to trigger endocytosis and more importantly activate kinases leading to specific signalling pathways (Jean-Charles et al., 2017). β-arrestins are well known regulators of G-proteins. Following GPCR activation, β-arrestin has been shown to

translocate from the cell membrane, binding to the receptor, uncoupling it from the G-protein promoting receptor internalisation and desensitisation (Gainetdinov et al., 2004). Interestingly, in HEK293 cells expressing P301S tau, we found a reduction in receptor internalisation following sulprostone exposure. We hypothesise that this could be secondary to changes in microtubule function and transport contributing to the reduction in EP3Re-Gi coupling noted, but changes in β-arrestin function could have similar implications. GPCR-β-arrestin complexes are able to instigate G-protein independent signalling cascades and have been shown to interact with kinases such as AKT, JNK and p38 (Jean-Charles et al., 2017; Reiter et al., 2012), kinases known to phosphorylate tau. This provides an alternative signalling mechanism, independent of EP3Re-Gi coupling, that could account for the changes that we observe on tau phosphorylation which do not correlate with Gi activity. Individual EP3 isoforms have been shown to internalise differently and this is believed to be determined by differences in C-terminus (Bilson et al., 2004). Although internalisation of EP3Re has not been previously characterised, EP3 I and f have been shown to internalise with β arrestin. Further characterisation of EP3Re by investigating β-arrestin recruitment and function would be important to determine if this signalling mechanism could be implicated in the changes observed in our models of disease. This discrepancy between impaired EP3Re signalling and an increase in EP3Re mediated tau phosphorylation in our models of disease could therefore be explained by differences in GPCR independent vs dependent signalling, with one being suppressed whilst the other drives tau pathology in tauopathies. Alternatively, this could be a protective mechanism in which EP3Re initially promotes tau phosphorylation which in turn suppresses EP3Re activity. In support of this hypothesis studies have shown an increase in PGE2 in early Alzheimer's disease, when it could be driving tau pathology, with lower levels in advanced disease

stages (Combrinck et al., 2006; Montine et al., 1999), once neurodegeneration is advanced.

6.4 Thermoregulation

The reduction in EP3Re function as measured by Gi coupling in our models of disease could have implications beyond tau pathology. An alteration in EP3Re function could be reflected in impaired thermoregulation and autonomic dysfunction as described clinically in tauopathies (Carrettiero et al., 2015; Engelhardt and Laks, 2008; Oliveira et al., 2019). EP3 expressing paraventricular neurons in the hypothalamus have been shown to mediate PGE2 central excitatory effects on the sympathetic nervous system including hypertension and tachycardia (Ariumi et al., 2002; Chen et al., 2012; Zhang et al., 2011). In our study we have shown EP3Re to be expressed in the paraventricular neurons of the hypothalamus and is thus likely to be one of the isoforms mediating PGE2 effects on the sympathetic nervous system. If the changes observed in EP3Re-Gi coupling in the presence of P301S tau do occur in human disease, this could contribute to the impairments in the sympathetic nervous system function associated with tauopathies (Oliveira et al., 2019).

Thermoregulation and generation of pyrexia is a well characterised function of EP3. Loss of thermoregulation has been demonstrated in Alzheimer's as well as normal ageing (Norman et al., 1985; Vandal et al., 2016) and could be associated with impaired EP3Re function as measured by Gi-coupling. EP3 receptors in the median preoptic nucleus have been shown to mediate PGE2 induced febrile response (Lazarus et al., 2007; Morrison and Nakamura, 2011). In our present study we found EP3Re to be expressed in the median preoptic nucleus as well as the dorsomedial hypothalamus, two critical areas in thermoregulation, and is thus likely to be one of the EP3 isoforms involved

in thermoregulation. Selective deletion of EP3 receptors in the mouse brain, and in particular the median preoptic nucleus, not only eradicates the pyrexial response, but also promotes hypothermia (Lazarus et al., 2007). Interestingly we show in SHSY5Y cells that hypothermia induced tau phosphorylation is dependent on EP3Re. Similar to sulprostone induced tau phosphorylation, this is independent of EP3Re-Gi coupling. The mechanism behind hypothermia induced tau phosphorylation has been largely attributed to inhibition of serine/threonine protein phosphatase 2 (PP2A) (Bretteville et al., 2012; Planel et al., 2004). However other mechanisms could also be involved. Diabetic murine models, based on impaired leptin signalling, display an increase in tau phosphorylation associated with a reduction in their basal temperature. These changes reverse on return to normothermia (El Khoury et al., 2016). In these mice it is notable that no deregulation of kinases or phosphatases was found, specifically PP2A. This suggests a mechanism independent of PP2A inhibition.

If EP3Re function is impaired in human disease, as we have shown it to be in our models of disease, then this could account for the changes in thermoregulation that have been noted in Alzheimer's (Vandal et al., 2016). With the proposal that hypothermia may even drive tau phosphorylation (El Khoury et al., 2016; Tournissac et al., 2017), a potential mechanism emerges linking impaired EP3 function and tau phosphorylation through deficits in thermoregulation and hypothermia.

6.5 Tau tangles and EP3Re

This study expanded from the finding that in tauopathies EP3Re is present in tau tangles. Although our *in vitro* experiments show EP3R/EP3Re signalling increases tau phosphorylation, it is unclear if EP3Re remains functional if bound to tau within the cytoplasm. Signalling cascades through GPCRs are traditionally thought to emanate

at the cell membrane, however, there is growing evidence that translocated GPCRs are capable of interacting with intracellular membranes, binding with different partners and instigating different signalling cascades compared to when bound to the cell membrane (Jong et al., 2018). Indeed, EP3 has been shown to signal not only from the cell membrane but also when translocated to the nuclear membrane (Bhattacharya et al., 1999). The significance of the physical interaction between EP3Re and fibrillary tau that we identify in this study is unclear. Tau aggregates have been known to sequester a number of proteins including molecular chaperones with resulting functional inhibition of chaperone-dependent events of protein folding and clathrin mediated endocytosis (Yu et al., 2019). The findings reported in this study could represent EP3Re being entrapped by aggregated tau rather than the receptor being an intrinsic component of tau tangles. Our interaction assays show no interaction between monomeric tau and EP3Re, nor do they show an association between the intracellular EP3Re C- terminus and the N- or C- terminus of tau. This leaves the possibility that an interaction could be occurring between tau and the EP3Re extracellular N- terminus, although due to time constraints we were not fully able to explore this possibility. If this proves to be the case, this proposes a potential role for EP3Re in tau propagation by internalising extracellular tau aggregates into the cell however, more studies and use of mass spectrometry of tangle extracts can determine the interaction specificity and nature of the interaction between EP3Re and tau in neurofibrillary tangles. If this interaction were confirmed it will provide important evidence for a role of EP3Re in tauopathies.

6.6 Limitations and future direction

In the present study we have described the distribution of EP3Re in human brain, the signalling mechanism associated with it, and the interaction between EP3Re and

tau. We, however, cannot state that this interaction is unique to the e isoform and experiments performed measuring tau phosphorylation in HEK293 cells would suggest that other isoforms may also contribute to increase tau phosphorylation. The study of individual isoforms is challenging due to the lack of commercially available antibodies. Further work examining EP3 RNA would allow us to identify all the isoforms present in the human brain and compare this to isoforms expressed in our primary cell lines and iPSC derived neurons. Furthermore, in iPSC derived neurons we were only able to study the effect of EP3 signalling on tau phosphorylation, and not specifically EP3Re. Attempts to silence EP3Re were unsuccessful due to the poor survival rate of the neurons. Genomic engineering using guide RNAs complexed with the nuclease Cas9 from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system has proven to be a highly valuable tool in molecular biology. Several studies have successfully used CRISPR-Cas9 in iPSC derived neurons, as well as primary cell lines, to model disease (Fang et al., 2006; Komor et al., 2016; Ortiz-Virumbrales et al., 2017; Srikanth et al., 2015). In contrast to siRNA, which reduces gene expression, CRISPR-Cas9 would provide irreversible gene knockout and a more specific mechanism to further study the role of EP3Re in tauopathies. This would allow us to determine the specific contribution of the e isoform to our results, although it would be difficult to exclude the possibility of a redundancy effect in EP3Re knockout neurons, whereby the function of EP3Re is overtaken by other EP3 isoforms.

Our studies in HEK293 cells and iPSC derived neurons was also limited to tau mutations involving P301 – P301L or P301S. To understand if our findings could be relevant across all tauopathies, further work will be required examining the relationship between EP3Re and tau in the presence of other tau mutations. Tau mutations fall into two major categories, those that influence alternative splicing of tau and those

that alter tau at the protein level (Goedert, 2003; Strang et al., 2019). The mutations used in our present study alter tau at the protein level disrupting its functions, such as axonal transport and microtubule binding, promoting filament formation and tau aggregation (Goedert and Jakes, 2005). P301 is situated in exon 10 and therefore P301S and P301L tau mutations are present in 4R tau isoforms. Although we show EP3Re to interact with aggregates consisting of both 3R and 4R tau, we do not know if the results of our functional assays, such as Gi-coupling, would be replicated with tau mutations outside of 4R tau. The P301L and P301S tau mutations are in the microtubule binding domain and thus reduce the ability of tau protein to interact with microtubules impairing microtubule extension and stabilisation (Dayanandan et al., 1999; Falcon et al., 2015; Goedert and Jakes, 2005). Disrupted microtubule function may contribute to the changes we see in EP3Re function, such as reduced receptor internalisation and Gi-coupling. Studying the impact of other tau mutations such as N279K, which has its effect at the RNA level by increasing exon 10 splicing, disrupting the 3R:4R tau isoform ratio, will be important to determine if our findings are relevant in other tau mutations. The association of EP3Re with tangles in sporadic Alzheimer's and PSP would support that the interaction is not specific for a disease, tau mutation or tau isoform.

Fully recapitulating a complex disease process in a two-dimensional (2D) cell culture system is challenging. Three-dimensional (3D) brain organoid systems generated from human pluripotent stem cells have shown great potential at being able to model the complex interactions between glial cells and neurons (Lancaster and Knoblich, 2014), which our two-dimensional cultures fail to capture. Our study is also limited to EP3Re neuronal signalling by the models we have available. Microglia are the resident immune cells within the central nervous system and are key mediators of

the neuroinflammatory process (Bachiller et al., 2018). Activated microglia produce pro-inflammatory factors, including PGE2 (Zhang et al., 2009). The possibility that microglial PGE2 secretion may influence or be influenced by EP3Re expression needs to be explored and the interplay between microglia and neurons with regards to EP3Re signalling further characterised. Other EP receptors have been shown to interact with microglial. PGE2-EP2 signalling has been shown to suppress beneficial microglial functions such as chemotaxis and amyloid clearance (Johansson et al., 2015). PGE2-EP3Re signalling could be exerting a similar effect. Future studies using 3D brain organoids would allow us to better understand the role of EP3Re signalling in non-neuronal cells, as well as how this could influence neuronal-glial interaction.

In summary, we have shown that EP3Re is widely distributed in the human brain, interacts with tau in tauopathies, and, signalling through EP3 and EP3Re increases tau phosphorylation in our cellular models of disease. This is independent of Gi coupling, the main EP3Re signalling pathway identified in our study. Further work is required to fully elucidate the mechanism is and understanding if targeting inflammatory prostaglandin signalling through EP3Re or other EP3 isoforms could ameliorate tau pathology.

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Appendix A

EP3RE-FLAG (Flag sequence in red)

GAATTCACCATGGACTACAAGGATGACGATGACAAAATGAAGGAGACCC
GGGGCTACGGAGGGGATGCCCCCTTCTGCACCCGCCTCAACCACTCCTAC
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TAU-VENUS

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P301STAU-VENUS

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NFAT-LUC

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FR-LUC

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