Understanding how Age and Biological Sex Influence the Development of Alzheimer's Disease

Jawza A. Almutairi

Cardiff University

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THESIS ABSTRACT

Alzheimer's disease is a highly complex neurodegenerative disease and multifactorial. Age is the most significant risk factor for Alzheimer's disease (AD), with cases doubling every five years after 65. Thus, one of the most challenging areas in AD research is understanding what happens to the brain when it ages. Such insights could aid in distinguishing individuals who are more susceptible to developing AD during ageing. Over the last 25 years, brain ageing studies have looked at thousands of human brains to investigate the neuronal basis of age-related cognitive decline. However, most of these studies enrolled adults over 60 years of age. Therefore, those studies overlooked the most significant period of neuroendocrine changes in a woman's life, the menopause transition period. In the menopause phase, females undergo a significant decline in ovarian sex steroid production, including approximately 90% of oestrogen (E2) production. It is well documented that E2 has a neuroprotection function in the brain. Thus, the dramatic loss of sex steroids during menopause impacts multiple biological systems in the body, including the brain. In addition, despite documented sex disparities in the risk for dementia, the effect of biological sex and sex hormones on human brain ageing and AD development is understudied. Thus, in this thesis, it was hypothesized that an inter-relationship between age and biological sex could impact brain structure and function during ageing and increase the susceptibility of women to develop AD.

In this thesis, AD biomarkers and their processing proteins, along with E2-associated proteins expression, were investigated in frontal cortical brain samples from young (20-30), middle-aged (45-55), and elderly (70-90) males and females with no history of dementia, and in AD samples (70-90). A sex disparity during brain ageing and AD in the expression of AD biomarkers was reported in the first two experimental chapters, with females exhibiting age-related upregulation in the levels of APP and its amyloidogenic enzymes. Also, Aβ overproduction was observed in both sexes with advancing age, but its levels were significantly higher in aged female samples compared to aged males. In addition, higher levels of tau and GSK3β were found in the aged female frontal cortex compared to the male frontal cortex. In AD samples, these sex disparities in AD biomarkers were also visible in higher Aβ levels and tau hyperphosphorylation in female AD patients compared to AD male patients. When E2-associated proteins were investigated, oestrogen receptor (ERα and ERβ), in male samples only ERβ and its downstream signalling molecules (Akt and ERK2) were upregulated in the

male frontal cortex with ageing, reported in chapter 5, while middle-aged female samples have shown a decline in the level of ERβ and an age-related decrease in ERα in chapter 6. In AD samples, ERβ expression declined in males in chapter 5, and in females, both ERα and ERβ were decreased in chapter 6. Thus, the decline of ER in middle-aged females and AD of both sexes samples could indicate a reduction in E2 neuroprotection function; E2 can regulate Aβ production, and it is the most significant neuroprotection function against AD. The neuroprotection of E2 against AD was illustrated in chapter 7, where the treatment of non-transfected female AD neuronal human induced pluripotent stem (hIPS) cells with E2 showed an apparent significant decline in Aβ levels.

An inter-relationship between brain ageing and biological sex in AD development was apparent in this project. The findings of this project could partly explain the sex-based variation in AD development. ER decline in the female frontal cortex during ageing and tremendous overproduction of Aβ might highlight the differences between the sexes in the age of onset of AD. Preclinical AD could be initiated earlier in females because of losing the neuroprotective function of E2 during the menopause transition phase. Also, the thesis findings could indicate how important it is to investigate both sexes separately and not neglect to report female findings in preclinical and clinical studies where male samples were predominant.

ABBREVIATIONS

^{18}FDG-PET: ^{18}F-Fluorodeoxyglucose Positron Emission.
3α-5α-THPROG: 3α-5α Tertrahydroprogesterone **.**
aa: Amino Acid.
AD: Alzheimer Disease.
ADAM Period: Androgen Deficiency in Ageing Males Period.
AICD: c-terminal intracellular domain of APP.
Apo: Apolipoprotein.
APP: Amyloid Precursor Protein.
AR: Androgen Receptor.
AβOs: Oligomeric Aβ.
Aβ: Amyloid Beta.
BACE: Beta-Amyloid cleaving enzyme/ **β**-secretase enzyme.
BBB: Blood Brain Barrier.
BDNF: Brain Derived Neurotrophic Factor.
cdk-5: Cyclin-dependent kinase 5.
CE: Coefficient of Error.
CSF: Cerebrospinal Fluid.
CTF: C-Terminal Fragment.
DHT: Dihydrotestosterone.
DPN: Diarylpropionitrile.
DV: Dependant Variant.
E2: Oestrogen.
EC: Entorhinal Cortex.
EGF: Epidermal growth factor.
EOAD: Early Onset Alzheimer Disease.
ER: Oestrogen Receptor.
ERE: Oestrogen Receptor Element
ERK: Extracellular-signal Regulated Kinase.
FAD: Familial Alzheimer Disease.
FC: Frontal Cortex**.**
FGF: Fibroblast Growth Factor.
GPER1, GPER30: G Protein-bound Oestrogen Receptor1.
GM: Grey Matter.
GSK-3: Glycogen synthase kinase 3.
hIPS: Human Induced Pluripotent Stem.
HRT: Hormone Replacement Treatment.
IDE: Insulin-Degrading Enzyme.
IL: Interleukin
IV: Independent Variant.
LOAD: Late Onset Alzheimer Disease
LTP: long-term potentiation.
MAPK: Mitogen-Activated Protein Kinase.
MBD: Microtubule-Binding Domain.
MCI: Mild Cognitive Impairment.
MHPG: Methoxy-4-Hydroxyphenylglycol.
MRI: Magnetic Resonance Imaging.
MTL: Medial Temporal Lobe.
ND: Non-Diseased.
NFT: Neurofibrillary Tangles.
NGF: Neuronal Growth Factor's.
NMDA: N-Methyl-D-Aspartate receptor.
OC: Oral Contraceptive.
ORX: Orchidectomy.
OVX: Ovariectomized.
PET: Positron Emission Topography.
PKC: Protein Kinase Chain.
PPT: Propyl-Pyrazole-Triol.
PR: Progesterone Receptor.
PSEN/PS: Presenilin.
RT: Room Temperature.
SAD: Sporadic Alzheimer Disease.
SNPs: Single Nucleotide Polymorphism.
STZ: Streptozotocin.
Tg Mice: Transgenic Mice.
TNF: Tumour Necrosis Factor.
Wnt: Wingless-related Integration Site.
βCTF: Beta C-Terminal fragment.

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POSTER PARTICIPATION

1- Poster at Alzheimer's Research in United Kingdom (ARUK) 2020, national conference_ Title: “Investigating the Relationship between Age and the Female Gender on the development of Alzheimer’s Disease**”.**

2- Poster at Alzheimer's Research in United Kingdom (ARUK) 2021, national conference_ Title: “**β**-Amyloid Precursor Protein and its Metabolites have Distinct Sex- Dependent Age-Related Profiles”.

3- Poster at the Alzheimer's Association International Conference (AAIC) 2021, Title: “Increased ERβ Expression During Ageing Could be Neuroprotective Against Alzheimer’s Disease Development in the Male but Not the Female Brain”.

CHAPTER 1 _ LITERATURE REVIEW

1.1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in patients, accounting for 60 to 70 % of dementia incidence (Alzheimer's_Association, 2021). It is a neurological condition that affects the elderly, about 50 to 75 % of all the cases reported are with ages of 85 and above (Jorm and Jolley, 1998, Qiu et al., 2009). AD is a progressive condition that affects the neurons, those mostly affected are located in the hippocampus and the cortex (Nussbaum and Ellis, 2003). The clinical symptoms of AD involve a decline in the ability of proper decision-making, memory erosion, and a reduction in an individual's cognitive capacity. Cognitive awareness is a fundamental component of human functioning. The human mind needs to think, process, understand, and make decisions. AD affects cognitive awareness, thereby affecting human consciousness (Carter et al., 2010). Alzheimer is fourth on the top list of causes of death in industrialized areas globally. The other three causes are cardiovascular disorders, cancer, and stroke (Alzheimer's_Association, 2021).

Age is the most common risk factor; cases are reported to keep doubling every five years for people older than 65 years old until reaching a prevalence of 40% in people aged above 85. Furthermore, the number of people living with dementia is projected to double every 20 years because of the continuing aging population globally. In 2021, it was estimated that AD cases where to rise from about 50 million to about 152 million in 2050 (Alzheimer's_Association, 2021).

AD is a burden socio-economically because it is a common disease in the elderly or aging population. Dementia costs the UK an estimated £23 billion a year. This cost is more than that of stroke, cancer, and heart disease treatment all put together (Alzheimer's_Association, 2021). Unfortunately, the cost is expected to treble because it is estimated that the number of people living with the condition will triple by the year 2050 (Powell and Baker, 2019).

The medial temporal lobe (MTL) is where the disease is first initiated, it constitutes the hippocampus and the entorhinal cortex where the disease was reported to initiate before progressing to the frontal and parietal lobes (Braak and Braak, 1991). The clinical progression of the disease is a gradual process as observed through signs and symptoms. The signs include a decline in the semantic and MTL-dependent episodic memory detected in the disease's preclinical stages (Hodges et al., 1990, Bondi et al., 2008). Due to an effect on the brain's cognitive functioning, diagnosis of conditions such as mild cognitive impairment (MCI) is

inevitable. MCI is a transitional stage from normal cognitive function to AD (Petersen et al., 1999). The proper diagnosis of AD depends on both biomarker and clinical criteria (McKhann et al., 2011). Hyperphosphorylated tau, which causes intracellular neurofibrillary tangles (NFT) and extracellular aggregation of amyloid plaques are the first two biological hallmarks indicating the condition, as first implicated by Dr. Alois Alzheimer in 1907 (McKhann et al., 2011).

1.2. Alzheimer's Disease Background

The neuropathological forms of AD are divided based on the disease age of onset and genetic predisposition into: Early onset AD (EOAD) and Late onset AD (LOAD). The LOAD accounts for the majority of AD cases, 95% of the total cases, and effect people who are older than 65 years old (Rabinovici, 2019). However, EOAD is the least form of AD, represents 5% of AD cases, and effect people younger than 60 years old, often in their 40s or 50s (Mendez, 2019). Genetically, AD divided into a very rare forms of Sporadic AD (SAD) and Familia AD (FAD). With SAD pathogenesis is likely started by a mix of genetic and environmental elements changes during the lifetime of effected individuals (Bettens et al., 2013). Familial AD (FAD) is an autosomal dominant acquired disease including changes in three genes, APP and the presenilin genes (PSEN1 and PSEN2) (Mendez, 2019). These FAD mutation forms are usually inherited from a parent and exhibiting a similar age of onset of EOAD (Mendez, 2019). However, actuality, the majority of EOAD is a non-familial, sporadic, form. The FAD is a very rare form of AD and accounts for less than 1% of AD cases, about 11% of EOAD is FAD (Mendez, 2019).

While SAD does not have a discrete genetic mutation background, its heritability has been assessed at 79% by examining the Swedish Twin Registry study (Gatz et al., 2006). The quick advancement in genetic sequencing procedures has prompted genome-wide sequence examination to identify genetic variations inside a population, giving a readout of any qualities wherein variations partner with a specific attribute like AD. The best-known genetic impact on SAD development is Apolipoprotein E (APOE), which has three isoforms, i.e. ε2, ε3 and ε4, that differ in allele prevalence with mean values across the population of about 6.4%, 78.3% and 14.5%, respectively (Eisenberg et al., 2010). The APOE ε4 allele contributes to increasing AD risk by 3 times in people that are heterozygous (ε3/ ε4) and 15 times in people who are homozygous (ε4/ ε4) (Ferrara et al., 2008). In contrast, the APOE ε2 allele decreases the risk

of developing the disease. However, it is more uncommon than the APOE ε4 (Corder et al., 1994, Eisenberg et al., 2010).

The importance of APOE ε4 in AD initiation is its involvement with amyloid overproduction (Shi et al., 2017), ε4 is reported to have a dose-dependent association with AD risk and age of onset (Liu et al., 2013a). Additional to APOE, a more than 30 risk loci genes involved in the immune response and microglial was discovered, refer to the review paper of (Griciuc and Tanzi, 2021) for more details. In addition to immunity response genes, some of risk gens were associated with amyloid overproduction include Phosphatidylinositol Binding Clathrin Assembly Protein (PICALM), amphiphysin II (BIN1) and Clusterin (CLU), as they have been involved in clathrin-mediated endocytosis (Liu and Niu, 2009, Thomas et al., 2016, Harold et al., 2009). Thus, highlighting the importance of studying the amyloid plaque's role in AD.

1.3 Amyloid Precursor Protein

Amyloid precursor protein (APP) is a type-1 transmembrane glycoprotein and the gene for this is located on chromosome 21 in humans (Sisodia et al., 1993). APP is widely expressed in multiple tissues, but it is found in highest concentration in the brain. Two of the major splice isoforms, APP_{751} and APP_{770}, exhibit protease inhibitor functionality, which is a consequence of the Kunitz domain. A third major variant, APP_{695}, is largely present in neurones, and this form does not contain the Kunitz protease domain (Zheng and Koo, 2006). APP proteolytic cleavage is associated with multiple cell functions, including cell signalling, neuronal development, and synapse communication (Hefter et al., 2020). These functions occur as a result of multiple fragments of APP which are produced via metabolism of the parent molecule (Hefter et al., 2020).

1.3.1 APP Metabolism

As seen in Figure 1.1, the rapid metabolism and cleavage of APP occurs via two pathways, specifically the amyloidogenic and non-amyloidogenic paths (Lee et al., 2008). Following APP translation in the endoplasmic reticulum, it is transported via the axon to the synaptic terminals, where it is located either at the cell surface membrane or in the endosomal compartments within the cell body (Koo et al., 1990). Metabolism of APP is dependent on both the location and the variety of enzymes involved. Generally, the cell surface-located APP molecules are cleaved through the non-amyloidogenic pathway (Parvathy et al., 1999). The

endosomal-located APP molecules are cleaved through the amyloidogenic pathway and its products are subsequently secreted during endosome recycling (Zhang et al., 2011b).

90% of APP processed through non-amyloidogenic pathway (Figure 1.1). In non-amyloidogenic pathway, APP is metabolised by α-secretase that cleaves inside the Aβ region (thus hindering its formation), leading to the release of a soluble ~110–120 kDa N-terminal APP fragment (sAPPα) from the surface of the cell. This pathway also releases αCTF (a C-terminal fragment). αCTF is 83 amino acids in length (CTF_{83}) and remains in the cell membrane having a relatively long half-life (Zhang et al., 2011b). The αCTF/CTF_{83} fragment can also be cleaved by γ-secretase leading to the release of a small, non-toxic 3kDa fragment, p3 (Zhang et al., 2011b, Counts et al., 2017, Kojro and Fahrenholz, 2005). On the other hand, in the amyloidogenic pathway, APP is first cleaved by β-site amyloid precursor protein cleaving enzyme (BACE) at the Aβ peptide sequence N-terminus, and the soluble sAPPβ fragment is released, leaving a 99 amino acid βCTF that is embedded in the membrane (CTF_{99}). From there, the βCTF/CTF_{99} fragment is then metabolised within its intra-membrane region by γ-secretase, releasing the Aβ peptide and the APP Intracellular Domain (AICD). The Aβ product is released into the extracellular matrix (ECM) where it is broken down by neprilysin and Insulin-Degrading Enzyme (IDE) (Zhang et al., 2011b, Counts et al., 2017, Kojro and Fahrenholz, 2005).

The α-secretase enzyme activity is controlled by the ADAM enzyme family, a membrane-bound disintegrin and metalloproteinase enzyme. Three members of this enzyme family have been indicated in this function, specifically ADAM9, ADAM10 and ADAM17 (Asai et al., 2003).

BACE1 was confirmed as the neuronal β-secretase over BACE-2 when secretion of Aβ was eradicated in BACE1-null neuronal cultures and consequently, it controls the rate of production of the peptide, Aβ (Cai et al., 2001). APP and BACE1 appear to be widely expressed, as they are produced by multiple cell types; however, the activity of β-secretase is highest in neuronal cells (Zhao et al., 1996). In addition, acidic conditions, such as those within endosomes, in which APP may be present, are favourable to BACE1 and enhance its activity (Huse et al., 2000). Fluorescence labelling immunoassays show that BACE1 and APP exist together within endosomes. Furthermore, inhibition of surface endocytosis is associated with

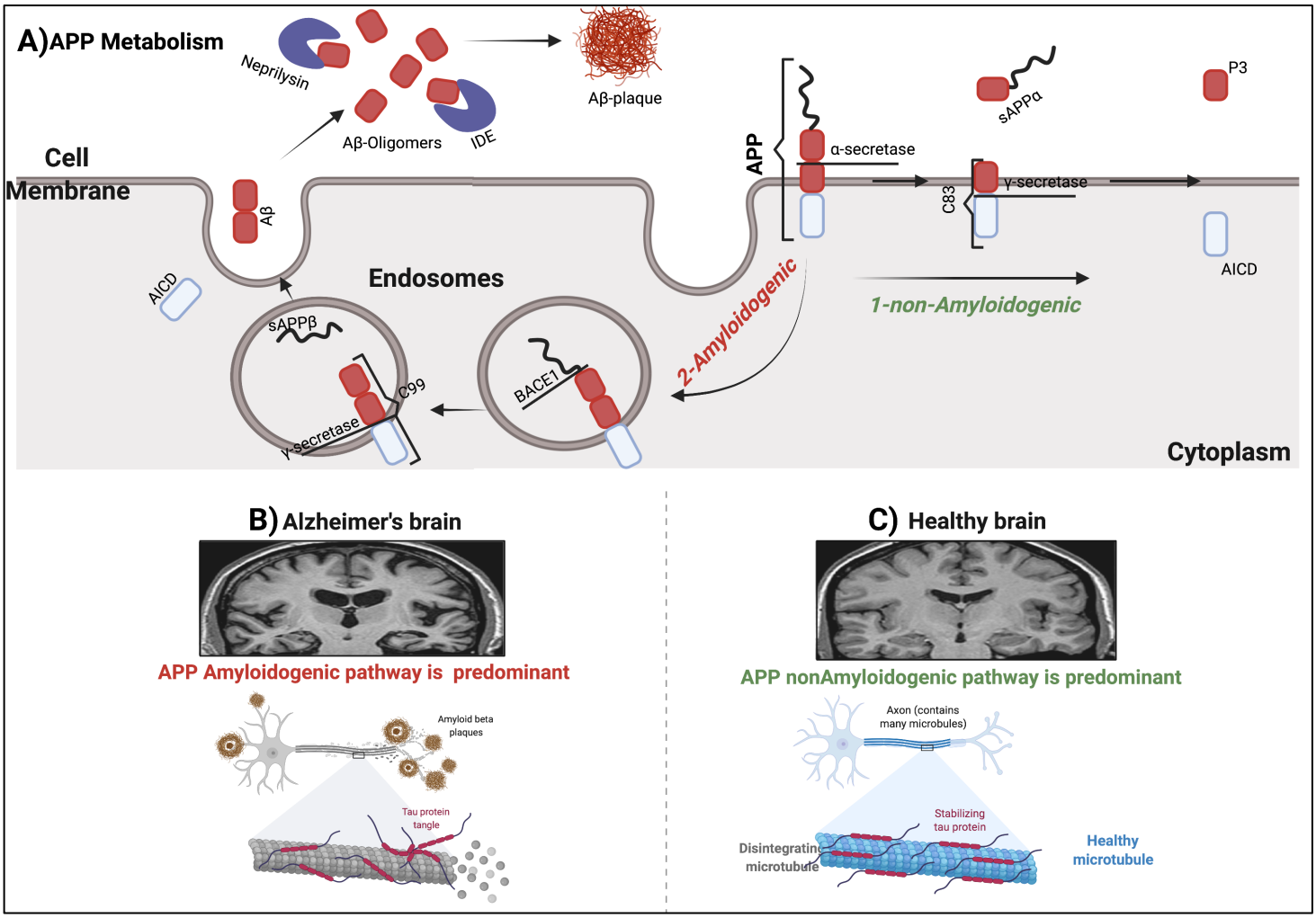


Figure 1.1 Schematic representation of APP metabolism. A) the non-amyloidogenic and amyloidogenic pathway of APP processing in the brain. **1-** In the non-amyloidogenic pathway, where APP is cleaved by α-secretase producing sAPPα and CTF_{83}/CTFα. Subsequently, the CTF_{83} fragment is further cleaved by γ-secretase producing two peptides, namely P3 and the c-terminal intracellular domain of APP (AICD). **2-** in the amyloidogenic metabolism, APP is cleaved by BACE1 (also termed β-secretase), producing sAPPβ and CTF_{99}/βCTF. The CTF_{99} is cleaved further by γ-secretase producing two peptides, Aβ and AICD. The Aβ product is released into the extracellular matrix (ECM) where it is broken down by neprilysin and IDE. Excess Aβ accumulates forming plaques in the ECM. **B)** In AD, the APP amyloidogenic processing pathway is predominant resulting in Aβ overproduction, subsequently the Aβ aggregates in the brain. **C)** In the healthy brain, approximately 90% of all APP processing occurs via the non-amyloidogenic pathway. The figure was drawn by the Bio-render Inc. App.

decreased Aβ production by 80%, and conversely impairing direct trafficking of APP to the cell surface increased Aβ secretion (Cataldo et al., 1997).

The–γ-secretase enzyme is comprised of a multiplex of enzymes, which includes either presenillin-1 or 2 (PS1, PS2), nicastrin, anterior phalanx defective 1 and presenillin enhancer 2 (Francis et al., 2002, Haass and Steiner, 2002). The functions of the individual constituents of this complex have not been described; however, targeted deletion of two transmembrane aspartate residues in PS1 inhibited cleavage of APP by γ-secretase. In addition, this resulted in a substantial decrease in Aβ production and corresponding increase in βCTF (Wolfe et al., 1999). It is likely that γ-secretase works in both non-amyloidogenic and amyloidogenic processing of APP, as it is present in both the cell surface and endosome (Fukumori et al., 2006).

However, recently it has been observed that metabolism of APP is substantially more complex than depicted, with multiple secretase enzymes and resulting proteolytic fragments being identified. Incorporating cleavage of the ectodomain of APP via delta (δ)- and ita (η)-secretase activity has been identified as driving amyloidogenic processing of APP. In transgenic APP mouse models, deletion of the δ-secretase gene led to lower levels of Aβ and improved biochemical, synaptic and cognitive outcomes (Zhang et al., 2015). In addition, η-secretase produces proteolytic fragments which have been associated with synaptic dysfunction (Willem et al., 2015, Baranger et al., 2016). The presence of these alternative APP metabolism pathways becomes increasingly significant in the potential therapeutic inhibition of BACE1. Interrupting the activity of BACE1 may lead to unintended consequences of increased levels of possibly neurotoxic APP fragments through the alternative metabolic pathways (Nhan et al., 2015, Andrew et al., 2016).

1.3.2 The Role of Amyloidogenic Products in the Brain

Researchers propose that APP may contribute to the formation and / or function of synapses. This is supported by the fact that the greatest expression of this protein occurs in the post-natal phase, one in which a substantial amount of synapses are generated (Wang et al., 2009). In addition, mice in which the APP gene has been knocked out exhibit nuanced changes in phenotype such as reduced brain size, body weight, movement, and grip strength (Zheng et al., 1995, Ring et al., 2007). The APP-KO mice at 10 months old and onwards exhibited a number of neurological impairments and sensitivities, including brain injury and seizures, and

disrupted synapse function and cognition (Steinbach et al., 1998, Corrigan et al., 2012, Dawson et al., 1999, Seabrook et al., 1999).

In cell cultures of hippocampal cells, APP has been shown to contribute to the growth and promotion of neurites (Qiu et al., 1995), and APP is also closely associated with neuronal function. Indeed, APP is metabolised by α- and β-secretases in a manner which is dependent on neuronal activity, indicating a defined role for APP (Nitsch et al., 1993, Fazeli et al., 1994, Kamenetz et al., 2003). However, previous studies conducted to investigate the role of APP in the nervous system have produced contrasting results. As an example, one group found that in neuronal cell cultures overexpression of APP reduced activity in the synapse, while another group found that overexpression improved plasticity of the synapse and enhanced memory (Kamenetz et al., 2003, Ma et al., 2007). Current understanding of the physiological actions of APP metabolites has been derived from isolation of the individual peptides and exposing neuronal cell cultures or rodent models to the fragments. The physiological actions of APP will be described below via reviewing the actions of post-metabolism peptides individually.

1.3.2.1 sAPP:

Using evidence derived from APP-KO mice and cell cultures, researchers concur that sAPPα confers protection to neurons and improves both plasticity of synapses and memory (Turner et al., 2003, Meziane et al., 1998). In brief, the addition of the sAPPα ectodomain recovered the morphology of dendrites, improved synapse plasticity and cognitive function (Ring et al., 2007, Tyan et al., 2012, Obregon et al., 2012, Hick et al., 2015). In these studies, it is possible that the soluble fragment (sAPPα) may modulate APP metabolism via direct association with BACE1 and switching the metabolism to the non-amyloidogenic pathway (Obregon et al., 2012). Others have demonstrated the *in vivo* effects of sAPPα treatment on improving LTP and spatial memory (Taylor et al., 2008).

Mice which have a conditional KO of ADAM10, the major α-secretase, are unable to produce sAPPα, and the subsequent loss of this protective mechanism is likely to play a pivotal role in the observed epileptic phenotype seen in the animals (Prox et al., 2013). It is important to note that inhibition of α-secretase cleavage of APP results in enhanced β-secretase activity, and consequently increased toxicity of the amyloidogenic pathway peptides (Wang et al., 2012a).

sAPPβ, which is homologous to sAPPα, with the exception of it lacking 16 terminal residues, does not replicate either the *in vivo* or *in vitro* LTP enhancing properties of sAPPα, clearly demonstrating that these effects are specific to the α-secretase cleaved product (Taylor et al., 2008, Hick et al., 2015, Tyan et al., 2012). sAPPβ neuroprotection actions against glucose deprivation, excitotoxicity, and Aβ, are reported to be 50- to 100- fold less potent than sAPPα (Chasseigneaux and Allinquant, 2012). However, sAPPβ has the ability to promote neurite and axonal outgrowth more efficiently than sAPPα, thus suggesting that sAPPβ is not deleterious in the brain per se (Chasseigneaux et al., 2011, Freude et al., 2011).

1.3.2.2 AICD

The action of the AICD is less well understood than that of sAPPα; some work has demonstrated that this domain may have similar functions to those of APP. This occurs because of a highly conserved 15aa region in the C-terminal, specifically the YENPTY domain that is recognised by adaptor proteins which facilitate linkage to actin in the cytoskeleton. Generation of APP-KO mice which lack these final 15aa residues shows structural impairment of the neuromuscular junction, poor plasticity at the synapse, and decreased spatial learning and memory (Klevanski et al., 2015), thus indicating a physiological role for AICD. The putative role for AICD may be in transcription regulation, given that following γ-secretase cleavage the peptide fragment is located to the nucleus and complexes with FE65 and Tip60, a nuclear adapter protein and histone acetyltransferase, respectively (Cao and Südhof, 2001, Schettini et al., 2010). Endoplasmic-based association between AICD and FE65 is important as it drives translocation of APP to the cell surface and enhances secretion of sAPPα and Aβ (Sabo et al., 2003). Excision of the conserved domain, YENPTY, results in a substantial decrease in processing of APP via the amyloidogenic pathway (Klevanski et al., 2015).

1.3.2.3 β-Amyloid

Dependent on the specific target site for γ-secretase, several isoforms of Aβ are produced comprising between 38 and 43 aa. The $A\beta_{1\text{-}40}$ form, which is soluble and present in the CSF at physiological concentrations (Vigo-Pelfrey et al., 1993), is the major form of secreted Aβ protein. While $A\beta_{1\text{-}40}$ is found in low nanomolar concentrations in body tissues and fluids (Mulnard et al., 2000, Shumaker et al., 2003), at higher concentrations it aggregates resulting in extracellular deposits, which are observed as plaques in AD (Glenner and Wong, 1984). Another isoform of Aβ, the 42 aa peptide ($A\beta_{1\text{-}42}$), represents 10% of the Aβ peptide and is

found largely in AD amyloid brain plaques. The $A\beta_{1-42}$ product is less soluble and more hydrophobic, and as a consequence, is more likely to form plaques (Burdick et al., 1992). Initiation of plaques by the 42-aa form is thought to lead to the additional accumulation of $A\beta_{1-40}$ (Qiu et al., 2009). Additional to those isoforms, in AD patients, the $A\beta_{1-38}$ peptide and shorter forms, comprising of 15, 16 or 17 aa of the N-terminal, are found in the brain (Mawuenyega et al., 2013). Following the discovery of Aβ in plaques of AD patients (Glenner and Wong, 1984), research efforts have been directed towards investigating neurotoxicity of this peptide; however, a potential neurotrophic effects was noted, which appear to be concentration-dependent. That is, high concentrations of Aβ result in retraction of dendrites and death in differentiated neurons, while lower concentrations were neurotrophic to undifferentiated cells (Puzzo et al., 2008). An *in vitro* study showed that the addition of a 28 aa peptide, representative of the N-terminal domain of Aβ improved survival of hippocampal pyramidal neuronal cultures (Yankner et al., 1990, Whitson et al., 1989). Since then, researchers have returned their focus to the physiological role of this peptide and its hormesis effect; where it is beneficial at low concentrations and toxic at high (Puzzo et al., 2008).

Electrophysiology experimentation to determine synapse plasticity in hippocampal samples has shown that picomolar concentrations of Aβ improve plasticity and memory, whereas nanomolar concentrations have a negative impact (Puzzo et al., 2008, Garcia-Osta and Alberini, 2009, Morley et al., 2010). In addition, injection of anti-Aβ antibodies to block the effects of Aβ resulted in memory issues and subsequent LTP induction in the hippocampal slices (Puzzo et al., 2011). This positive role at low concentrations may be attributed to the N-terminal domain, which is partly homologous with sAPPα (Lawrence et al., 2014, Portelius et al., 2011). In addition, an *in vivo* study using transgenic mouse models (Tg5469) with overexpressed human APP, approximately 6 times greater than endogenous APP, revealed enhanced spatial memory which was inhibited using anti-BACE1 antibodies, thus suggesting that enhancement in spatial memory could be partly mediated by Aβ (Ma et al., 2007).

In addition to the concentration-related action of Aβ activity on synapse plasticity, researchers have shown that incubation time affects the action, where a short incubation results in facilitation of plasticity, while a longer incubation leads to dysfunction (Koppensteiner et al., 2016). In these *in vitro* tests, which have been replicated *in vivo,* incubation of neurons with $A\beta_{1-42}$ at picomolar concentrations for minutes enhanced plasticity, while incubation at the

same concentration for hours reduced it. It is possible that this difference results from increased aggregation over time (Puzzo et al., 2015).

In humans, two studies of acute brain injury patients have noted that increase of the Aβ peptide levels in the interstitial fluid of the brain were correlated with improved neurological outcomes (Brody et al., 2008, Magnoni et al., 2012). Following brain injury in children, plaques can be identified; however, in the brains of long-term survivors there was no greater plaque formation noted compared to age-matched controls, irrespective of the persistent Aβ accumulation in the damaged axons (Graham et al., 1995, Cheng and Yakel, 2015).

Eradication of this protective measure may explain the increase in hypersensitivity of seizures observed in APP and BACE1 KO- mice (Steinbach et al., 1998, Hitt et al., 2010). In addition, in APP- and BACE1-KO mice, outcomes of stroke and cortical impact were observed to be worse than in non-KO animals (Mannix et al., 2011, Koike et al., 2012).

Disruption of the blood-brain-barrier (BBB) is well-established with AD and is a widely accepted aging process (Montagne et al., 2015). Given that amyloid plaques are comprised of vascular and inflammatory proteins, and are correlated with the occurrence of micro-haemorrhages (Cullen et al., 2006), it is possible that accumulation of Aβ leads to disruption of the BBB. However, there is great significance in the interaction of Aβ with the BBB from a physiological perspective. Evidence emerging from multiple clinical trials has demonstrated that anti-amyloid treatments have resulted in several unwanted inflammatory side-effects, including oedema and micro-haemorrhages (Sperling et al., 2012, Sevigny et al., 2016). In combination, these findings suggest that Aβ plays a physiological role in regulating the inflammation, supporting the integrity of the BBB, and a protective role against brain injury.

1.4 Pathophysiological Role of APP in AD

As noted in section 1.2 above, development of FAD is promoted by missense mutations in the APP gene which induce increased Aβ production (Haass et al., 1994, Wisniewski et al., 1991, De Jonghe et al., 1998, Van Nostrand et al., 2001). The increase in Aβ production is correlated with a transfer in the equilibrium from α-secretase activity to β-secretase activity (Citron et al., 1994, Haass et al., 1995), altering the balance towards greater amyloidogenic processing of APP (Citron et al., 1994, Haass et al., 1995). In addition to the missense mutations in APP which increase amyloid deposits, other mutations have been identified that result in

greater production of the $A\beta_{1-42}$ peptide, including presenilins (Citron et al., 1997) and neprilysins (Iwata et al., 2005), which in turn promotes the occurrence of early-onset and aggressive forms of AD (Kumar-Singh et al., 2006, Bentahir et al., 2006). $A\beta_{1-42}$ is more toxic than the alternative $A\beta_{1-40}$ form (Phillips, 2019). Researchers have demonstrated an inverse relationship between the $A\beta_{1-42}/A\beta_{1-40}$ ratio and age of onset age AD (De Jonghe et al., 2001).

1.4.1 Pathophysiological Roles of βCTF and β-Amyloid

1.4.1.1 βCTF/CTF99

The C-terminal fragment derived from β-secretase action on APP, βCTF, is both neurotoxic and disruptive to functionality of the synapse. In cases where this fragment was overexpressed *in vivo*, two major characteristics of amyloid pathology emerged, increased reactive gliosis and degeneration of the hippocampus (Berger-Sweeney et al., 1999). This fragment is upregulated in the brains of AD patients (Zhao et al., 2007).

It has been highlighted that the previous amyloid pathology associated βCTF overexpression could be a direct result of βCTF by its own . Simply reducing Aβ may not have an effect on βCTF, and consequently neuronal function may still be disrupted, proposing that this effect may be a contributing factor in the observed failures of clinical studies to improve cognition using anti-Aβ strategies (Cummings et al., 2014).

1.4.1.2 β-Amyloid

The association of Aβ with key synapse functions has been described above, it is clear that any interruption in these processes is likely to result in neuronal dysfunction. Furthermore, there is a large body of evidence that details a neurotoxic role for Aβ, resulting in disrupted plasticity of the synapse and impaired memory. However, these findings are described as a result of dosing with a high (>nanomolar) concentration or oligomers of the peptide, not physiological concentrations (Flood et al., 1994, Lesné et al., 2006, Townsend et al., 2006).

Persistent Aβ exposure is largely linked to synaptic depression, via either NMDA or metabotropic glutamate receptor (mGluR1) -dependent processes (Hsieh et al., 2006, Chang et al., 2006). However, it is vital to note that the biochemical disruption initiated by build-up of Aβ is greater than depression of synapses, as it includes dysfunction of mitochondria, a precursor in AD pathogenesis (Swerdlow and Khan, 2004, Reddy et al., 2009), which in turn

causes oxidative stress, excitotoxicity and neurodegeneration (Devi et al., 2006, Tillement et al., 2006, Roses et al., 2010). Vitally, these processes induce positive feedback, in which the increase in oxidative stress produces greater amounts of Aβ (Leuner et al., 2012).

The innate immune response within the brain occurs via the microglial cell population; these cells are phagocytes that are activated by the build-up of Aβ and they are stimulated to degrade the accumulation of peptides (Liu et al., 2012, Simard et al., 2006). The actions of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and -6, and tumour necrosis factor-alpha (TNF-α) overcome amyloid-associated pathology (Shaftel et al., 2007). However, drawing parallels with other aspects of Aβ-driven neuronal dysfunction, the neural inflammatory response perpetuates a deleterious positive feedback loop, which increases biochemical dysfunction as a consequence of further increased peptide levels (Goldgaber et al., 1989). Persistent inflammation and activation of microglial cells inhibits their ability to effectively remove accumulations of Aβ, a response that may be associated with downregulation of Aβ-binding scavenger receptors, and which delivers an outcome of increased neuroinflammation and neurotoxicity (Hickman et al., 2008, Meda et al., 1995). Epidemiological studies provide indicative data to suggest that anti-inflammatory therapeutics may demonstrate a protective role in AD (McGeer et al., 1996). This phenomenon has been observed in the APP/PS1 mouse models, to add to the theory that neuroinflammation which is stimulated by the presence and accumulation of Aβ peptide contributes negatively to the pathogenesis of AD (McGeer et al., 1996, Olmos-Alonso et al., 2016).

1.5 Disease Hypotheses

Many hypotheses about AD have been established, such as cholinergic neuron damage (Bartus, 2000), oxidative stress (Pratico, 2008), inflammation (Zotova et al., 2010), mitochondrial damage (Mancuso et al., 2007), BBB permeability (Sweeney et al., 2018), amyloid β (Aβ) cascade and tau hyperphosphorylation (Hardy and Allsop, 1991, Selkoe, 1991). This literature review will discuss only the two latter hypotheses, as they are most relevant to this thesis and have been studied in more depth compared to the others.

1.5.1 The Amyloid Cascade Hypothesis

The amyloid cascade theory has been central to AD research for over three decades. Developed by Selkoe initially (Selkoe, 1991) and underpinned by Hardy and Allsop (Hardy and Allsop, 1991), these two groups suggested that formation of amyloid deposits is the pivotal

event in the neuropathology of AD. The theory hypothesised that: “deposition of amyloid β protein, the main component of the plaques, is the initial solo causative agent of Alzheimer's pathology and that the neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition” (Cummings et al., 2014). There are multiple factors which contribute to the supposition that Aβ is the cause of the development in AD, including transgenic mice models with mutant human APP exhibiting sustained increases in the deposition of Aβ as time passes, and exhibiting behaviours similar to those observed in AD patients (Ashe, 2005). Furthermore, *in vivo* and *in vitro* studies showed that Aβ peptides cause toxicity to hippocampal and cortical neurones (Pike et al., 1991, Lambert et al., 1998, Hoshi et al., 2003, Deshpande et al., 2006). Additional, as reported above, familial mutations within the presenilin 1 and 2 genes causes disruption to the $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio which leads to highly aggressive forms of AD (Kumar-Singh et al., 2006, Bentahir et al., 2006).

Majority of therapies were developed based on this hypothesis and aims to reduce Aβ, however these therapies failed in clinical trials as a treatment for AD (Cummings et al., 2014). This has caused consternation in the field, and cast doubt on the theory, specifically as a result of several aspects that are inconsistent with the theory that Aβ is the central causative for AD progression. One of the major issues with the amyloid hypothesis is that the quantity of insoluble fibular plaques in the brain does not necessarily correlate with disease severity. The most significant marker of cognitive impairment is the reduction in the density of synapses. Multiple studies have identified amyloid plaques in apparently ‘normal’ human controls, who exhibited no cognitive decline markers (Katzman, 1986, DeKosky and Scheff, 1990, Terry et al., 1991, Dickson et al., 1995). Furthermore, imaging has revealed that approximately 30% of elderly subjects show positive amyloid deposits, while exhibiting normal cognitive behaviour (Jellinger, 1995, Jack Jr et al., 2009). Also, interestingly, a study of 300 individuals who were cognitively normal, reported that, while Aβ pathology and hippocampal atrophy were both associated with memory ability, there was no correlation between the two variables (Svenningsson et al., 2019).

Irrespective of the mounting evidence against the amyloid cascade hypothesis, it retains its position as the majority view on the pathogenesis of AD. The driving principle behind the development of the amyloid hypothesis was to direct the focus of AD research towards therapeutically beneficial targets, relating to scientific knowledge of the disease process (Hardy

and Selkoe, 2002). While there are, admittedly, issues with the original theory, it has been recently altered to include current data described below (Figure 1.2) (Selkoe and Hardy, 2016).

A clear correlation between soluble Aβ peptide concentration and synaptic loss associated with cognitive impairment indicates that insoluble fibrils may not be responsible for initiating the toxicity (Wang et al., 1999, McLean et al., 1999). This led to the introduction of an alternative theory, which suggests that soluble AβO peptides, oligomers comprising between 15 and 20 monomeric units, are the major promoters of neurotoxicity and dysfunction of the synapse (Hardy and Selkoe, 2002); a theory which merits further discussion.

As early as 2007, Shankar et al. noted that oligomers added at picomolar concentrations to rat organotypic slices resulted in synapse disruption (Townsend et al., 2006). Furthermore, in live rats, the infused oligomers prevented induction of LTP and memory performance (Lesné et al., 2006, Reed et al., 2011, Bao et al., 2012). The relationship between the concentration of oligomeric Aβ and the severity of clinical symptoms is greater than that between plaques and symptoms (Esparza et al., 2013). Testing of human cortical neurons *in vitro* was conducted to determine the toxicity of both AβOs and Aβ fibrils. In the main, the majority of AβOs rapidly located in the synapses, and the remaining population localised within cell membranes, thus indicating that soluble Aβ compounds are capable of stimulating toxicity within various locations of the cell. AβOs were acutely toxic and stimulated mitochondrial death pathways, activated programmed cell death and nuclear condensation. Alternatively, Aβ fibrils necessitated a prolonged, 10 day incubation prior to initiation of a similar response (Deshpande et al., 2006). Consequently, defining AβOs as the major toxic species promotes the main argument of the amyloid cascade hypothesis, while removing its most obvious detractors. However, this also moves amyloid plaques, the classic sign of AD pathology, to a currently undefined role in the condition. More recent hypotheses have suggested that amyloid plaques operate as inert collection points for soluble Aβ, which is sequestered until saturation is reached and thereafter oligomeric forms diffuse into the synapses (Benilova et al., 2012, Koffie et al., 2009). Indeed, assessing plaques in transgenic APP mice shows that a halo of soluble oligomers encases every plaque (Martins et al., 2008). Correspondingly, others have identified that the formation of plaques (fibrils) from oligomers (protofibrils) is a reversible process, and that, following formation, plaques may operate as a repository for neurotoxic forms of Aβ (Shankar et al., 2007).

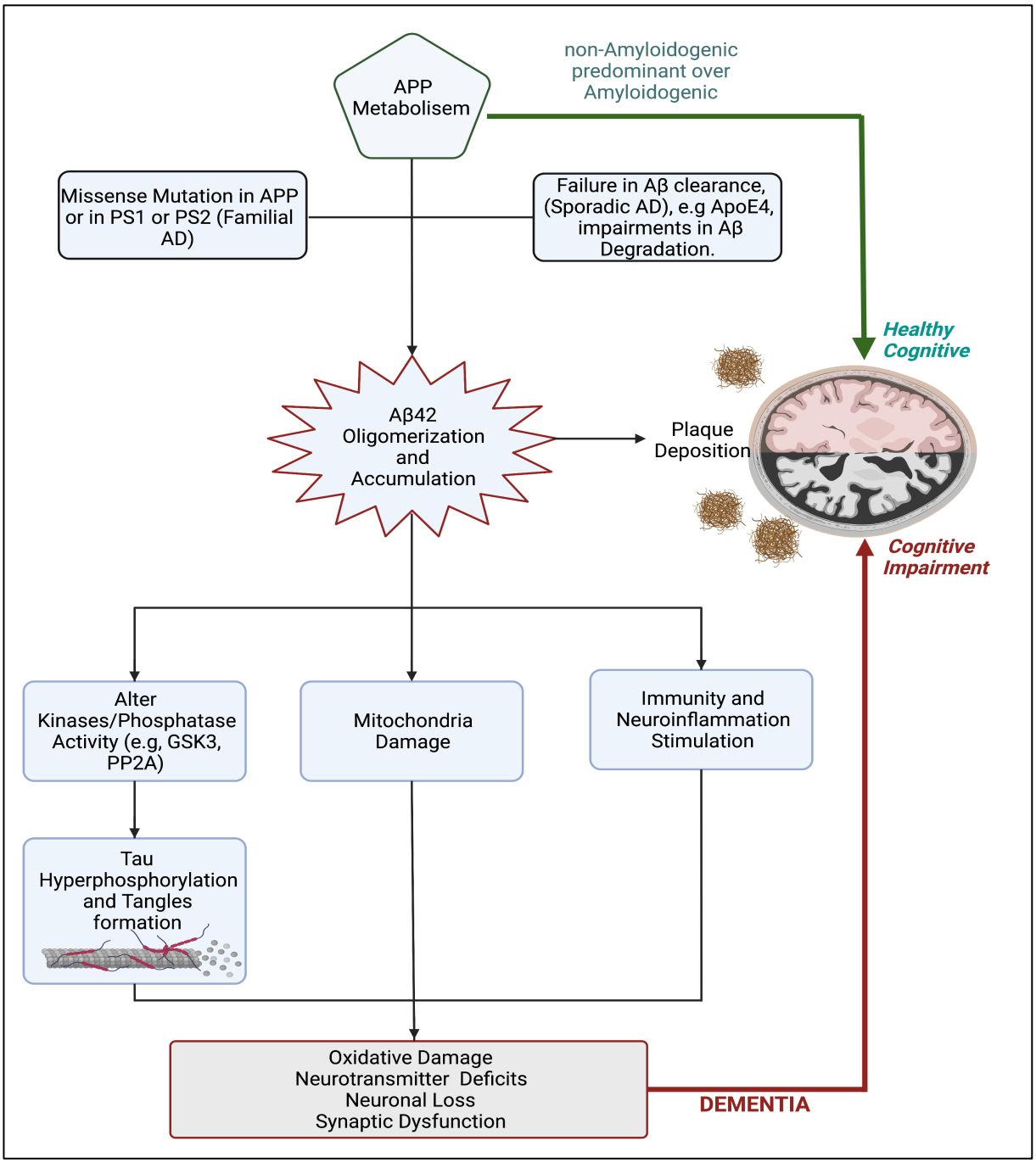


Figure 1.2 Updated Amyloid Cascade Hypotheses. The original amyloid cascade hypothesis, proposed by Higgins and Hardy in 1992, viewed amyloid deposition as directly leading to the other forms of neuropathology associated with AD. However, the hypothesis has been embellished with additional details that have been elucidated by studies into amyloid; for instance, plaque deposition is no longer considered as part of the pathogenic process. This diagram highlights the fact that Aβ performs a physiological, healthy cognitive function. However, as the peptide accumulates, it exerts a detrimental effect on synaptic function, causes mitochondrial damage, activates the immune system, and induces tau pathology. These processes culminate in the synaptic dysfunction and neuronal cell death that are hallmarks of AD. The scheme was adapted from (Selkoe and Hardy, 2016, Counts et al., 2017).

1.5.2 The tau Hypothesis

Tau is a microtubule-associated protein exclusively found in neurones, and it is fundamental for assembly and stabilization of microtubules (Weingarten et al., 1975). It is present in six main isoforms in the human brain ranging from 352-441 aa. For decades, many studies have demonstrated the normal physiological involvement of tau at different subcellular sites: it regulates axonal maturation, extension and transportation (Dawson et al., 2001, Vershinin et al., 2007, Yuan et al., 2008, Ittner et al., 2009), participates in dendritic synaptic plasticity (Frandemiche et al., 2014, Qu et al., 2017) and aids in maintaining the integrity of RNA and DNA (Sultan et al., 2011, Violet et al., 2014).

The various neurophysiology functions of tau have been widely investigated and a fine regulation is required to maintain the normal structure and function of tau (Dickson et al., 2011). Although tau has been characterized as a "natively unfolded" protein with a low aggregation tendency (Mukrasch et al., 2009), biochemical studies in AD demonstrated that the deposition of insoluble tau can aggregate into NFTs, and that process depends upon alterations in the phosphorylation domains in the microtubule-binding domain (MBD) of tau (Takahashi, 2008). While in healthy brain two phosphates per one tau molecule are normally activated (Kanemaru et al., 1992), analysis from AD brains revealed the presence of about eight phosphates per one molecule of tau (Kopke et al., 1993). From that finding, tau hyperphosphorylation has been connected to protein misfolding and deposition in the diseased brain (Smith, 2002).

Dephosphorylation of tau in the brain is processed by protein phosphatase (PP) PP2A (Liu et al., 2005). While, phosphorylation of tau is carried out by kinases, such as cyclin-dependent kinase 5 (cdk-5) and glycogen synthase kinase-3β (GSK-3β) (Sandra et al., 2014). In AD, while PP2A activity is reported to be reduced by half, GSK3β activity is increased, leading to tau hyperphosphorylation and memory deficiencies, (Martin et al., 2013a). GSK3 consists of two homologous proteins which are paralogs, GSK3α and GSK3β. An *in vivo* study using GSK3β, showed that degeneration of hippocampal neurones was stimulated by phosphorylated tau and colocalized with NFTs, GSK3β activity also increased with Aβ peptides and caused tau hyperphosphorylation in the hippocampus (Martin et al., 2013b) and anti-GSK3β was shown to protect neurons from Aβ toxicity (Martin et al., 2013a).

While aggregation of Aβ fibril plaques in the AD brain is considered an early event, formation of NFTs is thought to be more a proximate cause of later neuronal malfunction and

death (Hardy and Higgins, 1992, GÛmez-Isla et al., 1997, Bennett et al., 2004). NFT distribution is usually limited to the medial temporal lobe at early stages of AD pathology (Braak I-II), whereas at later stages (Braak V-VI) NFT spreads outside the temporal lobe and toward Aβ-rich sites such as the limbic system and association area of the cortex, simultaneously associated with actual cognitive dysfunction (Braak et al., 2011, Schˆll et al., 2016, Schwarz et al., 2016, Sepulcre et al., 2016, Brier et al., 2016, Wang et al., 2016b). An *in vivo* study discovered that, when human AD-tau was injected into transgenic mice brains bearing Aβ plaques, the ability of Aβ plaques to mediate tau pathogenesis was manifested (Almeida et al., 2006, He et al., 2018).

Aβ plaques have been shown to cause the accumulation of endogenous soluble tau within dystrophic axons surrounding the plaques and to facilitate rapid tau accumulation into fibrils as neuritic plaque tau (NP-tau). Thereafter, NP-tau acts as an enriched source for secondary tau seeding, which might translocate endogenous soluble tau accumulation from axons to neuronal somas and dendrites, leading to the development of NFTs. A protein correlation study with "surface plasmon resonance" found that tau has an affinity constant for Aβ nearly 1000-fold higher than for another tau molecule (Guo et al., 2006). Parallel to these preclinical findings, a clinical study demonstrated an association between Aβ plaques and NFT formation (Sperling et al., 2019). These findings suggest that the significant cognitive decline observed in aged individuals is associated with abnormalities of both Aβ and phosphorylated tau and not related to the subjects' age (Sperling et al., 2019, Vos et al., 2013, Soldan et al., 2016, Gomar et al., 2016, Schindler et al., 2017, Clark et al., 2018).

1.6 Current Treatments

It has been almost two decades (2003) since the first AD treatment was approved. Those approved drugs are anticholinesterase inhibitors, an NMDA antagonist, and a combination of anticholinesterase and NMDA inhibitors. In mild to moderate stages of AD, the anticholinesterase inhibitors donepezil (Aricept; Pfizer), rivastigmine (Exelon; Novartis) and galantamine (Reminyl; Janssen) tend to be prescribed (Birks, 2006). These drugs inhibit breakdown of acetylcholine (Ach) via blocking the enzyme, acetyl cholinesterase (Birks, 2006). The NDMA receptor inhibitor, memantine (Ebixa; Eli Lilly), is used in moderate to severe cases of AD (Tariot et al., 2004). It acts to prevent the excitotoxicity which is dependent on NMDA receptor activation and results from the presence of excess glutamate in the extracellular environment and disruption to the calcium balance (Danysz and Parsons, 2003).

The combined treatment with memantine and donepezil were superior in improving cognitive ability than treatment with donepezil alone (Tariot et al., 2004). While these treatments reduce some of the cognitive issues related to AD, they are not able to stop the progression of the disease, clearly as they do not act on the underlying pathology. As a consequence, there have been substantial efforts directed towards defining the pathology of AD, and mechanisms to disrupt this pathology.

Following the discovery of the amyloid cascade hypothesis, many AD therapeutics have entered clinical trials, supported by strong pre-clinical data, however most of these candidate drugs didn't pass the clinical trial tests (Uddin et al., 2020, Liu et al., 2019b). The predominant strategic therapy was directed towards Aβ through either: (i) the application of small molecules to inhibit the enzymes that cleave APP, or (ii) immunotherapeutics directed towards Aβ with the intention of improving its clearance via immune mechanisms (Uddin et al., 2020, Liu et al., 2019b).

Another strategy for treating AD was immunotherapy stimulation against Aβ, a common pharmacological approach as it confers a high level of specificity and targeting due to high affinity antibodies. In immunotherapy, immune molecules are used to induce a therapeutic effect. This may be active, which uses the patient's own immune system, while a passive therapy uses an infusion of antibodies from other sources that have been designed to specifically target the protein of interest. Given that AD patients exhibit fewer endogenous anti-Aβ antibodies within their serum and CSF than the normal population, the peptides and plaques are an obvious target for this treatment (Du et al., 2001).

A further putative target for AD therapy derived from the amyloid theory is to target tau. Given that tau pathology as regards to AD incorporates disrupted phosphorylation, researchers have indicated that kinase and phosphatase enzymes are potential targets; however, these are likely to result in difficulties with selectivity and lead to unwanted off-target effects (Lovestone et al., 1994).

In June 2021, the first anti-Aβ drug was post-approved by the USA-FDA, aducanumab (Aduhelm®) (Cavazzoni, 2021). The drug acts through stimulating the body's immune system to remove plaques from the brain. Nonetheless, as a consequence of the 3 decades of repeated failures of anti-amyloid therapies in clinical trials with AD patients, the effectiveness of this drug has been questioned. In particular, the criteria for the approval were based on the ability

of aducanumab to clear plaques from the brain, even though there was no proof of cognitive improvement or halt in disease progression. Thus, many medical centres and researchers criticised this approval. Also, not enough clinical data were published to back up the effectiveness of this drug against AD. It has been estimated that, by 2030, there may be enough data that could show if aducanumab has any beneficial effect on cognitive improvement in AD patients (Walsh et al., 2021). Nonetheless, even with all the recent failures of anti-tau and anti-Aβ drugs in clinical trials, the increases in Aβ and tau oligomers in the brains of AD patients are still considered as a pathogenic biomarker of the disease.

It is therefore proposed by many experts in the field (Thomas et al., 2011, Egan et al., 2019), that a comprehensive up-to-date evaluation of AD causative factors is essential to prevent further depletion of resources and time in developing unsuitable anti-Aβ therapies.

The causes of AD are still unclear. AD is a multifactorial neurodegenerative disease (James and Bennett, 2019). A combination of genetic, lifestyle and environmental factors might affect brain function over time (Armstrong, 2019). Other causative risk factors include: ethnicity (south Asia and Africa) (Weiner, 2008), medical conditions (cardiovascular and depression) (Breteler et al., 1991), advancing age (Weiner, 2008), and biological sex (Waters et al., 2021). The latter two risk factors will be discussed further below as they are most relevant to this thesis.

1.7 Ageing and Cognitive Health

The events and features that determine the onset and progression of the pathophysiological changes to the brains of dementia and AD patients are multiple and diverse. Ageing is one such factor; rather than being a uniform process, parts of the body age differently. Much of the ageing process is not observable, although atrophy of the skin is an overt manifestation (Chambers and Vukmanovic-Stejic, 2020). In contrast to skin that ages readily, brains are quite robust, as the process does not result in the significant loss of neurons and glial cells or acute cognitive damage (Wilson et al., 2020, Pelvig et al., 2008, Fabricius et al., 2013). Instead of widespread neuronal loss or damage, studies suggest that, during the normal process of brain ageing, particular circuits of neurons undergo minor functional and morphological changes (Prolla and Mattson, 2001). In spite of this, the most significant risk factor for AD and other neurodegenerative diseases, is ageing (Farfel et al., 2019). The effect of ageing in AD exceeds that of heritable factors. As described previously, the increased incidence of AD as

age advances is dramatic. In people aged 65–74 years, 3% have AD; this rises to 17% in those aged 75-84 years and peaks at 32% in those over the age of 95 years (Farfel et al., 2019). In a normally ageing brain, any loss of neurons is counterbalanced by enhanced growth of dendrite branches. As the dendritic branches extend, they make compensatory synaptic contacts (Dickstein et al., 2007). However, this growth and compensatory process does not occur in patients with age-related neurodegenerative disorders (Herms and Dorostkar, 2016).

The neural health and cognitive performance of an individual as they progress from childhood to late adulthood is referred to as their life-course cognitive health. Cognitive health is not a constant as it actively changes throughout the individual's life. It is well recognised that cognition during childhood exerts effects in later life to the extent that the cognitive performance at 90 years of age can be predicted by intelligence tests administered to pre-teen children (Walhovd et al., 2014). However, long-term cognitive function is also influenced throughout life by ongoing behaviour and experiences. Recently, there has been a paradigm shift in the approach to normal brain ageing. In the past, declining cognitive function in the elderly was considered a normal manifestation of the ageing brain that most people lucky enough to reach old age would experience to some extent (Grady, 2008). In detail, decades of cognitive studies highlighted the existence of age-associated deficits in the speed of cognitive processing and function, particularly the ability to control behaviour and in episodic memory. Researchers also indicated that, while some cognitive functions decline with age, others remain relatively intact and might even improve with age, for instance semantic knowledge (Grady, 2008). As such, cognitive ageing is regarded as different to neurodegeneration, with the implication that age is the primary factor that determines outcomes. Research now focuses on 'optimal/healthy' ageing rather than considering cognitive degeneration to be normal ageing (Lockhart et al., 2014).

Since the ageing process in the brain fluctuates throughout life, and because there is an overlap in the manifestation of cognitive decline due to ageing and that of AD, it is important to try to distinguish between normal cognitive ageing and neurodegenerative disease (Van der Linden and Juillerat Van der Linden, 2018, Walhovd et al., 2014). A critical point is that AD is a disease state, not a normal part of aging (Nelson et al., 2011), and it is important to recognise that age alone in itself does not cause AD. The implication is that as the brain becomes older and less resilient, subtle changes take place that increase the susceptibility of the brain to age-related pathologies (Pathological Ageing) (Nelson et al., 2011).

Although brains are privileged organs, they do not exist or function independently of the rest of the body and their structure and function are influenced by events and the lifestyle choices made by its owner as well as the wider environment. For example, multiple studies have examined the adverse effects that excessive alcohol consumption, obesity and smoking can have upon cognition (Baumgart et al., 2015, Rusanen et al., 2011, Matloff et al., 2020, Luchsinger et al., 2012). Erickson et al. stated that the health of the brain is determined by a combination of intrinsic factors, including genetics and health behaviour, with extrinsic factors, such as environment, lifestyle and social effects (Erickson et al., 2014). There is agreement among researchers that age-associated neurological disorders, such as dementia, are the product of complex interactions between various intrinsic and extrinsic factors, rather than developing in any single period during life (Walhovd et al., 2014). For example, being well educated and adopting a healthy lifestyle is associated with delaying the onset of AD, not preventing the development of AD (Rusanen et al., 2011, Matloff et al., 2020, Luchsinger et al., 2012, Wada et al., 2018).

Throughout a person's life, their brain undergoes constant change and development. As a consequence of experiences and the natural ageing process, dendrites undergo morphological changes, extending new branches leading to new synaptic connections, whilst retracting others, removing synaptic connections; also, new neurons may form, as others die (Killgore et al., 2013). Using neuroimaging techniques to examine ageing brains, selective changes reflecting a decline in neurons have been identified in addition to a compensatory neural recruitment. While older adults exhibit cortical thinning and decline in the integrity of white matter (Park and Reuter-Lorenz, 2009), along a reduction in all of dopaminergic activity, hippocampal and occipital functional activity (Goh and Park, 2009), it was reported that there were compensatory increases in the frontal regions functional activity giving these adults better behavioural performance (Goh and Park, 2009). The results of structural imaging studies suggest that introducing intensive physical and cognitive interventions can potentially counteract age-related neural atrophy (Walhovd et al., 2016). The conclusion that can be drawn from such results is that, in response to ageing, the brain undergoes morphological changes, called the age-adaption process, that enable additional parts of the brain to process data or to undergo structural remodelling. These adaptations epitomise neuroplasticity, which is the brain's capacity to enhance function and potentially grow extra cells to meet increased usage demands (Goh and Park, 2009). However, it is unclear whether these adaptions are manifestations of healthy brain ageing processes or whether they are indicative of mechanisms that protect

against cognition decline diseases. To illustrate the point, the frontoparietal activity in patients with preclinical AD pathology and undamaged cognitive function is greater than in similar patients whose cognition is diminished (Elman et al., 2014).

Altogether, a large body of research consistently reports age to be just one of multiple factors responsible for the development of neurodegenerative diseases. However, it is still unclear what effect age has on brain health, and the mechanisms that cause particular cell types or parts of the brain to be affected over others. For example, in AD patients, it is mainly the hippocampus that is affected (Braak and Braak, 1998), in Parkinson's disease (PD), it is the substantia nigra (Parent and Parent, 2010), in Huntington's disease (HD), it is the striatum (Graybiel, 1998) and in myotrophic lateral sclerosis (ALS) it is the primary motor cortex and the spinal cord (Marini et al., 2018). Despite the advances in molecular genetics and pathophysiology research into neurodegenerative disorders, understanding the mechanisms and factors involved in selective neuronal vulnerability phenomena remains a stubborn problem to resolve.

1.7.1 Clinical Stages of AD

The clinical staging of AD is divided into three phases: pre-symptomatic/preclinical, prodromal, and symptomatic/clinical dementia (mild-, moderate-, and sever-AD dementia) (Jack et al., 2010). Individuals at the pre-symptomatic phase present with AD-associated pathological changes but without any cognitive impairments. The prodromal phase refers to mild cognitive impairment (MCI), a transitional stage from normal cognitive function to AD (Petersen et al., 1999). The final phase is where the pathological impairments of the disease are widespread in several domains of the brain causing loss of function (Jack et al., 2010).

The use of disease biomarkers for early diagnosis has a long history, many studies have shown that AD biomarkers can be used to predict the conversion from MCI to AD. These studies used imaging, PET, and CSF biomarkers to predict the disease course (Yuan et al., 2009, Mattsson et al., 2009, Visser et al., 2009). However, the main criteria for the accurate diagnosis of early AD depends on both biomarkers and memory impairment (McKhann et al., 2011).

According to the amyloid cascade hypothesis, Aβ and NFT are the main hallmarks of AD. However, neurodegenerative symptoms, especially synapse loss, are more important than these hallmarks in the pathological lesions of AD, manifesting as atrophy, gliosis, and neuronal

loss (Jack et al., 2010). Nonetheless, a theoretical model of the pathological stages of the AD biomarkers has been established (Jack et al., 2010). In this model, Aβ deposition becomes abnormal earlier than any manifestation of neurodegeneration and AD clinical symptoms (Figure 1.3). This is followed by abnormalities in tau as a biomarker of neurodegeneration in later stages of the disease (Jack et al., 2010). It is important to highlight that the biomarker of tau dysfunction is a better indicator of AD initiation and neurodegeneration than the biomarker of Aβ deposition. Jack et al. provided a time-dependent assumption showing that abnormalities in the AD biomarkers would typically precede the clinical symptoms (Figure 1.3) (Jack et al., 2010).

Furthermore, the pre-symptomatic/preclinical AD state could be a possible explanation for the failure of anti-Aβ drugs in clinical trials. When the disease pathology diagnosed (clinical AD), patients may be past the point where anti-amyloid drugs are effective, since Aβ accumulation were initiated before AD clinical diagnosis (at the preclinical stage of AD) (Jack et al., 2010, Palmqvist et al., 2017). This is supported by the fact that the current anti-Aβ drugs nominated for clinical studies were successful in animal models representing preclinical AD pathologies (Ohno et al., 2004, Laird et al., 2005, Kobayashi et al., 2008). Thus, early treatment intervention of AD at pre-symptomatic/preclinical stage might delay the age-related cognitive decline and the development of AD. However, the question which is yet to be investigated is when is the critical age window(s) where preclinical AD stage might manifested at?

With Aβ plaque deposition identified in 20-40% of healthy cognitive elderly individuals (Shaw et al., 2009, Mintun et al., 2006, Bouwman et al., 2009), these data support the assumption that Aβ plaque deposition alone, even in abundant quantities, is not sufficient to initiate dementia. Thus, abnormalities of Aβ deposition precede AD clinical symptoms (Fagan et al., 2007, Fagan et al., 2009, Li et al., 2007, Stomrud et al., 2007). In addition, using imaging, Aβ deposition was found in widespread areas of the brain in the pre-symptomatic phase. In contrast, tau was found to be confined to the entorhinal cortex in the pre-symptomatic phase (Braak stage I-II), but in symptomatic individuals NFTs were found to be far more widespread in the brain (Jack et al., 2010, Knopman et al., 2003, Savva et al., 2009). In addition to the AD hallmarks, MRI atrophy was found to be lowest in cognitively normal individuals, moderate in MCI, and greatest in individuals diagnosed with AD (Jack Jr et al., 2009). Thus, Aβ deposition in cognitively normal individuals might have normal MRI structure, implying that Aβ load can

accumulate before MRI changes in the AD pathological stages are seen (Jack Jr et al., 2008, Josephs et al., 2008).

Identification of the pre-symptomatic phase is hypothetical rather than a statement, as some individuals in this phase die without showing any clinical symptoms (Knopman et al., 2003, Price and Morris, 1999, Savva et al., 2009). Nonetheless, the hypothetical assumption is that an individual who is asymptomatic for AD, but with pathological hallmarks, would eventually become symptomatic if they lived long enough. The AD pre-symptomatic phase might be initiated 15-20 years prior to the emergence of the clinical symptoms of the disease (Sperling et al., 2011, Sperling et al., 2013). In addition, while Aβ plaque-bearing elderly individuals had normal cognitive function, after their death, clinical diagnosis revealed a similar pathological signature to those with AD (Counts et al., 2017). These results suggest a heterogeneous pre-symptomatic stage of AD which could be initiated earlier in individuals younger than 60 years old.

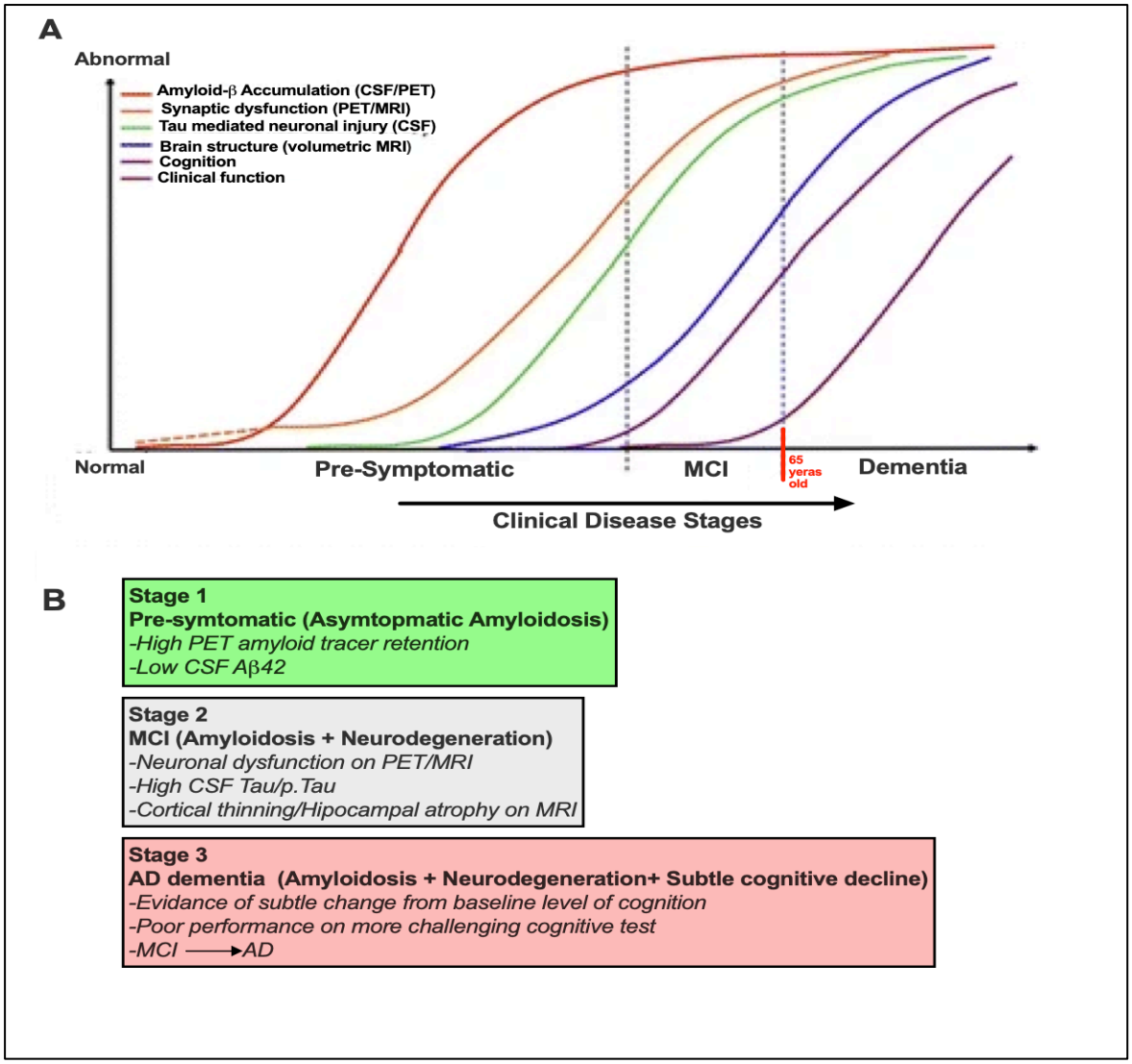


Figure 1.3 Theoretical Stages of AD Biomarkers. A) AD biomarkers became abnormal in a temporally ordered manner as the disease progress. Firstly, Aβ deposition started initially at an age before the appearance of clinical symptoms and reaches a plateau after AD diagnosis. Secondly, the neuronal injury and degradation biomarkers started to increase latel and are correlate with the disease severity. Finally, MRI is the last abnormal biomarker seen and it correlates to cognitive performance. Each of these biomarkers follows a non-linear sigmoid time course which indicates a variance in the maximum effect of each biomarker over the course of AD progression. **B)** Hypothetical clinical staging of AD, with the possibility that the first two stages, 1 & 2, do not progress to AD. Stage 3 individuals might be more likely progress to clinical AD. Taken and adapted from (Counts et al., 2017, Jack et al., 2010).

1.8 Biological sex Disparities in AD

A person's biological sex is a significant yet neglected demographic variable influencing disparities in AD. According to latest published figures of dementia cases in UK and USA; about 61-65% of cases are females (Alzheimer's Association, 2021, Prince et al., 2014). The same pattern was also reported worldwide through the latest world health organization (WHO) global dementia status report (World_Health_Organization, 2021). This sex discrepancy in the disease incidence is believed to be an indirect result of the longer lifespan of the female sex, making them more likely to reach age at risk for developing AD (Fratiglioni et al., 1997, Gao et al., 1998, Plassman et al., 2008). In the United States (US), the Alzheimer Association reported a greater lifetime risk of developing AD among females at ages 45 and 65 years (Alzheimer's_Association, 2021). Table.1.1 defines some of the terms used in this section.

Anatomically, numerous publications have documented that brain structure and physiology demonstrate differences according to sex. The most obvious disparity between the brain anatomy of males and females is the approximately 10% increased skull dimensions and volume of the brain in males (Giedd et al., 2012). Given that, females may exhibit a rapid and more extensive structural loss in the early AD phases than men, who are more robust owing to a larger cognitive reserve (Mielke et al., 2014). The notion of cognitive reserve originated from the description by Katzman et al., who noted that individuals with larger brains have an improved ability to tolerate a higher disease burden with a similar degree of cognitive activity (Katzman et al., 1988).

Table 1.1 Definitions of medical terms used in the thesis to differentiate between them.

Term	Definition	References
Sex	"Biological differences such as chromosomal, gonadal, or hormonal differences."	(Regitz-Zagrosek and Oertelt-Prigione, 2012)
Gender	"Psychosocial and cultural differences between men and women, e.g. access to education and occupation."	(Regitz-Zagrosek and Oertelt-Prigione, 2012)
Susceptibility	"It features subjects who have a priori disadvantages and who are at risk of developing a certain condition_(morbidity)."	(Kottow, 2003)
Vulnerability	"It features subjects with no priori disadvantages but who are at risk of severe damage when a certain condition affects them_ (mortality)"	(Kottow, 2003)

Incidence	"Refers to the occurrence of new cases of disease or injury in a population over specified period of time"	(Crichton, 2000)
Prevalence	"The proportion of persons in a population who have a particular disease or attribute at a specified point in time or over a specified period of time"	(Crichton, 2000)

The cognitive reserve theory was substantiated by a post-mortem study which noted that females had a greater chance of a clinical label of AD than males with a similar degree of pathology. Although the generally larger dimensions of head size may indicate a greater brain reserve in males, research has uniformly demonstrated a more rapid rate of age-related volume loss in males as opposed to females in cognitively normal subjects (Coffey et al., 1998, Oguro et al., 1998, Gur et al., 2002, Pfefferbaum et al., 2013). Nevertheless, in patients with MCI and AD, brain volumes have been reported to decrease at a faster rate in females than in males (Skup et al., 2011). Thus, it is expected that males might be able to withstand a greater level of disease than their female counterparts. However, female are more likely to live longer than male with AD (Alzheimer's Association, 2021, Prince et al., 2014). Preclinical trials have demonstrated that, while females are highly susceptible to the pathology-associated characteristics of AD, males appear more vulnerable to AD per se (Dubal et al., 2012, Stern et al., 1997).

In addition to skull dimensions, other sexual dimorphisms of brain anatomy and metabolic function are also present (Giedd et al., 2012, Luders et al., 2009, Good et al., 2001). For instance, when evaluating abnormalities of cerebral metabolism related to the cognitive dysfunction seen in dementia, two publications have reported that males have more marked metabolic deficits than females at equivalent levels of cognitive impairments, implying that the greater brain reserve in the former might aid them in withstanding the severe disease progression (Perneczky et al., 2007b, Perneczky et al., 2007a). Also, with regards to brain anatomy, females have a larger proportion of grey matter in a number of brain areas; in males, white matter is present in higher proportions (Cosgrove et al., 2007). Furthermore, functional imaging modalities, e.g. ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET), which measures cerebral metabolism, and resting state functional magnetic resonance imaging (MRI), which illustrates cerebral connectivity, have revealed marked disparities between the two sexes (Cosgrove et al., 2007, Biswal et al., 2010). In general, cerebral blood supply and connectivity are greater in females in the parietal association cortex, while in males it was higher in the visual and motor areas of the cortex (Hsieh et al., 2012, Gur et al., 1995). These

findings corroborate the presence of differential brain operation and behaviours between the two sexes. A number of imaging studies have demonstrated that the variations identified previously impact the disparity in how certain cognitive tasks are conducted; for instance, males outperform females on occupations necessitating visual orientation. Importantly, in view of the contemporary theory that areas of high connectivity within the cerebrum accumulate amyloid deposits (Buckner et al., 2005), there is an evident requirement to explore disparities between the sexes in the pathogenesis of AD. This necessity has been emphasised by the National Institute of Health Office of Research on Women's Health (Ronquillo et al., 2016), and in fact, research to evaluate the implication of sexes in AD has accelerated over the last few years.

Sex dissimilarities in MCI have been identified in several studies. Overall, a larger incidence of MCI is seen in females later in life. Uniformly, males have a greater incidence of the non-amnestic form of MCI (Roberts et al., 2012, Caracciolo et al., 2008, Beinhoff et al., 2008). Amnestic MCI refers to cognitive dysfunction that encompasses the memory domain; non-amnestic MCI excludes the memory domain but impacts alternative areas, e.g. those responsible for executive operations, visuospatial appreciation and language (Petersen, 2004). In addition to MCI sex disparities, there may also be different patterns of shrinkage according to sex, with men experiencing most shrinkage in the frontal and temporal lobes, whilst in women the hippocampus and parietal lobes are affected most (Murphy et al., 1996).

Whether this sex-disparity in AD cases was related to the increased longevity of females or if additional elements heightened the risk for women remains obscure. However, there is still a gap pertaining to the biochemical explanation for the sex disparities reported in AD incidence which is in need of investigation (Schäfer et al., 2007). The next subsection will therefore offer theories founded on substantial data which postulates that sex should be recognised as a risk factor for predisposition to, development of and resilience to AD.

1.9 Female sex as a Risk Factor for Developing AD

Rocca et al. evaluated three classes of factors pertaining to the risk of being female for AD. The first category denoted those elements that arise equally in both sexes but have a more robust influence on females, i.e. genetic. The second group exert an equivalent influence on each sex but arise more frequently in a certain sex because of cultural or social backgrounds, e.g. education and employment opportunities (gender factor). The third class are those that are

limited to females, e.g. ovariectomy or sudden E2 absence at a certain life stage (Rocca et al., 2014).

1.9.1 Genetic Factors

Autopsies have shown exaggerated amyloid plaque deposition and NFT formation in the brains of individuals who carry the ApoE ε4 allele (Schmechel et al., 1993, Ohm et al., 1995). *In vivo*, Raber et al. demonstrated that expression of human ApoE ε4 in female transgenic mice (Tg mice) increased their vulnerability to learning difficulties in the water-maze task (Raber et al., 1998). In clinical studies, meta-analysis concluded that ApoE ε4 was an age-dependent risk factor for AD in both sexes, however, the risk was greater in females in the 65-75 age range than male subjects (Neu et al., 2017). ApoE ε4-carrier females displayed more prominent alterations in the connectivity configuration of neural networks (Damoiseaux et al., 2012), a higher degree of tauopathy (Corder et al., 1993), diminished cerebral metabolism and augmented cerebral atrophy (Sampedro et al., 2015), and an inferior memory performance (Fleisher et al., 2008). Additionally, the impact of the ε4 allele on AD cerebrospinal fluid (CSF) biomarkers was more prominent in females than in males (Altmann et al., 2014).

Additional genes and single nucleotide polymorphisms (SNPs) have been demonstrated to heighten the risk and advancement of AD in a specific sex. A big global multi-national study documented that the Met66 allele of the Brain-Derived Neurotrophic Factor (BDNF) gene, which impedes BDNF transport, is linked with an augmented risk of AD in females but adds no extra risk to males (Fukumoto et al., 2010). This observation makes biological sense since the expression of BDNF is to some extent governed by oestrogen (Sohrabji et al., 1995). In the presence of the MeT66 allele, post-menopausal females have diminished BDNF transport and expression, thus leading to an elevated risk of AD. Also, Karch & Goate studied 20 genetic loci on autosomal chromosomes associated with a heightened risk of AD (Karch and Goate, 2015), and noted that several, such as the Serpin genes, demonstrated a more robust relationship with amyloidosis particularly in women (Deming et al., 2018).

The physiological pathways that underlie these findings have not yet been elucidated, largely owing to the fact that the biological consequences of most of these genetic polymorphisms remain unclear. The majority of studies recognising disparities between the sexes connect this phenomenon to sex hormone concentrations. For instance, some of the benefits of E2 have been associated with ATP Binding Cassette Transporter 1 (ABCA1)-mediated mechanisms, which govern intracellular cholesterol and phospholipid management

(Srivastava, 2002). Nonetheless, with ongoing recognition of variations between the sexes for AD risk associated with SNPs, an improved understanding of the physiological changes that contribute to sex disparities is necessary.

1.9.1.1 Molecular Factors

Additional elements that may play a role in the sex differences associated with AD encompass the cognate receptors of neuronal growth factor (NGF), e.g. Tropomyosin receptor kinase A (trkA) and p75 receptors, which are associated with neuronal longevity (Counts et al., 2011). These receptors were investigated in the nucleus basalis of male and female individuals who were healthy, or had MCI, or mild-moderate AD. It was determined that the receptors exhibited a differential shift along the course of the pathology, which varied between the sexes. Males and females with AD had diminished trkA receptor levels compared to the cohorts who were healthy or had MCI. In contrast, the low-affinity p75 receptor was diminished solely in females with AD (Counts et al., 2011, Salehi et al., 2000). The lower frequency of receptors associated with neuronal survival in women is in keeping with the notion of females' augmented susceptibility to AD. In contrast, a similar percentage of the high-affinity trkA receptors in males and females could offset the more significant reduction in low-affinity p75 receptors in women, thus preserving some neurotrophic signalling.

In addition to NGF cognate receptors, apolipoprotein D (ApoD) expression varies between the sexes during the normal brain ageing process. It protects against stress and conditions associated with ageing and neurodegenerative disease. In AD, ApoD expression is elevated in both males and females (Ordóñez et al., 2012). An age-dependent rise in cellular ApoD expression is observed in a number of brain regions in healthy females without evidence of degeneration or apoptosis; this is not evident in healthy males. Since ApoD exerts neuroprotective activities which promote stimulation of glial cells (Muffat and Walker, 2010) and the scavenging of lipid oxidation products in the surroundings of amyloid plaques (Li et al., 2015), this protein has two potential routes through which it may influence the sex discrepancies in AD. The rise seen in healthy elderly females may reflect the premature requirement for this neuroprotective function towards disease-inducing elements, such as Aβ oligomers and the early manufacture of fibrils. However, a deferred ApoD neuroprotective reaction may occur in males, i.e. the level only rises when AD is already manifest.

1.9.2 Gender Factors

Gender-related factors, e.g. degree of education, can inter-relate with the influence of the ApoE4 genotype. Females who carry the E4 allele, but who have a high degree of education at a young age, exhibit a lower likelihood of dementia (Wang et al., 2012c). Furthermore, activities that stimulated cognitive function, e.g. endeavours that necessitated complicated engagements with both data and people, bestowed a reduced dementia risk (Karp et al., 2009). Educational extent, type of job held and cognitively engaging hobbies in the middle years comprise intellectual enhancement that may retard cognitive impairment and the manifestation of dementia (Vemuri et al., 2012, Vemuri et al., 2014).

The influence of lifelong hobbies, education and mental challenges during work are mostly sex-related and dictated by history. Traditionally, males achieved more from an educational perspective than females and, in some low- and middle- income nations, this disparity is still very much in evidence (Petrongolo, 2019). Similarly, jobs that require a high degree of intellectual input have been conventionally limited to males, e.g. managerial positions in public or private enterprises, and top-level political or academic positions, amongst others, although in several nations gender distribution in such posts is now more equivalent (Tabassum and Nayak, 2021). In this regard, adjustments in sociocultural mindsets that have been arising in multiple nations over the latter half century may change future predictions of the impact of gender on AD (Tabassum and Nayak, 2021, Rocca et al., 2011).

1.9.3 Sex-Restricted Factors

Some of the risk factors for developing AD mentioned above are found equally in both sexes but show greater risk for females. So, what drives these risk factors to be more robust in influencing females. A point which had been noticed in preclinical studies is that when AD animal models are treated with E2, the risk factors for various molecules are diminished, e.g., genetic and metal homeostasis. That led to the theory that E2 is a restricted factor for women and that life alterations in its levels could increase their susceptibility for AD (Rocca et al., 2014). Indeed, it was reported for breast cancer treatments that the long-term inhibition of E2 synthesis using aromatase inhibitors was associated with long-term cognitive impairments, especially in breast cancer survivors (Cheung et al., 2013, Marbouti et al., 2020, Li et al., 2016).

One of the elements limited to females which will affect reproductive hormone levels and could be linked with a greater likelihood of AD is ovariectomy. Studies have demonstrated

that bilateral ovariectomy prior to menopause was associated with an elevated risk of cognitive impairment and dementia (Rocca et al., 2014). Bove et al. conducted a cohort study on the link between menopause arising from operative intervention, cognitive dysfunction and AD. Early menopause was linked with a more rapid deterioration in cognition, particularly in relation to episodic and semantic memories, and augmented production of amyloid plaques in individuals with AD. Supplementation with E2 in the perimenopausal phase decelerated the rate of cognitive loss (Bove et al., 2014). It has therefore been postulated that bilateral ovariectomy at a young age in females leads to a sudden fall in E2 levels which appears to precipitate pathological mechanisms that trigger degenerative and cerebrovascular injury.

1.10 Neuroprotective Functions of Oestrogen

Life alterations in reproductive hormone concentrations is a sex-limited factor which can significantly impact cerebral activity, although it is not yet evident whether this has any link with the risk of AD. A population-based study, which amalgamated data from Korea and Greece (Jang et al., 2018), noted that multigravida women who had had over four full-term pregnancies had twice the risk of AD of women who had undergone a lesser number or only partial pregnancies. This observation remains the subject of debate. A British study comprising a lower number of subjects reported that rising gestation numbers were associated with a diminished likelihood of AD (Fox et al., 2018). Results from a big prospective cohort study comprising 15,754 women added weight to the relationship between reproductive life and the risk of dementia. An older age of menarche, menopause prior to the age of 47 years and a shorter reproductive span were demonstrated to raise the incidence of dementia (Gilsanz et al., 2019). Additional research is required to validate the above findings and to elucidate whether cognitive complications and hormonal changes associated with pregnancy are connected (Abheiden et al., 2015, Fields et al., 2017).

E2 has numerous functions over and above its influence on sexual determination and reproductive activities. The marked decline in E2 levels is a major component of the menopause, with well-established adverse effects on the female body, such as loss of bone density and cardiovascular impacts (Christiansen, 1993, Stampfer and Grodstein, 1994). E2 also has a vital effect on the central nervous system (Galea et al., 2013, Cooke and Woolley, 2005, Brinton, 2009, Gibbs, 2010, Simpkins et al., 2010, De la Fuente et al., 2004a, Borras et al., 2007, De la Fuente et al., 2004b, Fuente et al., 2005), being able to enhance neurogenesis in a number of cerebral regions, e.g. the hippocampal dentate gyrus, which plays a role in

learning and memory (Galea et al., 2013). A neuroprotective influence of E2 has been reported in animal models including promoting dendritic spine formation within the hippocampus, (Cooke and Woolley, 2005), upregulation of LTP (Brinton, 2009), modulation of some neurotransmitters (Gibbs, 2010) and inhibiting apoptosis through its effect on mitochondrial activity (Simpkins et al., 2010). In females rats, E2 can also augment immunocompetence (De la Fuente et al., 2004a, Keller et al., 2001) and amplify antioxidant enzyme expression, e.g. reduced glutathione (Borras et al., 2007), which may maintain immune integrity during ageing (De la Fuente et al., 2004a, De la Fuente et al., 2004b, Fuente et al., 2005, Guayerbas et al., 2004).

1.11 Sex hormones and AD

It has been highlighted that, during critical points of ageing, the organizational impact of the sex steroid hormones in neural growth and maturation may change in the brain (Bowers et al., 2010, Gore, 2008, Krohmer and Baum, 1989, Slob et al., 1980, Weisz and Ward, 1980). Also, the manipulation of sex steroid signalling processes during essential early life points leads to enduring neural alterations (Bakker and Baum, 2008, Isgor and Sengelaub, 2003, McCormick et al., 1998). Thus, the variations between males and females in their susceptibility to neurodegenerative conditions may be a result of sexual dimorphisms that are established during development, together with disparities in the brain and circulating steroid hormones levels, E2, progesterone, testosterone and their breakdown products.

In females, it is well documented that E2 and progesterone serum concentrations are diminished significantly and rapidly as a consequence of the menopause (Sherman et al., 1976), and a decline in their supply to the brain and thus a decline in the neuroprotective effects in postmenopausal women was postulated (Bonomo et al., 2009, Henderson, 2006b). In contrast, the age-dependent fall in the male primary sex steroid hormone, testosterone, is only slowly progressive (Morley et al., 1997). The variation in the steepness of the decline in sex hormones in the two sexes could contribute to the higher incidence of AD in females compared with males (Pike et al., 2009).

Previous studies have shown that females suffering from AD have lower concentrations of circulating (Manly et al., 2000) and brain (Rosario et al., 2011, Yue et al., 2005) E2 than age-matched control subjects. Additionally, experimental ovariectomy enhanced the accumulation of Aβ in wild-type mice (Petanceska et al., 2000) and a number of mouse models of AD (Carroll and Pike, 2008, Carroll et al., 2007, Levin-Allerhand et al., 2002, Xu et al.,

2006, Gibbs, 2010), and this could be inhibited or rectified by administration of E2. However, ovariectomy and E2 supplementation failed to be accompanied by substantial alterations in Aβ overproduction in several of the AD animal models (Golub et al., 2008, Green et al., 2005, Yue et al., 2005). Yue at al. showed that ovariectomy failed to augment Aβ concentrations in the APP_{23} mice model. However, the levels of Aβ rose when the strain was crossed with an aromatase-deficient strain which inhibited the majority of E2 generation from sources outside the gonads (Yue et al., 2005).

1.11.1 Preclinical and Clinical Studies of the Neuroprotective Functions of Oestrogens and Progesterone

Oestrogens have a number of well-defined neural functions that have been postulated to influence their proposed protective activities with respect to AD. These neuroprotective roles can be divided into three overall groups: (i) enhanced cognition through the regulation of several mechanisms, such as spine density (Cooke and Woolley, 2005) and neurotransmitter systems (Gibbs, 2010) and LTP (Foy et al., 2010); (ii) protection against neuronal apoptosis (Simpkins et al., 2010, Suzuki et al., 2009); and (iii) suppression of certain facets of the disease process underlying AD, such as accrual of Aβ and tau hyperphosphorylation (Pike et al., 2009). Since the abundant aggregation of Aβ is broadly recognised as a major aetiological component in the initiation and advancement of AD pathology (Hardy, 2009, Hardy and Selkoe, 2002, Selkoe, 2011), the capacity of E2 to diminish Aβ levels may form its most significant mode of neuroprotection (Carroll and Rosario, 2012, Pike et al., 2009).

The first finding associating E2 with an Aβ-reducing function was the recognition that E2 had the ability to shift APP processing towards the non-amyloidogenic pathway (Gandy, 2003) and enhance lowering of BACE levels (Singh et al., 1999). In a number of cellular systems, E2 stimulated synthesis of sAPPα which, in turn, was able to diminish the quantity of Aβ produced (Amtul et al., 2010, Desdouits-Magnen et al., 1998, Jaffe et al., 1994, Manthey et al., 2001, Thakur and Mani, 2005). Whether E2 acts directly through stimulating the non-amyloidogenic pathway or if it alters the amyloidogenic pathway is still unclear (Greenfield et al., 2002). Some studies have shown that APP processing relies on ERK1/2 and/or pathways requiring PKC (Desdouits-Magnen et al., 1998, Manthey et al., 2001). Both these downstream signalling pathways are activated by E2. Furthermore, E2 has the ability to regulate the transcription of a number of secretase enzyme constituents that participate in APP processing, e.g. downregulation of BACE (Amtul et al., 2010, Bernstein et al., 2010, Nord et al., 2010).

Further elucidation is required to determine whether this action is reliant on an E2 receptor, facilitated by the traditional genomic functions of E2, or influenced by transcriptional regulation within the ERK1/2 pathway downstream. Of note is that, in several models described above, E2 modulates APP processing to diminish Aβ synthesis.

Current studies are highlighting the fact that E2 and potentially progesterone may mitigate the accrual of Aβ through another mechanism, i.e. augmentation of Aβ clearance. The equilibrium between the manufacture of Aβ and its removal dictates the steady-state for Aβ concentrations. The proteolytic activity of Aβ breakdown enzymes represents one of a range of essential processes that participate in the clearance of Aβ (Eckman and Eckman, 2005). Cerebral levels and the function of neprilysin are enhanced by E2 (Liang et al., 2010, Xiao et al., 2009). Additionally, E2 promotes insulin degrading enzyme activity (Amtul et al., 2010, Zhao et al., 2011) and stimulates microglia phagocytosis of Aβ (Singh et al., 1999). E2 has also been reported to amplify transthyretin expression (Amtul et al., 2010, Oliveira et al., 2011, Quintela et al., 2009) which attaches to and sequesters Aβ, thus precluding the formation of neurotoxic plaques (Schwarzman et al., 1994). Further work has demonstrated that a reduction in neurone numbers arising from the toxic effects of Aβ is mitigated by E2, which stimulates the anti-apoptotic Bcl-2 protein, together with simultaneous inhibition of the pro-apoptotic isoform expression (Pike, 1999). E2 also reduced hyperphosphorylated tau concentrations, a function which is reliant on the involvement of kinases and phosphatases, e.g. GSK-3β, Wnt, and PKA (Zhang et al., 2008).

Since the findings in both female humans and mice connect reduced E2 levels to AD and the multiple neuroprotective roles of E2 appear to be pertinent to AD, utilisation of E2-based hormone replacement treatment (HRT) in postmenopausal women is a potentially encouraging method to diminish the likelihood of AD. Preliminary results from a number of human observational and clinical trials (Henderson and Buckwalter, 1994, Hogervorst et al., 1999, LeBlanc et al., 2001, Paganini-Hill, 1996, Zandi et al., 2002), cited in (Craig and Murphy, 2010) and a retrospective analysis (Kim et al., 2021), although inconsistent (Haskell et al., 1997), have suggested that HRT initiation during menopause phase is linked with a decreased likelihood of AD.

Although there is a plethora of data to substantiate the neuroprotective functions of E2 with respect to AD, this theory is still the subject of debate. A significant study that contested this hypothesis is the Women's Health Initiative Memory Study (WHIMS) (Rapp et al., 2003b,

Shumaker et al., 2003). This was a component of a large-scale multicentre clinical trial that assessed the endpoints linked with HRT comprising conjugated equine E2 either as a sole agent or in conjunction with progestin medroxyprogesterone acetate. The data determined that HRT offered no retardation of cognitive impairment in females that had notable dysfunction, and in fact, heightened the likelihood of dementia.

A number of reasons to explain the WHIMS trial findings have been put forward with respect to the possible hazards and advantages of HRT (Gleason et al., 2005, Henderson, 2006a, Maki, 2004). The disparity between the empirical data demonstrating that E2 has an advantageous effect on the nervous system and the WHIMS clinical results emphasising the contradictory opinion, have supported the view that some major facets of HRT remain problematic. These include the formulation of E2, the use of conjugated equine oestrogens as opposed to E2 per se, the delivery route, e.g. oral or transdermal, and whether it should be offered continuously or in cyclic form (Gleason et al., 2005, Henderson, 2006a).

There is a plethora of results from studies that seem to reinforce these views. Carroll et al. investigated the use of continuous or cyclic delivery of progesterone together with E2 in 3xTg-AD mice following ovariectomy (Carroll et al., 2010b). Of note, the Aβ-reducing activity of E2 was mitigated when administered with continuous progesterone but restored when progesterone was delivered in a cyclic manner. It is possible that the most important elemental addition to the differential clinical endpoints of HRT is the age when therapy is commenced. The 'window of opportunity' hypothesis (Craig and Murphy, 2010, Whitmer et al., 2011) states that HRT has a greater likelihood of offering a beneficial neural impact when prescribed at the perimenopause as opposed to several years after menopausal onset, as generally occurred in WHIMS.

The perimenopause is a state of natural evolution to the menopause, when there is a steep drop in hormone levels, particularly of E2 and progesterone (Maki, 2013, Rocca et al., 2010). This changeover period is presumed to be essential to the neuronal protection impact of E2 (Daniel and Bohacek, 2010, Sherwin, 2007). Indeed, previous animal studies have shown that numerous advantageous effects of E2 are ameliorated in older females [cited in (Pike et al., 2009). Clinical findings have implied that the likelihood of AD is diminished when HRT is commenced in mid-life but potentiated when HRT is prescribed late in life (Whitmer et al., 2011). Nonetheless, an integral issue that may underlie the disparity in the preclinical and

clinical results is the lack of an appropriate animal model that naturally simulates the human menopausal hormonal scenario (Dubal et al., 2012).

Alternative sex steroid hormones, e.g. progesterone, may additionally participate in the variations relating to AD between the sexes. A recent study showed that the neuroprotective ability of E2 in non-Tg and Tg-AD mice is under the control of progesterone (Carroll et al., 2010a, Carroll et al., 2010b, Jayaraman et al., 2012). Neuroprotective effects against AD have been ascribed to progesterone, e.g. regulating of γ-secretase activity (Jung et al., 2013), and enhanced Aβ clearance catalysed by insulin degrading enzyme (Jayaraman et al., 2012).

Progesterone has also been shown to influence tau protein phosphorylation in cell culture studies, animal models and humans (Dang et al., 2013). In contrast to E2, progesterone failed to influence α-secretase (Jung et al., 2013, Amtul et al., 2010), although one study demonstrated that progesterone delivery to ovariectomised rodents stimulated BACE gene expression downregulation (Zhao et al., 2012). In a Tg-AD mouse model, use of progesterone enhanced performance in novel-object recognition and T-maze challenges (Frye and Walf, 2008). In this study, progesterone notably decreased Aβ concentrations, and also worked with E2 to mutually potentiate a neural protection effect. In contrast, a further study demonstrated that continuous progesterone delivery failed to affect Aβ levels and ultimately suppressed the protective effects of E2 (Carroll et al., 2010b). Lastly, oxidative injury arising from glutamate- (Nilsen and Brinton, 2003) and Aβ-provoked (Goodman et al., 1996) toxicity in hippocampal cell cultures was markedly ameliorated by progesterone. In conclusion, there is considerable evidence to support a part played by progesterone in the neuroprotective activity of the sex steroid hormones.

1.11.2 Males and the Andropause in AD

Despite the fact that both the prevalence and incidence of AD is greater in females, males also undergo a significant age-dependent rise in the risk of AD. In an analogous manner to females, the likelihood of AD in males is markedly impacted by a natural age-dependent fall in the major male sex steroid hormone, testosterone. Males do not experience a true andropause that mimics the swift decline in hormone levels and reproductive ability typical of the female menopause. Instead, the normal ageing process in the male sex is characterised by a slow decrease in bioavailable testosterone, which falls by approximately 2-3% annually once the age of 30 years is reached (Feldman et al., 2002, Muller et al., 2003). Since testosterone can be converted to E2, males do not exhibit the steep drop in E2 levels seen in females following the

menopause. The age-associated diminution in serum testosterone gives rise to a clinical entity referred to as androgen deficiency in ageing males (ADAM). This describes the augmented risk of pathology and abnormal activity of androgen-responsive tissues, such as the skeleton, muscle, adipose and cardiovascular systems (Morley et al., 1997, Kaufman and Vermeulen, 2005).

The brain is also an androgen-responsive tissue and is impacted by the age-associated fall in androgen levels. Assays of cerebral testosterone concentrations and its major breakdown product, dihydrotestosterone (DHT) in both rodents (Rosario et al., 2009) and humans (Rosario et al., 2011, Rosario et al., 2004) have shown that this organ is especially vulnerable to age-linked androgen deficit. In keeping with the recognised lower brain androgen concentrations, ADAM period features several neural adaptations such as decline in mood and libido (Morley et al., 1997) and, in some instances, impairment in some facets of cognition (Janowsky, 2006). In particular, the age-associated fall in androgens has been linked with an elevated likelihood of AD.

Antagonists to androgen such as those used for the treatment of malignancy have been connected to a heightened likelihood of AD, in keeping with findings noted in females (Wagner et al., 2020). Androgen deprivation treatment in prostate cancer causes a lasting drop in free testosterone levels and has been reliably related to a rising risk of AD over a decade of follow-up (Nead et al., 2016, Jayadevappa et al., 2019), along with reported rise in serum Aβ concentration of prostate cancer patients treated with androgen antagonist (Almeida et al., 2004, Gandy, 2003).

Males with AD demonstrated reduced serum (Moffat et al., 2004, Hogervorst et al., 2003, Hogervorst et al., 2001) and brain testosterone levels compared with age-matched healthy males (Rosario et al., 2011, Rosario et al., 2004). Significantly, the decline in testosterone seemed to occur prior to clinical (Moffat et al., 2004) and neuropathological (Rosario et al., 2011, Rosario et al., 2004) manifestations of AD, implying that androgen loss is a precursor event that is likely to be an aetiological factor and not a consequence of the AD pathological process. Of note, is that in males with preclinical AD, cerebral testosterone concentrations are negatively correlated to soluble brain Aβ. This finding implies a potential mechanism associating testosterone with the risk of developing AD (Rosario et al., 2011). However, brain E2 and oestrone levels have no relation to AD in males, and show no relationship with cerebral concentrations of Aβ, suggesting sex variations in the associations between AD likelihood and the sex steroid hormones (Rosario et al., 2011).

In a similar manner to oestrogens, androgens engage in several protective activities within the brain that may be pertinent to their theorised protective function against the pathogenesis of AD. These activities encompass: (i) encouragement of neurone growth and regeneration of axons and synaptic function (Brännvall et al., 2005, Huppenbauer et al., 2005); (ii) protection against neurone cell damage caused by oxidative stress or Aβ toxcicity (Toro-Urrego et al., 2016, Lei and Renyuan, 2018); and (iii) regulation of AD-associated disease processes, such as Aβ aggregation (Gouras et al., 2000). Foe the lateral neuroprotection function, *In vivo*, androgen depletion in male rats through orchidectomy (ORX) was associated with increases in Aβ production while treatment of these animals with dihydrotestosterone (DHT) decreases Aβ levels in their brains (Ramsden et al., 2003). In addition, the natural age-associated fall of brain DHT that is typically evident by 13 months of age in rats was associated with a parallel rise in brain Aβ concentrations (Rosario et al., 2009).

Analogous to the management of Aβ by oestrogens, androgens may diminish Aβ concentrations by influencing both Aβ synthesis and clearance. Early research suggested that testosterone lowers Aβ by shifting APP processing in the direction of the non-amyloidogenic pathway (Gouras et al., 2000). Given that it is established that E2 has an equivalent effect on APP, it is not unexpected that a degree of the testosterone control of APP metabolism was determined to be reliant on its aromatase-catalysed reaction to generate E2 (Goodenough et al., 2000). Nevertheless, many of the Aβ-reducing activities of androgens arise autonomously from E2 pathways. In a mouse aromatase knockout model of AD, increased testosterone was related to a lower Aβ load and diminished expression and function of β-secretase (McAllister et al., 2010). In keeping with the combined influence of the E2 and androgen pathways, research performed recently on the Aβ-reducing impact of the sex steroid hormones showed that Aβ was notably reduced in ORX 3xTg-AD by testosterone, DHT and E2; testosterone had the most potent effect and E2 the weakest (Rosario et al., 2010).

A further mechanism via which androgens suppress the accretion of Aβ is by promoting Aβ removal. Studies in neuronal cultures (Yao et al., 2008) and animal models (McAllister et al., 2010, Yao et al., 2008) have suggested that the reduction of Aβ levels produced by androgens was connected to amplified neprilysin expression; this enzyme seems to be key to the risk of AD (Wang et al., 2010). Androgens achieve this through androgen receptor (AR)-mediated classic genomic pathway (Yao et al., 2008) that incorporates androgen response factors in the neprilysin gene (Shen et al., 2000). A number of studies have additionally

demonstrated that testosterone therapy enhances cognitive abilities in males (Tan and Pu, 2003, Cherrier et al., 2005).

Interestingly, sex differences seem to exist between E2 and androgen neuroprotective effects. Whereas in females, for instance, oestrogens govern spine density, androgens exerted a more potential neuroprotection effect than oestrogens in the male brain in certain regions such as the hippocampus (Hajszan et al., 2008, Leranth et al., 2003).

1.12 Thesis Hypothesis and Aims

One of the greatest challenges in the field of neuroscience is to grasp what happens to the brain as it ages. A critical strategy for AD prevention is to distinguish between individuals undergoing normal ageing and those who are at risk of developing AD. Since the development of the amyloid hypothesis, over the last 3 decades several human brain imaging studies have tried to investigate the correlation between Aβ plaques and neuronal activity in age-related cognitive dysfunction in both sexes (Counts et al., 2017, Jack et al., 2010). Also, most AD studies enrol adults over 60 years of age, ignoring the longitudinal nature of AD. These studies overlook the most significant period of neuroendocrine changes in a woman's life which is the menopause transition period, where ovarian sex steroids undergo a significant decline in their production, with up to a 90% decline in oestradiol. This dramatic endocrine change influences multiple biological systems including brain activity (Morrison et al., 2006)

Furthermore, the data pertaining to the contentious preclinical, clinical and epidemiological aspects of the sex variations in AD should not be assumed to be a straightforward result of increasing age or longevity, i.e. studies should not discard the notable sex discrepancies that arise throughout brain growth and differentiation which are founded on considerable evidence, and particularly, results from *in vivo* models. Also, studies on AD should consider sex-specific infirmities and susceptibilities over the life time and not simply what makes one sex more prone to AD than the other in a specific age group (Mazure and Swendsen, 2016).

Although a number of these sex disparities could be attributed to the sex hormones (Witte et al., 2010, van Amelsvoort et al., 2001, Lentini et al., 2013), the precise ways in which they affect the human brain is not yet fully elucidated, since both males and females express E2 and testosterone. Nonetheless, the drop in sex steroid hormones associated with ageing is unquestionably one of the underlying causes of the sex disparities linked to AD. Even though

the individual hormones have a potential protective influence with respect to the risk of AD, female hormones exhibit a more precipitous drop because of the menopause and thus the susceptibility to AD is greater in this sex. In contrast, males are less susceptible to the decline in the protective properties of E2 but may be prone to additional elements associated with AD, e.g. oxidative stress.

To date, no clinical study had investigated the relationship between brain ageing and the biological sex differences in the development of AD. In this project, it is ***hypothesised*** that there is an inter-relationship between ageing and biological sex which affects many different aspects of brain function and contributes to the increased susceptibility of women to the development of AD.

The first aim was to explore the levels of AD biomarkers during human brain ageing and in AD in males and females. This aim is described in the first two experimental chapters, Chapters 3 & 4, where APP, its metabolic enzymes and by-products were investigated, along with tau and its phosphorylation enzymes.

The second aim was to investigate the expression of oestrogen-associated proteins and associated downstream signalling pathways in the same brain samples. The work for this aim is described in Chapter 5. In Chapter 6, work continued on this aim to determine the cellular localization of oestrogen-associated proteins in female brains during ageing and in AD.

The third aim was to take the key findings of the four experimental chapters, Chapters 3 to 6 and explore their functional relevance using human stem cell-derived neurones. A Pilot study for this aim on neurones from control and AD stem cells are presented in Chapter 7.

CHAPTER 2 _ EXPERIMENTAL METHODS

Overview: This Chapter identifies the criteria used to select the human brain samples and the process of preparing them for investigating the expression of proteins of interest throughout the thesis. In addition, biochemical techniques to identify and quantify targeted protein levels in human brain and stem cells along with genomic studies are described here. Methods which are specific to individual experiments are discussed in the relevant chapter.

2.1 Antibodies and Reagents

All chemical, reagents and materials were purchased from Sigma–Aldrich, Poole, UK or Fisher Scientific, Leicester, UK unless otherwise specified.

2.2 Human Brain Samples

Fresh frozen human frontal cortex brain samples from individuals of both sexes with no recorded history of dementia who died due to different reasons and samples from AD patients were obtained from the Newcastle Brain Tissue Resource, the Sudden Death Edinburgh Brain and Tissue Bank, the Cambridge Brain Bank and the Manchester Brain Bank. For the ageing groups, the MRC Brain Bank database, which lists details on all brain samples held by Brain Banks in the UK, was used to confirm that the subjects did not suffer from any ante-mortem neurodegenerative disease nor presented with any neuropathological symptoms of dementia or AD. In addition, human brain samples were used representing different periods in life including young before the start of any signs of AD pathology such as Aβ accumulation and after the start of the menstrual cycle where oestradiol is detectable in serum (20-35 years old) (Henderson, 2009), middle age, representing perimenopause age (Danforth et al., 1998) and during the possible initiation of early pre-clinical stages of AD (45-55 years old) (Jack et al., 2010) and old age, representing post-menopause (Nordin et al., 1990) and when clinical symptoms of AD are often manifested (>70 years old) (Table 2.1). For the comparisons with AD patients, the non-diseased (ND) age-matched controls used were the same old samples with age > 70. The AD samples (Table 2.2) used were at Braak stage V-VI (determined by the Brain Banks), i.e. late stage AD, the most severe stage of the disease where Aβ aggregation and tau hyperphosphorylation invade the neocortex and affect motor and sensory functions in addition to memory (Braak et al., 2006). Thus, these sample groupings will enable me to draw conclusions about the fate of my proteins of interest during ageing and how they may be involved in the development of AD. In addition to the age grouping method, male and female samples were investigated separately to control for the effect of sex in any age-related changes,

followed by comparing proteins of interest identified in the ageing studies between the male and female groups to see if sex could be a factor affecting these changes.

For the brain samples used, the mean age ± SD for each group of male samples was 25.6 ± 3.1 years, 50 ± 2.4 years, 75.1 ± 7.3 years and 78.4 ± 5.5 years for young, middle, old and AD subjects, respectively. For the female subjects, the mean age ± SD was 27.3 ± 6.9 years, 44 ± 4.2 years, 80 ± 9.8 years, and 80.8 ± 7.4 years, for young, middle, old and AD subjects, respectively. The mean post-mortem interval for each age group investigated was 53.6 ± 26 hours, 70.78 ± 20 hours, 41.8 ± 23 hours, and 60 ± 25.3 hours, respectively for female samples, while for male samples the average PMI was 52.8 ± 20.2 hours, 46.5 ± 3.43 hours, 53.4 ± 20.6 hours, and 49.5 ± 13.5 hours, respectively (Table 2.1 and 2.2). All samples were stored at -80 ^{o}C prior to use. All procedures were performed under the U.K. Human Tissue Act (2004).

Table 2.1 Summary of the sample identifiers, ages, sex, and post-mortem intervals along with the samples pH when collected and the Braak tangle stage of the human frontal cortex brain samples

Brain Bank Sample Identifier	**Medical Research Council Identifier**	**Age (years)**	**Sex**	**Post-mortem interval (Hours)**	**pH^{-}**	**Braak Tangle stage**	**Cause of Death**	**Experiments**	
								W.B	**IHC**
Young Subjects									
SD023/08	BBN_2442	24	Female	47	6.4	-	Suspension by ligature	✓	✓
SD020/06	BBN_2360	20	Female	94	6.39	-	Combined drug poisoning	✓	
SD006/10	BBN_2503	32	Female	63	6.3	-	Acute decompensation of mitral valve disease and cardiomyopathy	✓	✓
SD001/08	BBN_2420	35	Female	44	5.9	-	Depression	✓	✓

							Increased alcohol intake		
SD009/06	BBN_2349	24	Female	88	6.62	-	Suspension by ligature	✓	
SD036/08	BBN_2455	20	Female	40	6.5	-	Suspension by a ligature	✓	✓
NP16-266	BBN110.29591	27	Female	31	6.06	-	Anorexia with Alcoholism	✓	✓
NP013/2016	BBN004.28581	36	Female	22	-	-	medication overdose	✓	
SD042/12	BBN_7270	29	Male	44	6.46	-	Suspension by ligature	✓	
SD014/09	BBN_2473	26	Male	44	6.3	-	Methadone and diazepam toxicity	✓	
SD021/10	BBN_2518	27	Male	40	6.2	-	MDMA (ecstasy) toxicity	✓	
SD041/05	BBN_2335	24	Male	51	6.3	-	Chronic alcoholism	✓	
SD027/06	BBN_2367	25	Male	41	6.45	-	Acute hydrocarbons poisoning	✓	
SD019/06	BBN_2359	20	Male	36	5.6	-	Hyperglycaemic coma	✓	
SD033/08	BBN_2452	22	Male	95	6.6	-	Combined effects of hypertensive heart disease and	✓	

							ischaemic heart disease Obesity		
SD030/11	BBN_2569	30	Male	71	6.4	-	Unascertained	✓	
SD008/12	BBN_3771	25	Male	53	6.4	-	Suspension by ligature	✓	
SD025/05	BBN_2322	28	Male	38	6.61	-	Suspension by a ligature	✓	
MiddleAge Subjects									
SD031/15	BBN_26124	40	Female	89	-	-	Suspension by a ligature	✓	✓
SD005/15	BBN_24479	46	Female	76	-	-	Urosepsis, Long term suprapubic catheter, Advanced MS, Immobility	✓	
SD037/14	BBN_23395	41	Female	50	6.4	-	Unascertained	✓	
SD031/14	BBN_22618	41	Female	40	6.6	-	Suspension by ligature	✓	
SD061/13	BBN_19591	40	Female	77	6.4	-	Bronchial asthma	✓	✓
SD042/13	BBN_18393	46	Female	49	6.5	-	Myocardial infarction.	✓	✓
SD017/13	BBN_14397	45	Female	93	6.36	-	Coronary artery atherosclerosis	✓	✓

SD016/13	BBN_14396	44	Female	70	6.74	-	Unascertained	✓	✓
DMP12/16	BBN_6067	53	Female	93	-	-	Unascertained	✓	
SD038/12	BBN_4176	51	Male	45	6.39	-	Hameopericardium, Ruptured cardiac aneurysm	✓	
SD023/13	BBN_15221	53	Male	45	6.1	-	Coronary artery thrombus And atherosclerosis	✓	
SD003/11	BBN_2542	49	Male	44	6.2	-	Ischaemic heart disease	✓	
SD023/12	BBN_3785	50	Male	45	6.3	-	Ischaemic heart disease.	✓	
SD015/10	BBN_2512	52	Male	47	6.2	-	Coronary thrombosis, Ischaemic heart disease.	✓	
SD037/11	BBN_2576	48	Male	46	6.2	-	Coronary artery atherosclerosis	✓	
SD033/10	BBN_2530	51	Male	52	6.3	-	Ischaemic and hypertensive	✓	

							heart disease.		
SD012/11	BBN_2551	53	Male	42	6.3	-	Coronary artery atherosclerosis, Ischaemic heart disease.	✓	
SD044/12	BBN_7271	46	Male	52	6.59	-	Coronary artery atherosclerosis, Ischaemic heart disease.	✓	
SD025/13	BBN_15223	47	Male	42	6.5	-	Coronary atherosclerosis	✓	
Elderly Subjects									
20060093	BBN_7403	78	Female	23	6.47	-	Metastatic Oesophageal Carcinoma with Broncho-oesophageal Fistula	✓	
20070049	BBN_7415	72	Female	27	-	-	Pulmonary oedema, Hypertension, Bronchopneumonia	✓	

20130020	BBN_13407	97	Female	21	6.4	II*	Chest infection	✓	
20130656	BBN_18821	99	Female	5	6.14	II*	Congestive heart failure	✓	
20141901	BBN_24279	80	Female	25	6.1	II*	Pancreatitis/ Peritonitis	✓	
20090088	BBN_7482	94	Female	50	6.05	II*	Unascertained	✓	
SD013/09	BBN001.35405	77	Female	85	6.2	-	Increasing frailty, Supranuclear palsy	✓	✓
SD030/09	BBN_2489	71	Female	70	6.1	-	Coronary artery atherosclerosis	✓	✓
SD027/11	BBN_2566	79	Female	46	6.5	-	Haemopericardium, Cerebrovascular disease	✓	✓
SD030/12	BBN_4169	71	Female	41	6.52	-	ischaemic and hypertensive heart disease.	✓	✓
SD014/13	BBN_14395	74	Female	41	6.3	-	Pulmonary thrombo-embolism	✓	✓
20090067	BBN_7477	74	Female	67	-	I*	Lung Ca.	✓	
SD016/11	BBN_2555	74	Male	66	6.3	-	Ischaemic heart disease	✓	
SD034/08	BBN_2453	70	Male	50	6.2	-	Pulmonary embolism,	✓	

							Deep vein thrombosis, Hypertension.		
SD025/10	BBN_2522	75	Male	47	5.4	-	Ischaemic heart disease, Coronary atherosclerosis	✓	
SD023/10	BBN_2520	74	Male	74	6.4	-	Ischaemic heart disease, Coronary artery atherosclerosis	✓	
SD015/12	BBN_3778	70	Male	74	6.9	-	Hypertensive and ischaemic heart disease. Type 2 Diabetes Mellitus.	✓	
SD036/12	BBN_4174	75	Male	78	6.43	-	Ischaemic heart disease, Coronary artery atherosclerosis.	✓	

20050087	BBN_7111	68	Male	54	-	-	Bowel Cancer	✓	
20040091	BBN_7386	75	Male	64	-	-	MI| Chest Infection| Skin Ca (Left Temporal)	✓	
20130894	BBN_19192	80	Male	16	6.36	II*	Prostate cancer	✓	
20131187	BBN_19217	88	Male	26	6.1	II*	Unascertained	✓	
20140411	BBN_24265	88	Male	28	6.3	II*	Chronic lymphocytic leukaemia	✓	
20030123	BBN_7341	64	Male	64	-	-	Ascending aortic aneurism	✓	

- Some samples did not have any pH values recorded by the Brain Bank.
* **Braak Stage I-II**, the individual shows some Aβ precipitation in their brain but are cognitively normal. They do not have AD (Braak et al., 2006).
W.B.= Western Blot
IHC= Immunohistochemistry

Table 2.2 Summary of the sample identifiers, ages, sex, post-mortem intervals, along with the samples pH when collected and Barak stage of the AD human frontal cortex brain samples

Brain Bank Sample Identifier	**Medical Research Council Identifier**	**Age (years)**	**Sex**	**Post-mortem interval (Hours)**	**pH⁻**	**Braak Stage**	**Experiments**	
							W.B	**IHC**
20090100	BBN_7485	80	Female	32		VI	✓	
20100098	BBN_7494	86	Female	69	-	VI	✓	
20110681	BBN_7562	90	Female	90	-	VI	✓	

20119990	BBN_7617	89	Female	65	6.11	VI	✓	
20140747	BBN_24271	80	Female	10	6.07	VI	✓	
DPM10/15	BBN_5766	76	Female	52	5.35	VI	✓	✓
DPM11/28	BBN_3466	71	Female	64	-	VI	✓	✓
DPM14/05	BBN_19684	69	Female	60.5	-	VI	✓	✓
DPM15/21	BBN_25109	79	Female	97	-	VI	✓	✓
DPM18/17	BBN_24287	88	Female	61	6.54	VI	✓	✓
20101151	BBN_7535	92	Male	40	-	VI	✓	
20070062	BBN_2606	76	Male	64	5.9	VI	✓	
20030116	BBN_7340	83	Male	48	-	VI	✓	
20090026	BBN_2612	83	Male	63	-	VI	✓	
20100483	BBN_7509	78	Male	37	-	VI	✓	
20121029	BBN_12991	68	Male	71	5.7	VI	✓	
20130871	BBN_19191	76	Male	53	5.89	VI	✓	
20100557	BBN_7511	85	Male	29	6.1	VI	✓	
DPM10/31	BBN_3426	75	Male	50.5	5.6	VI	✓	
20141909	BBN_24287	85	Male	39	-	VI	✓	

- Some samples did not have any pH record in the Brain Bank.

There is controversy around the impact of pH and long PMI on sample quality and protein levels (Ferrer et al., 2007). However, when the available pH values were compared between each age for male and female samples separately, no differences were observed (Appendix I). On the other hand, PMI was significantly higher in middle age against the elderly aged female samples (Appendix II). Thus, in each age group of both sexes, the correlation between PMI and protein expression data was calculated, and accordance to (Appendix III) there were no significant correlations for PMI for each age group in both sexes with majority of the investigated proteins in the thesis. Therefore, it was assumed that pH and PMI did not affect the results obtained in this thesis.

2.3 Human Induced Pluripotent Stem Cell (hIPSC) -Derived Cortical Neurons

Neural precursor cells obtained from dermal fibroblasts of a 67-year-old healthy female donor (ax0019) and 87-year-old AD female donor (ax0111) were purchased from Axol Bioscience Cambridge, UK. The AD patient was reported to be homozygous for ApoE ε4 (ApoE ε4 HOM). All the medium and reagents used for the culture of these cells were from Axol unless otherwise specified.

Cells were initially expanded to get enough cells for differentiation. The expansion step was conducted using NeuroCult™-XF Proliferation Medium (StemCell Tech., Cat. 05761) according to Axol's recommended protocol (Axol Protocol Weblink). Before thawing the cells, the tissue culture plate surface was pre-coated with SureBond XF (ax0053) at a volume of 200 µl/cm^2 so that the cells would adhere to the plate and incubated for 4 hours. All incubations were done at 37 ^{o}C in 5% CO_2. Next, the stem cells were seeded at a density of 75,000 cells/cm^2 using neural plating medium (ax0033) and incubated for 24 hours. The next day the medium was changed to NeuroCult™-XF Proliferation Medium freshly supplemented with 20ng/ml hEGF and hFGF2 (ax0048 & ax0047). The proliferation medium was changed every other day to remove any debris or dead cells and incubated until the cells were 80% confluent and ready for passaging.

For cell passaging, first the medium was removed, then cells were washed with PBS three times and discarded. A 3ml warm trypsin is added to the cell and incubated for 5 minutes at 37 ^{o}C in 5% CO_2. The flask is then banged hard on the side after the incubation to make sure the cells are detached from the flask surface. The detached cells were aspirated with warm NeuroCult™-XF Proliferation Medium at a concertation of 5:1 to the trypsin volume. The cell suspension was transferred to a corning flask and centrifuged at 200x g for 5 minutes. The supernatant was discarded, and cells were resuspended with a 2ml neural plating medium (ax0033). Finally, cells volume was determined with hemocytometer and were seeded at density of 75,000 cells/cm^2.

The previous method of cell culturing, the 75000 cells/cm, were expanded up to 3 passages as above to get sufficient cells for the experiments then seeded on 24- and 12-well plates to do the experiments. 13mm glass coverslips were used in the 24 well plates and, according to Axol's protocol, the coverslips were first pre-coated with 0.1mg/ml Poly-D-

Lysine (Sigma Aldrich, P7405) for 5 minutes then the coating reagent was removed, and the coverslips was dried at room temperature (RT) for 2 hours. The coated coverslips were then precoated with 200 µl/cm^2 SureBond-XF for 1 hour; the double coating aids in cell attachment to the glass surface during differentiation. The 12-well plates were coated only with 200 µl/cm^2 SureBond-XF for 4 hours. A density of 70,000 cell/cm^2 of stem cells was seeded using neural plating medium and incubated for 24 hours to allow cell attachment to the surface. This was followed by changing the medium to NeuroCult™-XF Proliferation Medium, free from growth factors, and incubating for 24 hours. The day after, the cells were ready for differentiation.

For differentiating the precursor cells to mature cortical neuron cells, the proliferation medium was changed to pre-warmed neural differentiation-XF medium (ax0034) and incubated for up to 20 days. Half of the differentiation medium was changed every 2 days and the differentiation process took 20 days to produce mature neuronal cells. After differentiating, cells were maintained with Neural Maintenance-XF Medium (ax0032) for up to 1 month until use, changing half of the maintenance medium every 2 days.

2.4 Protein Extraction

2.4.1 Tissue Protein Extraction

100mg of each brain sample was homogenised in 2% sodium dodecyl sulphate (SDS) with 1% protease inhibitor cocktail II (Calbiochem®, Millipore) at 75mg/ml wet tissue weight. The tissues were homogenised with a Precellys 24-Dual (Bertin Technologies, Montigny- le-Bretonneux, France) at 5500 rpm for 2x30 seconds. Then the homogenates were rotated overnight at 4 °C. The next day, homogenates were transferred to centrifuge tubes and centrifuged at 100,000g/28,300 rpm for 1 hour at 4 °C (Beckman Coulter, Optima LE-80K, Rotor 50.4Ti). The supernatant was then diluted with 1:3 EC Sodium Buffer (20mM Na_2HPO4/NaH_2PO_4, 0.4M NaCl, 0.2mM EDTA, 0.05% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% (w/v) bovine serum albumin (BSA), 0.05% (w/v) NaN_3 at pH 7). Samples were aliquoted and stored at -80 °C. Further, only for the old and AD samples, the insoluble pellet was dissolved in 70% formic acid at 150mg/ml original sample wet weight. Then the samples were centrifuged as above for 1 hour at 4 °C. After that, the supernatant was diluted with 1:20 neutralizing buffer (1M Tris, 0.5M Na_2HPO_4 at pH 11) and stored at -80 °C.

2.4.2 *Cell Lysis of Neuronal hIPS cells*

The experiment was conducted at 4 °C. First, the medium was removed completely, and cells were incubated with approximately 40 µl/cm^2 lysis buffer (50mM Tris base, 50mM EDTA-disodium salt, 150 mM NaCl, 1% (v/v) Triton at pH 7.5) and 1% protease inhibitor cocktail II (Calbiochem®, Millipore) for 15 minutes. The resulting cell suspension was transferred to a cold Eppendorf tube and centrifuged at 10,000 x g at 4 °C for 30 minutes. The supernatant was removed and stored at -80 °C.

2.4.3 *Bicinchonic Acid Protein Assay*

A bicinchonic acid (BCA) protein assay (PierceTM LOT.TG268884, ThermoScientific, UK) was used for all protein concentration determinations. Bovine serum albumin standards were prepared from 1 - 0.01 mg/ml in a serial dilution. 25µl standards and negative blank control (distal H_2O) were loaded in duplicate in a 96 well plate, 1 and 5 µl samples along with sample blanks (2% (w/v) SDS) were tested in duplicate. 200µl BCA Working Reagent consisting of 50 parts of Reagent A and 1 part of Reagent B was then added to all wells. The plate was mixed thoroughly for 30 seconds on a plate shaker, followed by incubation at 37 °C for 30 minutes. The plate was then read using a spectrophotometer (Infinite f50, Tecan) at 492 nm. Graphpad Prism-8 was used to generate a standard curve from the BSA standards which was used to determine the protein concentration of each sample.

2.5 Western Blot (WB) - Reducing Conditions

Samples were diluted 1:3 with 3x Sample Buffer (6.3mM Tris-Base, 20% (v/v) Glycerol, 0.6% (w/v) sodium dodecyl sulphate (SDS) (BioRad, UK), 2% (v/v) bromophenol blue, 7.5% (v/v) β-mercaptoethanol) and heated at 70 °C for 10 minutes for the first use in Western blot analysis. Samples were then re-frozen for later use when they were briefly thawed at 70 °C for 5 minutes before loading to resuspend the SDS to inhibit metabolism of sample proteins by proteases. 8µl of the molecular mass marker (Precision Plus Protein Standards marker, Bio-Rad Laboratories, Hercules, California, USA) and 10-20 µg samples were loaded onto 10% polyacrylamide gels (3.33% (v/v) acrylamide, 1.25% (v/v) 3M Tris.HCl, 0.1% (v/v) SDS, 0.05% (v/v) Ammonium persulfate (APS) (BioRad), 0.005% (v/v) Tetramethylethylenediamine (TEMED)) and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer (25mM Tris base, 190 mM glycine, 0.05% (w/v) SDS, pH 8.3) for 2 hours. The separated proteins were then blotted on to

0.2µm nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK) at 38.4mA for 60 min. Following that, the membranes were washed in Tris-buffered saline with Tween 20 (TBST, 2 mM Tris base, 15 mM NaCl, 0.1% (v/v) Tween-20, pH 7.5) and blocked for 1 hour at room temperature in 5% (w/v) non-fat milk (NFM) powder in TBST (Tesco). Membranes were incubated in 1% NFMT with primary antibodies prepared as described in Table 2.3 either overnight on a roller at 4 ^{o}C or for 90 minutes at RT. The membranes were washed as above in TBST and then incubated for 1 hour at RT with anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.4) according to the primary antibody species (Table 2.3) prepared in 1% NFMT. After that, membranes were rewashed in the same fashion with TBST. Bands were visualised with Super Signal, West Dura, or ECL (Perbio Science, UK) for 5 minutes then bands were detected using a Syngene® G BOX gel imaging system linked to automatic control software (Syngene, Frederick, MD, USA). Membranes probed for total proteins were stripped and re-probed for the appropriate phosphorylated protein by incubating the membrane with already prepared stripping buffer (Fisher, UK, 46430) for 8 minutes. All membranes were then stripped and re-probed with mouse monoclonal anti-β-actin primary antibody in 1% NFMT at room temperature for 60 min and detected with ECL as above.

Table 2.3 Primary and Secondary Antibody dilutions and suppliers for Western Blotting.

Primary Antibodies	**Species**	**Source**		**Dilution**	**Secondary Antibody Dilution**
		Manufacturer	**Code**		
Anti-APP	Mouse	Millipore, Watford, UK	MAB348	1:500	1:10,000
Anti-total GSK-3 α/β	Mouse		05-412	1:500	1:10,000
Anti-Phospho GSK-3 α/β (Tyr279/216)	Rabbit		ST1013-10T	1:500	1:15,000
Anti-total tau	Rabbit	Dako, Hamburg, Germany	DK-2600	1:50,000	1:50,000
Anti-Phospho tau	Mouse	Generous gift from Prof.	PHF-1	1:250	1:5,000

		Peter Davies, Albert Einstein College of Medicine, Bronx, NY, USA.			
Anti-Total Akt	Rabbit	Cell Signaling Technology, Beverly, MA, USA	9272	1:500	1:7,000
Anti-Phospho Akt (Ser473)	Rabbit		4060	1:500	1:7,000
Anti-Total MAPK (Erk 1/2)	Rabbit		4695	1:500	1:15,000
Anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit		4370	1:1000	1:15,000
Anti-PP2A	Rabbit		4953T	1:500	1:10,000
Anti-ERα	Rabbit	Abcam, Cambridge, MA	ab32063	1:200	1:2,000
Anti-Phospho ERα (Ser118)	Rabbit		ab32396	1:200	1:2,000
Anti-ERβ	Rabbit		ab3576	1:1000	1:3,000
Anti-Phospho ERβ (Ser105)	Rabbit		ab62257	1:500	1:3,000
Anti-PS-1	Rabbit		ab76083	1:500	1:15,000
Anti-PS-2	Rabbit		ab51249	1:500	1:10,000
Anti-ADAM10	Rabbit		ab1997	1:1000	1:15,000
Anti-BACE1	Rabbit		ab263901	1:500	1:30,000
Anti-Androgen receptor	Rabbit		ab133273	1:500	1:3,000
Anti-BCL-2	Rabbit		ab32124	1:1000	1:1,000

Anti-BAX	Rabbit		ab182733	1:200	1:5,000
Anti-G-protein Coupled Receptor 30	Rabbit		ab39742	1:500	1:3,000
Anti-Progesterone receptor	Mouse		ab2765	1:200	1:2,000
Anti-Androgen receptor	Rabbit		ab133273	1:500	1:3,000
Anti-Caspase3	Rabbit		ab136812	1:250	1:10,000
Anti-β-Actin conjugated with horseradish peroxidase (HRP)	Mouse	Sigma-Aldrich	A3854	1:15,000	-

Table 2.4 Secondary Antibodies with horseradish peroxidase (HRP) for Western Blotting

Secondary Antibodies	**Species**	**Source**	
		Manufacture	**Code**
Anti-Rabbit	Goat	Vector Laboratories	PI-1000
Anti-Mouse	Horse		PI-2000

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

Sandwich ELISAs, a highly sensitive quantification method, were performed for the detection of APP using the DuoSet ELISA (R&D Systems, DY850 and DY008, Abingdon, Oxon., UK) and for its amyloidogenic fragments, sAPPβ, βCTF, soluble and insoluble isoforms of Aβ, Aβ40 and Aβ42, and the non-amyloidogenic fragment sAPPα (all IBL International, Stratech Scientific Limited, UK). The ELISA procedures were performed according to the supplier's protocol and guidelines and described below. The data were

normalised to the total protein concentration as ng/mg for APP, sAPPβ and sAPPα, pg/mg for Aβ and pmol/mg for βCTF.

2.6.1 Amyloid Precursor Protein (APP)

All materials and reagents were bought from R&D Systems, unless otherwise specified. To prepare the plates, 96-well microplates (DY990) were coated with 100 µl capture antibody at a concentration of 4 µg/ml, diluted with phosphate buffer saline (PBS, DY006). The plate was sealed and incubated overnight at RT. The next day, wells were washed 4 times with washing buffer (WA126) followed by an essential step to remove any washing buffer through inverting the plate and blotting against clean paper towel. Unbound sites in each well were blocked with 300 µl of reagent diluent (DY995) for 1 hour at RT. Wells was washed as above ready for protein detection.

The standard was prepared in a two-fold serial dilution in reagent diluent at 20 ng/ml to 0.625 ng/ml. Samples were diluted in reagent diluent at 1:40. 100 µl of standard and samples were loaded in duplicate on the plate and incubated at RT for 2 hours. Plates were washed as above, and the detection antibody was prepared with reagent diluent at 300ng/ml and 100µl/well was loaded and incubated for 2 hours at RT. Wells were washed again as before. Streptavidin-HRP enzyme was prepared at 1:200 with reagent diluent, loaded at 100 µl/well and incubated in the dark at RT for 20 minutes. Wells were washed as above and 100 µl enzyme substrate solution 1:1 (DY999) was loaded in each well and incubated at RT for 20 minutes in the dark. The colour change reaction was then stopped by adding 50 µl/well of stop solution (DY994) and tapping the plate to ensure thorough mixing. Finally, the plate was read at 540 nm and at 450nm using a plate reader (Infinite f50, Tecan). The reading at 540 nm was subtracted from 450nm reading to correct for any optical imperfections in the plate.

2.6.2 sAPPα, sAPPβ and βCTF

Kits were bought from IBL International (sAPPα (No. 27734), sAPPβ (No. 27732) and βCTF (No. 27776)). Standards were prepared in a two-fold serial dilution from 12 pmol/L to 0.19 pmol/L for βCTF and from 50 ng/ml to 0.78 ng/ml for both sAPPα and sAPPβ. For the samples, dilutions of 1:4, 1:2 and 1:3 for sAPPα, sAPPβ and βCTF, respectively, were prepared, 100 µl of standards and samples were loaded in duplicate on precoated plates (precoated with Anti-Human sAPPα (2B3) for sAPPα, Anti-Human sAPPβ (R101A4) for

sAPPβ, and with Anti-Human APP-C for βCTF) and incubated overnight at 4 ºC. The next day, plates were washed 4x with 350µl washing buffer. 100 µl/well of labelled detection antibody was added and incubated for 30-60 minutes at 4 ºC. Plates were washed 5x 350µl with washing buffer, the chromagen was added at 100 µl/well and incubated in the dark for 30 minutes at RT. The colour change reaction was then stopped by adding 100 µl/well of stop solution. Finally, plates were read at 450nm against the reagent blank within 30 minutes of adding the stop solution.

2.6.3 Soluble and Insoluble Beta Amyloid (Aβ)

Commercial ELISA kits (IBL International, Stratech, UK) were used to determine the levels of soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ (Aβ40 #27713 and Aβ42 #27711). Standards were prepared in a two-fold serial dilution in "Assay Buffer" from 500 pg/ml to 7.81 pg/ml for $A\beta_{1-40}$ and from 800 pg/ml to 12.5 pg/ml for $A\beta_{1-42}$. The method from here on was similar for both soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$. Samples were prepared at a dilution of 1:5 in "Assay Buffer". 100 µl/well of samples and standards were loaded onto precoated microtiter plates (precoated with Anti-Human $A\beta_{35-40}$ (1A10) for $A\beta_{1-40}$, and with Anti-Human $A\beta_{38-42}$ (44A3) for $A\beta_{1-42}$) and incubated overnight at 4 ºC. The next day, plates were washed thoroughly 4x with 350µl washing buffer and aspirated. A labelled detection antibody (HRP-conjugate enzyme) was loaded at 100µl/well and incubated for 1 hour at 4 ºC. Plates were washed again 5x350 µl with washing buffer and aspirated thoroughly. The chromogen was loaded at 100 µl/well and incubated up to 30 minutes at RT in the dark. A 1:1 acid "stop solution" were added to stop any further colour change. Finally, plates were read at 450 nm against the reagent blank within 30 minutes of adding the stop solution.

2.7 Histological and Cell Immunostaining

2.7.1 Immunohistochemistry Staining (DAB-IHC)

4µm paraffin-embedded sections of frontal cortex samples were used to quantify proteins of interest (Table 2.5) and to investigate their location in the cells during ageing or in AD. A total of 5 samples from each individual female group (Tables 1.1 and 1.2) were used. First, sections were deparaffinized by incubation in xylene 2x6 minutes followed by rehydrating the tissue through a graded alcohol series (100%, 95%, 70%, 50% ethanol) for 3 minutes each and washed with $Di.H_2O$ twice for 3 minutes. It is critical that the sections do not get dehydrated

through the previous steps. Antigens were retrieved through boiling the sections in 0.01 M citric acid, pH 6, at 100 ^{o}C for 30 minutes using a microwave (Power-6). Sections were cooled under a running tap for 5 minutes and washed with 0.1M PBS for 10 min (0.1M PBS: 0.14 M NaCl, 0.0964M Na_2HPO_4 and 0.0215M NaH_2PO_4). Endogenous peroxidase was deactivated through incubating sections with 20% MeOH and 1.5% H_2O_2 for 30 minutes. Sections were washed for 3 x 5 min in PBS containing 0.03% Tween (PBST) followed by blocking with serum blocking buffer for 30 minutes, the blocking serum buffer consisted of the appropriate serum for the secondary antibody, 3% (Table 2.6) in PBS containing 1% (w/v) BSA and 0.1% (v/v) Triton X-100. After that, the Avidin/Biotin blocking kit was used (VectorLab, SP-2001) according to the manufacturer's protocol, where, after serum blocking and without washing, 4 drops of avidin were added to 1ml blocking serum buffer and incubated with the sections for 30 minutes. Sections were washed 3x5 minutes with 0.1M PBS and 0.03% (v/v) Tween-20 (PBST) then incubated with the primary antibody (Table 2.5) prepared at a pre-optimized concentration in 1ml of the serum blocking buffer containing 4 drops of biotin from the blocking kit to block non-specific antibody binding for 48-72 hours at 4 ^{o}C in a humidified container.

The sections were washed again 3x5 minutes with PBST before incubating with biotinylated secondary antibody (Table 2.6) in blocking serum buffer at 1:100 for 2 hours at RT. Then they were washed for 3x5 minutes in PBST and incubated for 45 minutes with Vectastain Elite ABC solution (Avidin/Biotin linked HRP VectorLab, PK-4000) prepared in 0.1M PBS containing 0.1% Triton X-100. Next, they were washed 3x5 minutes with 0.05M Tris.Base non-saline buffer (0.05M TNS, pH 7.4) and stained with DAB (1:1 mixture of 0.1% of 3,3'- Diaminobenzidine tetra- hydrochloride (DAB) (Sigma D5637) and 0.0036% hydrogen peroxide, both prepared in 0.05M TNS) until a brown colouration was visible to the naked eye. DAB was washed off with Dis.H_2O for 5 minutes and sections were dried overnight at RT.

The next day, sections were rewashed with Dis.H_2O for 5 minutes and subjected to nuclear counterstain using 0.5% Methyl Green (Sigma Aldrich, M8884) for up to 30 minutes then washed with Dis.H_2O. They were then dehydrated by passing through an ascending concentration of alcohol baths (95%, 2x100% of ethanol) for 2-3 seconds each and cleared twice with xylene solution for 6 minutes each. Finally, slides were cover-slipped using Dibutylphthalate Polystyrene Xylene (DPX) solution (Sigma Aldrich, 06522) and dried at RT.

Table 2.5 Primary and secondary antibody dilutions and suppliers for immunohistochemistry

Primary Antibodies	Species	Type	Source		Dilution	Secondary Antibody Dilution
			Manufacturer	Code		
FOXG1	Rabbit	mAb	Abcam, Cambridge, MA	ab196868	1:100	1:100
CX3CR1	Mouse	mAb		ab184678	1:50	1:100
GFAP	Rabbit	mAb		ab68428	1:100	1:100
Anti-ERα	Rabbit	mAb		ab108398	1:20	1:100
Anti-Phospho ERα (Ser167)	Rabbit	mAb		ab31478	1:20	1:100
Anti-ERβ	Rabbit	pAb		ab5786	1:100	1:100
Anti-Phospho ERβ (Ser105)	Rabbit	pAb		ab62257	1:25	1:100

Table 2.6 Biotinylated secondary antibodies for immunohistochemistry

Secondary Antibodies	Species	Source	
		Manufacturer	Code
Anti-Rabbit	Horse	Vector Laboratories	BA-1100
Anti-Mouse	Goat		BA-9200

Pictures were captured using a Leica DM6 B upright microscope connected to an electronic box CTR6 LED and an automatic controller for slide scanning (ProScanTM III, PRIOR). The automatic microscope was attached to a PC installed with a Visio Pharm image

capturing software and Intel® HD Graphic Control Panel. The latter software was set at an 1280x1024 image resolution and 32 bits color depth.

2.7.2 Immunocytochemistry Fluorescence Staining (ICC-F)

Human neuronal cells seeded on coverslips of 24-well plates (Section 2.3) were fixed with 2% formaldehyde for 15 minutes and washed three times with 0.1M PBS. This was followed by blocking non-specific binding sites with rabbit serum blocking buffer, the same as used in IHC, for 30 minutes at RT. After that, without washing the cells, rabbit serum blocking buffer and an appropriate concentration of primary antibody (Table 2.7) was added and cells were incubated overnight at 4 ºC. Next day, cells were washed 3x5 minutes with 0.1M PBST (0.1M PBS, 0.1% (v/v) Triton X-100) and incubated with 1:270 fluorescent secondary antibody (Table 2.8) for 2 hours at RT in the dark to prevent photobleaching. After that, cells were washed 3x5 minutes with 0.1M PBST and then washed with diH_2O to remove any salts. Finally, coverslips were dried at RT for 1 hour in the dark and mounted on sterile slides with anti-fade mounting media (Vectashield, H-1200) with DAPI and stored at -20 ºC. Images were captured using an Evos^{TM} M7000 Imaging System in a dark room.

2.7.3 Optimization of immunoassays

IHC procedures were optimized to detect the target and get the best possible image. The process of optimization involved using a series of antibody concentrations and comparing these with a negative antibody control and/or incubating the primary antibody for a longer period. Also, for IHC, hematoxylin was used as a counterstain at first, but it gave purple nuclei which were difficult to distinguish from the DAB brownish staining. Therefore, methyl green was used as a counterstain for all future work.

Table 2.7 Primary and secondary antibody dilutions and suppliers for immunocytochemistry

Primary Antibodies	**Species**	**Type**	**Source**		**Dilution**	**Secondary Antibody Dilution**
			Manufacturer	**Code**		
FOXG1	Rabbit	mAb		ab196868	1:100	1:270

CX3CR1	Mouse	mAb	Abcam, Cambridge, MA	ab184678	1:50	1:270
GFAP	Rabbit	mAb		ab68428	1:100	1:270
Anti- ERα	Rabbit	mAb		ab108398	1:20	1:270
Anti-Phospho ERα (Ser167)	Rabbit	mAb		ab31478	1:20	1:270
Anti- ERβ	Rabbit	pAb		ab5786	1:100	1:270
Anti-Phospho ERβ (Ser105)	Rabbit	pAb		ab62257	1:25	1:270

Table 2.8 Fluorophore-Conjugated secondary antibodies for Immunocytochemistry

Secondary Antibodies	**Species**	**Source**		**Label**	
		Manufacturer	**Code**	**Fluorophore**	**Color**
Anti-Rabbit	Horse	Vector Laboratories	DI-1088	DyLight 488	Green
Anti-Mouse	Horse		DI-2488	DyLight 488	Green

2.8 Data analysis

Data were first analyzed in Microsoft Excel for calculating the mean, standard deviation, standard error of the mean, coefficients of variance and confidence intervals. After that, Graphpad Prism8 (Version 8.4.1) was used to do all the statistical analyses as described below.

2.8.1 Quantifying ELISA and Western Blot data

Protein expression obtained from ELISAs were quantified using the standard curve calculated with Graphpad Prism8 and then normalized against total protein concentration. For Western blots, the intensity of the bands was quantified using ImageJ Fiji

(https://imagej.net/Fiji.html#Downloads) after subtracting the background to remove the interference of noise on band measurements. The expression of proteins within each age group was analysed across more than one Western blot. Since the samples were rune in more than one gel, all protein bands were normalised against β-actin levels to control for protein loading and then expressed as the relative density of the same young healthy female human brain sample. For the phosphorylated and total proteins, after they were normalized to β-actin and standardized against the young human brain sample, the ratio of phosphorylated to total protein relative density was calculated.

2.8.2 Statistical Analyses

Data are presented as mean ± SEM with p values < 0.05 considered significant and highly significant ** p values < 0.002 and *** p values< 0.001. Initially, all data were checked for normal distribution with Shapiro-Wilkes tests and equal variances for comparing between more than 2 groups was tested with Bartletts test, while equal variance for two groups was tested with the F-test. Violation of these tests was considered for p values < 0.05. Data which were considered to violate normality and equal variance tests were subjected to transformation, either by taking the square root or logarithm, according to the direction the data were distributed through a normal QQ plot and reassessed. Data which did not show any violation of normality tests were analysed through parametric methods, two-way analysis of variance (ANOVA) was used followed by Tukey post-hoc test, or Students' two-tail t-tests (independent samples and paired samples) where appropriate. However, for data which even after transformation were not normally distributed, non-parametric tests were used; Kruskal-Wallis H Test for two-Way ANOVA, Mann-Whitney U for Independent Samples t-test, and Wilcoxn-Signed Rank for Paired-Samples t-test.

CHAPTER 3 _ Exploring the Levels of APP, its Metabolic Enzymes and By-Products in Male and Female Frontal Cortex during Ageing and in Alzheimer Disease

3.1 Introduction

Several preclinical and clinical AD studies have investigated the APP amyloidogenic trajectory with the consideration of sex as an independent variable in their methodology. Some of these studies will be summarized below.

3.1.1 *Preclinical Studies*

In vivo, animal models of AD evidence sex variations in neuropathology with females demonstrating more premature and more pronounced alterations than males (Callahan et al., 2001, Lee et al., 2002). There are few studies that have incorporated male and female subjects and have employed non-Tg rodents, such as the streptozotocin (STZ) model. In these animal models, the STZ was deployed via intracerebral infusion to trigger behavioral and neurochemical changes akin to facets of AD in a non-Tg model (Torrão et al., 2012, Hoyer, 2002). Biasibetti et al. have shown that AD- adaptations of the STZ model were reliant on sex and were more marked in the male hippocampus (Biasibetti et al., 2017). On the other hand, Bao et al. demonstrated that, following STZ infusion, females exhibited heightened resistance to induced learning and had memory dysfunction (Bao et al., 2017).

However, in most of the animal work that has evaluated sex discrepancies in AD, Tg models have been utilised. In all ages, although unaffected in males, spatial memory was potently impacted in APPβ/PS1 Tg females. Synaptic connectivity loss and a compaction of hypertrophic astrocytes were related to the memory disruption (Richetin et al., 2017). Additionally in this model, Wang et al. documented markedly raised cerebral concentrations of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in females compared to males over time, at 4, 12 and 17 months of age. At the latter two ages, the burden of amyloid plaques was notably greater in females; no deposits were identified before 4 months. Of note was that the animals exhibited sex variations in Aβ levels before the time that would equate to the human menopause, i.e. during the age range of 4-12 months (Wang et al., 2003).

Additional research using Transgenic (Tg) mice models, i.e. overexpressing APP_{23} (Sturchler-Pierrat et al., 2000, Bayer et al., 2003), overexpressing APP_{695} (Callahan et al., 2001), APPβ/PS1 (Ordónez-Gutiérrez et al., 2015), and APPβ/tau (Lewis et al., 2001) have noted equivalent themes. These studies reported that elevated Aβ concentrations were present in a number of brain regions in females, together with a higher degree of deposition and development of amyloid plaques and more pronounced neurodegenerative findings when

judged against males of a similar model (Wang et al., 2003, Callahan et al., 2001). However, one study reported no notable sex disparities in Aβ in 3xTg-AD mice of all ages (Clinton et al., 2007), which is in contrast to the previous studies.

3.1.2 Clinical Studies

Amyloid identification using PET and assay of $A\beta_{1-42}$ levels in the CSF are well-recognised biomarkers of brain amyloidosis. In sizeable cross-sectional studies (Mattsson et al., 2017, Ferretti et al., 2018) no uniform variations between the sexes have been documented to date in amyloid concentrations in normal individuals or in those with MCI or AD. Nevertheless, it should be noted that some regional variations have been described in elderly humans with subjective memory issues. Despite appearing cognitively intact, PET demonstrated a greater amyloid burden in the anterior cingulate cortex of males compared with females (Cavedo et al., 2018). Despite the level of Aβ, there is some debate as to whether a more precipitous cognitive loss is seen in females compared with males both from MCI to AD, and when dementia is clearly apparent (Lin et al., 2015, Chapman et al., 2011, Hebert et al., 2000).

A number of evidential threads have shown that the influence bestowed by a disease biomarker on risk and disease course is higher in females than males. This was demonstrated initially by Koren et al. in the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort, especially in the MCI phase, where AD biomarkers were identified in both sexes, i.e. CSF levels of $A\beta_{1-42}$, but with more marked hippocampal atrophy and an accelerated loss of cognition noted in females compared to males (Koran et al., 2017). Additional corroborating data were demonstrated by Buckley et al. who reported that cognitively normal females accruing increased amyloid levels exhibited more rapid cognitive loss than males (Buckley et al., 2018). If these data are verified, they could suggest that amyloidosis is linked with different rates of pathological advancement in the two sexes. Despite the presence of an equivalent quantity of amyloid in the elderly brains of both sexes, females of a similar age manifest a higher rate of cognitive impairment (Buckley et al., 2018).

The clinical studies above enrolled elderly patients with clinical symptoms of MCI or AD, and, since AD is a longitudinal disease in nature, the question arises as to whether there is a sex-disparity in Aβ production during the preclinical stage of the disease. Interestingly, a recent study conducted by Rahman et al. investigated Aβ levels in the healthy brains of middle-aged males and females, aged 40 - 65 years, using PET imaging. This study reported a sex

disparity with females showing 30% more Aβ in their brains than male s(Rahman et al., 2020). While this finding might suggest that females are more suspectable to Aβ overproduction in their brains as an after effect of losing E2 during the menopause transition phase, the study did not provide information about the serum level of E2 in their subjects (Rahman et al., 2020). A wider age range is required covering pre- and post-menopausal ages to explore if there are correlations between E2 levels and Aβ production.

3.2 Hypothesis and Aims

The clinical and preclinical observations above suggest that the disease course may vary between the two sexes during brain ageing. female might manifest AD signs in earlier age than the male due to the significant dropdown of steroid hormones the female undergo during menopause transition phase.

Hypothesis: Females will have an earlier build-up of AD biomarkers in their frontal cortex at an earlier age compared to age-matched males.

The aim of this chapter is to explore if there are sex differences in APP metabolic pathways during frontal cortex ageing and in AD. This aim was investigated through the following objectives:

1) Determine the cortical levels of APP and its metabolites in brain samples from 'healthy' individuals without neurodegeneration aged 20 to 90 years old and in non-diseased (ND) vs AD age-matched samples.
2) Investigate sex differences in APP processing pathways in each age and disease group.

3.3 Experimental Methods

All the methods used in this Chapter have previously been detailed in Chapter 2.

3.3.1 Samples

Samples used in this chapter were prepared and grouped as described in Chapter 2, Tables 2.1 & 2.2. Briefly, the subjects were divided into three different age groups: young, middle-age and elderly group, and an additional fourth group with AD. The selected ages were young (18 – 35), middle-aged (40 - 55), elderly (68 – 99) and AD (68 - 99) years old. The mean age

± SD for the young, middle-aged, elderly and AD male samples was 26.5 ± 1.9 years, 50.5 ± 1.9 years, 75.1 ± 7.3 years and 78 ± 5.6 years, respectively. While for the female subjects it was 28 ± 6.9 years, 42.3 ± 2.9 years, 85.7 ± 10.9 years and 85 ± 5.9 years, respectively. The mean PMI was 47.9 ± 16.3 hours for male, and 55.17 ± 24.59 hours for female samples.

Also, for examining the effect of disease status on the levels of targeted proteins, the same elderly subjects used as above were used as a non-diseased (ND) group and were compared to age-matched AD subjects for each of the markers.

3.3.2 Experiments and Target Proteins

Western blot (WB) used all the samples groups to explore the expression of APP, ADAM-10, PS1, PS2, and BACE-1 (refer to Table 3.1 for expected band molecular masses). For exploring the impact of ageing on protein expression with WB, male and female samples were run separately, while for investigating the impact of sex, both sexes for each ageing group were run together on the same gel.

Table 3. 1 Expected WB band molecular masses for the proteins investigated in Chapter 3.

Proteins	Expected Size (kDa)	References
APP	110--130	(Alsaqati et al., 2018)
ADAM-10	Pro-/immature ADAM-10 (80) Mat-/mature ADAM-10 (70)	(Alsaqati et al., 2018)
BACE1	70	(Hemming et al., 2009)
PS1	18	(Hicks et al., 2020)
PS2	23	(Alsaqati et al., 2018)

The expression of proteins in all the samples was analysed across more than one gel. Therefore, all protein bands were expressed as the relative density of a standard human brain sample, a young female sample, for exploring proteins levels during ageing and for the ND vs AD experiments.

Quantitative sandwich ELISAs were used to investigate levels of APP, sAPPα, sAPPβ, soluble $A\beta_{1-40}$ and soluble $A\beta_{1-42}$ in all samples for the ageing groups and in AD, whereas insoluble $A\beta_{1-40}$ and insoluble $A\beta_{1-42}$ were determined by ELISA only in ND and AD samples of both sexes.

During WB analysis of ADAM-10, one middle-aged female sample and one young and one old male sample were removed from the analysis, due to violations in data distribution of the results (described in Chapter 2, Section 2.9).

To measure the band intensity of immature (pro-ADAM-10) and mature (mat-ADAM-10) forms of the ADAM-10 protein, images were enlarged by 75% to aid in selecting bands of interest without overlapping between pro-ADAM-10 and mat-ADAM-10.

3.3.3 Statistical Analysis

ELISA and WB data were normalized then analyzed as described in Chapter 2, Section 2.9. All data in this chapter were parametric and were analysed using a factorial two-way ANOVA to compare the main effects of age and biological sex [independent variants (IV)], or the disease state and biological sex (IV), as well as the interaction effects of those factors against each other [Sex*Age or Sex*Disease state Interaction]. If the lateral interaction [Sex*Age or Sex*Disease state Interaction] is significant it indicates that male and female presented with different patterns in protein expression throughout ageing or in the disease state, thus to investigate that further, a one-way ANOVA would be conducted for each sex separately. The statistical results of two-way ANOVA will be included in the figures legends, while the post-hoc test results will be discussed more deeply in the results section.

A correlation testing between sAPPα and sAPPβ, βCTF and Aβ and $A\beta_{1-40}$ and $A\beta_{1-42}$ within each age group was performed using Pearson's correlation coefficient, $p<0.05$.

3.4 Results

3.4.1 APP, and its processing enzymes were altered and showed sex differences with ageing and in AD

3.4.1.1 Female Samples:

APP expression was measured by WB (Figure 3.1a) and ELISA (Figure 3.1b,c) and its levels increased significantly with age ($p<0.05$). Since the secretase enzymes are essential for APP processing and Aβ production, it was also investigated whether their expression was changed during ageing with WB. Both forms of ADAM-10, the main α-secretase candidate, did not alter in the brain with age (Figures 3.2a & 3.2b). The β-secretase enzyme, BACE1, was investigated as an indicator of amyloidogenic APP processing activity during brain ageing.

Interestingly, it presented a similar finding to APP where a significant age-related increase was seen during frontal cortex ageing ($p<0.05$, Figure 3.3a & 3.3b). In addition, presenilin-1 (PS1) and presenilin-2 (PS2), components of γ-secretase, both showed significant age-related increases, $p<0.02$ and $p<0.05$, respectively, using WB (Figures 3.4a, 3.4b & 3.4d).

In AD, the levels of APP (Figure 3.1a & 3.1c), its secretase enzymes, ADAM-10 (Figure 3.2a and 3.2c), BACE1 (Figure 3.3a and 3.3c) PS1 and PS2 (Figure 3.4a, 3.4c and 3,4e) were not altered compared to ND subjects.

3.4.1.2 Male Samples:

APP (Figure 3.1a & 3.1b), mature and immature ADAM-10 (Figure 3.2a & 3.2b), PS1 and PS2 (Figure 3.4a, 3.4b & 3.4d) did not show any alteration of their levels during male brain ageing. However, a strong significant upregulation of BACE1 with age was identified ($p<0.002$, Figure 3.3a & 3.3b).

In AD, in contrast to females, the level of PS2 was higher in AD male subjects ($p<0.05$, Figure 3.4e). However, APP, BACE1, PS1 and ADAM-10 levels did not show any change (Figure 3.1c, 3.2c, 3.3c & 3.4c).

3.4.1.3 Sex Disparities:

Protein expression was compared between males and females for young, middle-aged and elderly subjects separately. No differences were found in the levels of APP (Figure 3.1c). Elderly female subjects displayed higher levels of PS1 (mean= 1.892± 1.39) and PS2 (mean= 1.451±1.08) than age-matched male subjects (mean= 1.0003±0.47, $p<0.05$) and (mean= 0.675±0.51, $p<0.001$), respectively (Figure 3.4b & 3.4d). However, BACE1 levels were higher in elderly males compared to females (Figure 3.3b), and no differences in ADAM10 were seen between the sexes (Figure 3.2).

For AD subjects, APP and its metabolic enzymes did not show any sex disparity (Figure 3.1c, 3.2c, 3.3c, 3.4c & 3.4e).

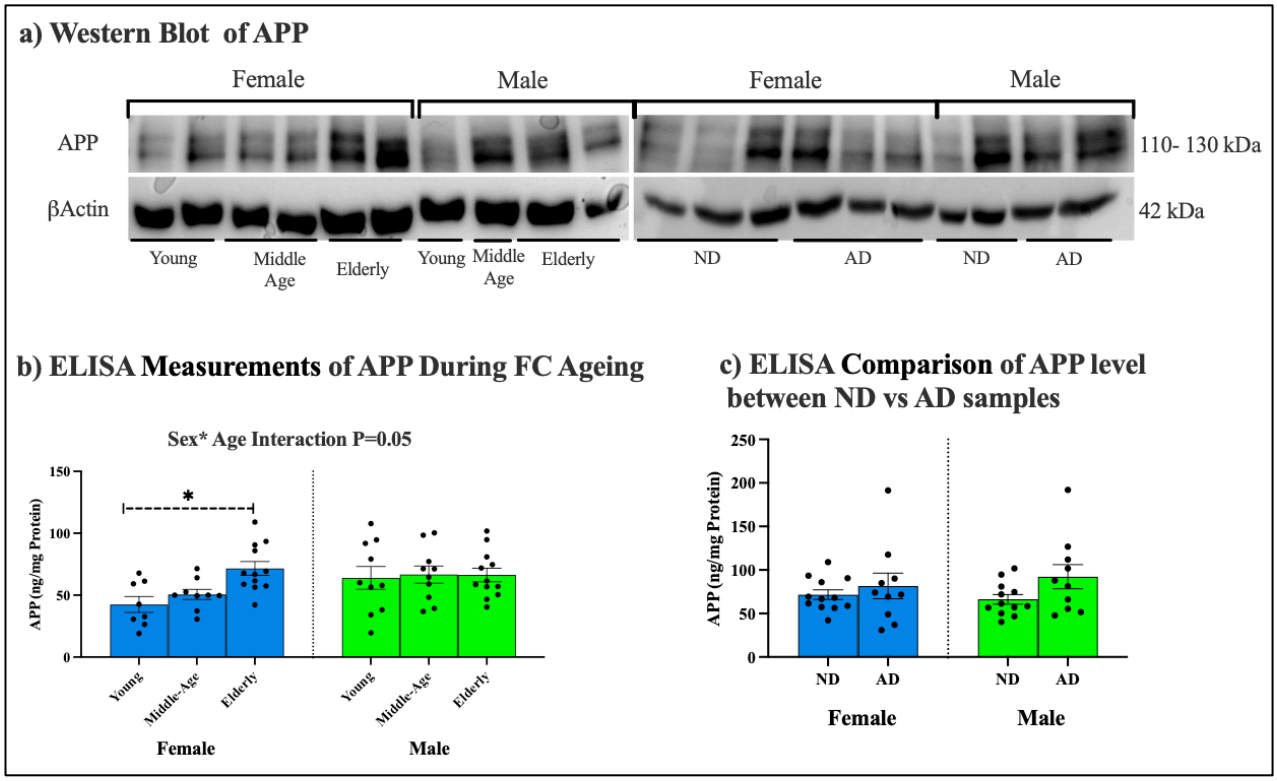


Figure 3.1 Comparison of the expression of APP in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=12) subjects and between ND (male n=12, female n=12) and AD (male n=10, female n=10) subjects. **a)** representative Western blotting data for APP with the housekeeping protein, βactin, **b)** ELISA quantification of APP expression in the brain during ageing, with two-way ANOVA showing a significant increase of APP levels only in female with ageing (Sex* Age $F(2, 55)= 3.17$, $P=0.05$). **c)** ELISA quantification of APP expression between ND vs AD samples, two-way ANOVA show no effect of sex or disease state on APP levels. Data are presented as mean ± S.E.M. *$p<0.05$.

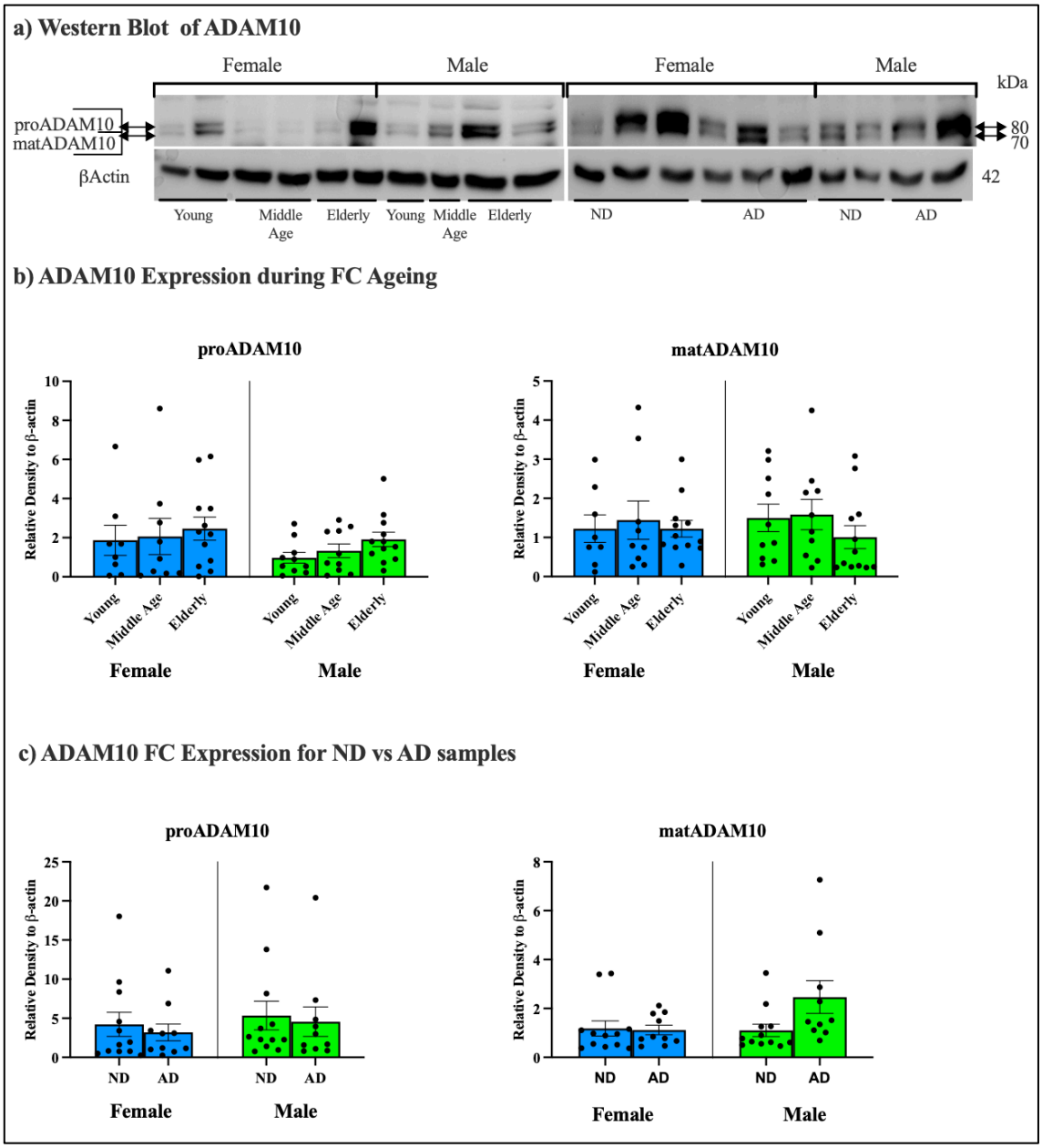


Figure 3. 2 Comparison of the expression of mature (mat) and immature (pro) ADAM-10 in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=12) subjects and between ND (male n=12, female n=12) and AD (male n=10, female n=10) subjects. **a)** representative Western blotting data for ADAM-10 with the housekeeping protein βactin, **b)** Densitometric analysis of ADAM-10 expression during brain ageing, with two-way ANOVA showing neither biological sex or age factorial effects were significant during frontal cortex ageing. **c)** Densitometric analysis of ADAM-10 expression between ND vs AD samples, two-way ANOVA show no effect of sex or disease state on ADAM-10 levels. Data are presented as mean ± S.E.M.

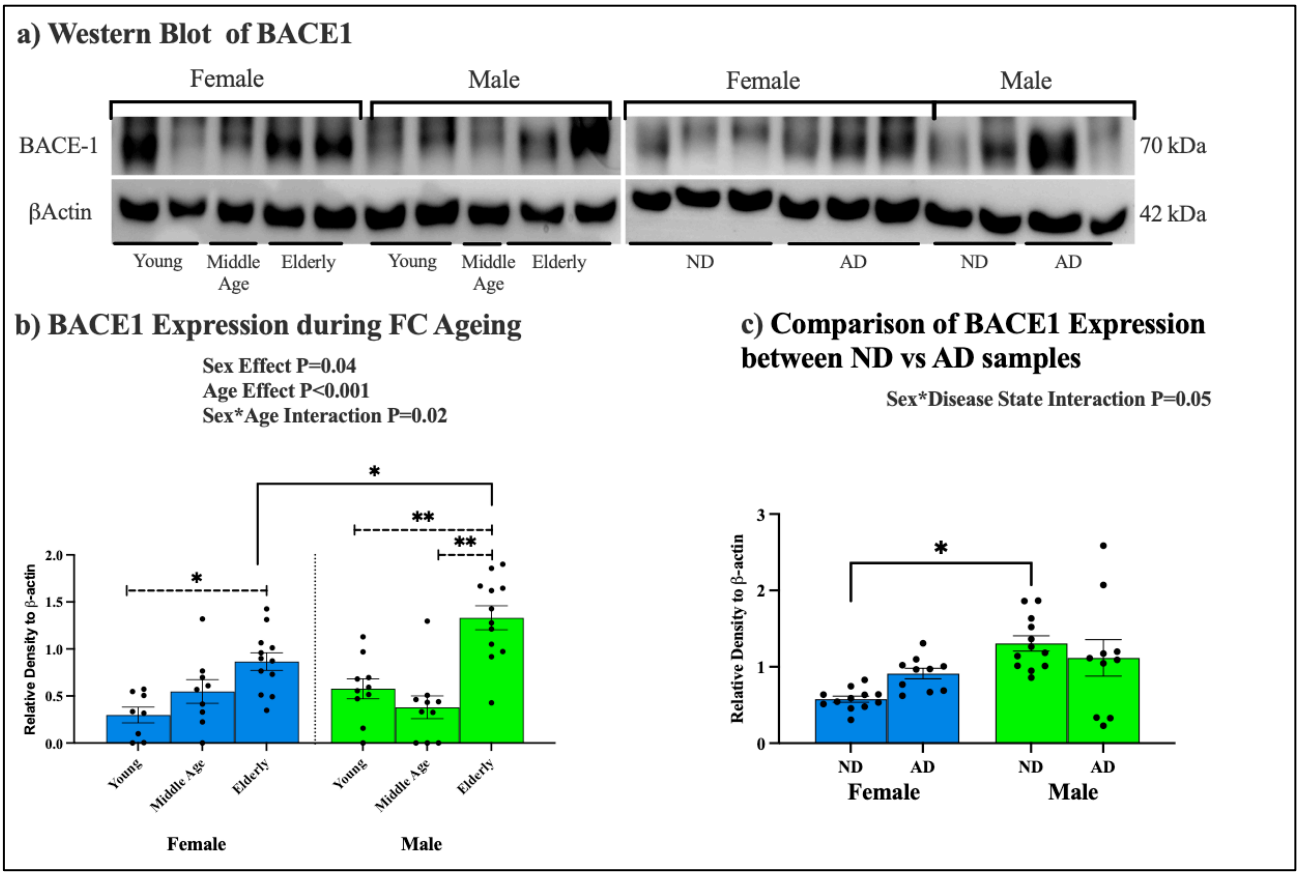


Figure 3.3 Comparison of the expression of BACE1 in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=12) subjects and between ND (male n=12, female n=12) and AD (male n=10, female n=10) subjects. **a)** representative Western blotting data for BACE1with the housekeeping protein βactin. **b)** Densitometric analysis of BACE1expression during brain ageing, with two-way ANOVA showing a significant increase of BACE1 levels in both male and female with ageing and a higher level of BACE1 was calculated in elderly male samples compared to elderly female (Sex*Age $F(2,55)=4.21$, $P=0.02$), (Age $F(2, 55)= 23.56$, $P<0.001$) and(Sex $F(1,55)= 4.26$, $P= 0.04$) **c)** Densitometric analysis of BACE1expression between ND vs AD samples, with two-way ANOVA showing a significant higher levels of BACE1 in ND male samples compared to ND female (Sex*Disease State $F(1, 40)= 4.26$, $P=0.05$). Data are presented as mean ± S.E.M. *$p<0.05$, **$p<0.002$.

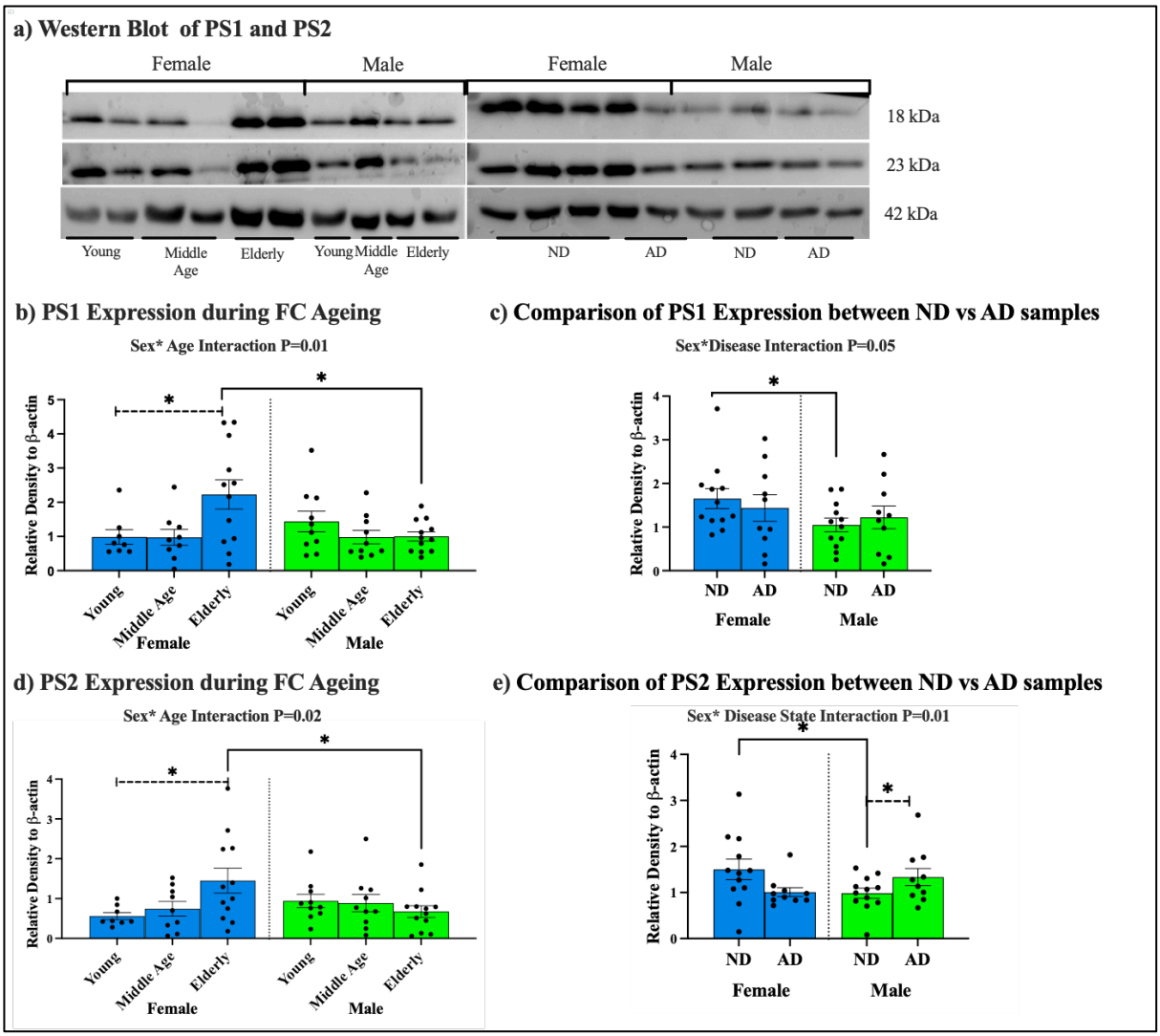


Figure 3.4 Comparison of the expression of PS1 and PS2 in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=12) subjects and between ND (male n=12, female n=12) and AD (male n=10, female n=10) subjects. **a)** representative Western blotting data for PS1 and PS2 with the housekeeping protein βactin, (**b&d)** Densitometric analysis of PS1 and PS2 during ageing, with two-way ANOVA showing a significant increase of PS1and PS2 levels only in female samples with ageing along with higher levels of these proteins in elderly female compared to elderly male subjects (Sex*Age $F(2, 55)=5.04$, $P=0.01$ and $F(2,55)=4.36$, $P=0.02$, for PS1 and PS2 respectively).**(c&e)** Densitometric analysis of PS1 and PS2 between ND vs AD samples, with two-way ANOVA showing a significant higher levels of PS1and PS2 in ND female samples compared to ND male samples along with increase in PS2 levels in AD male samples (Sex*Disease State $F(1, 40)=4.01$, $P=0.05$ and Sex*Disease State $F(1,40)=6.47$, $P=0.01$, for PS1 and PS2 respectively) Data are presented as mean ± S.E.M. *$p<0.05$, **$p<0.002$.

3.4.2 APP metabolic fragments, sAPPα, sAPPβ and βCTF were altered and showed sex differences with ageing and in AD

3.4.2.1 Female Samples

APP non-amyloidogenic and amyloidogenic metabolic by-products, sAPPα and sAPPβ, were measured by ELISA (Figure 3.5c & 3.5c). While the absolute levels of both sAPPα and sAPPβ did not change during ageing, their inter-relation did. In the middle-aged brains, levels of sAPPα were significantly correlated with those of sAPPβ ($r^2 = 0.541$, $p< 0.02$, Figure 3.6b) but no correlation was observed between sAPPα and sAPPβ in young or elderly subjects ($r^2 = 0.0120$, $p> 0.05$ for young samples and $r^2 = 0.68$, $p> 0.05$ for elderly samples, Figure 3.6a & 3.6c, respectively). βCTF levels were significantly increased with ageing as measured by ELISA ($p<0.05$, Figure 3.8).

In AD brains, the absolute levels of sAPPα were significantly lower in AD compared to ND subjects ($p<0.05$), however, levels of sAPPβ showed no change from ND to disease state (Figure 3.5b). Furthermore, a significant positive correlation of sAPPα to sAPPβ was apparent in female AD samples ($r^2 = 0.871$, $p< 0.001$, Figure 3.6d). When the slope of the AD regression plot was compared to the slope of the middle-aged subjects, no difference between the two slopes was identified ($p=0.76$, Figure 3.6c). βCTF expression was significantly higher in AD compared to ND subjects ($p<0.05$, Figure 3.8).

3.4.2.2 Male Samples

Regarding sAPPα and sAPPβ, sAPPα expression decreases during ageing ($P=0.02$, Figure 3.5a), while no alternation in sAPPβ was detected. In addition, an alteration in their inter-relation were detected. In the young male brains, levels of sAPPα were positively correlated with those of sAPPβ ($r^2 = 0.558$, $p< 0.05$, Figure 3.7a) but no significant correlation was observed in middle-aged or elderly subjects (Figure 3.7b & 3.7c). Also, the level of sAPPβ was higher than sAPPα in elderly subjects ($p<0.05$, Figure 3.5a). No significant change with age was observed with βCTF levels (Figure 3.8).

In AD, sAPPα and sAPPβ levels were unchanged compared to ND subjects (Figure 3.5b and 3.5c), but a significant correlation between these fragments was found in AD subjects ($r^2 = 0.385$, $p= 0.05$, Figure 3.7d). The slope correlation of the AD subjects was significantly

different to the slope for the young subjects ($p=0.05$, Figure 3.7e). βCTF expression was significantly higher in AD compared to ND subjects ($p<0.05$, Figure 3.8).

3.4.2.3 Sex Disparities

During brain ageing, males had higher levels of sAPPα (mean=12.77± 5.77) and sAPPβ (mean= 13.24±4.53) in young subjects than in age-matched females (mean= 8.626±1.58, $p<0.05$ and 7.581±4.72, $p<0.05$), respectively (Figure 3.5a). On the other hand, elderly females had higher levels of βCTF than males ($p<0.002$, mean in female = 3.33±1.52, mean in male = 1.59±0.857, Figure 3.8)

In AD, no changes between the sexes were seen for any of the APP metabolites; sAPPα, sAPPβ, and βCTF (Figure 3.8).

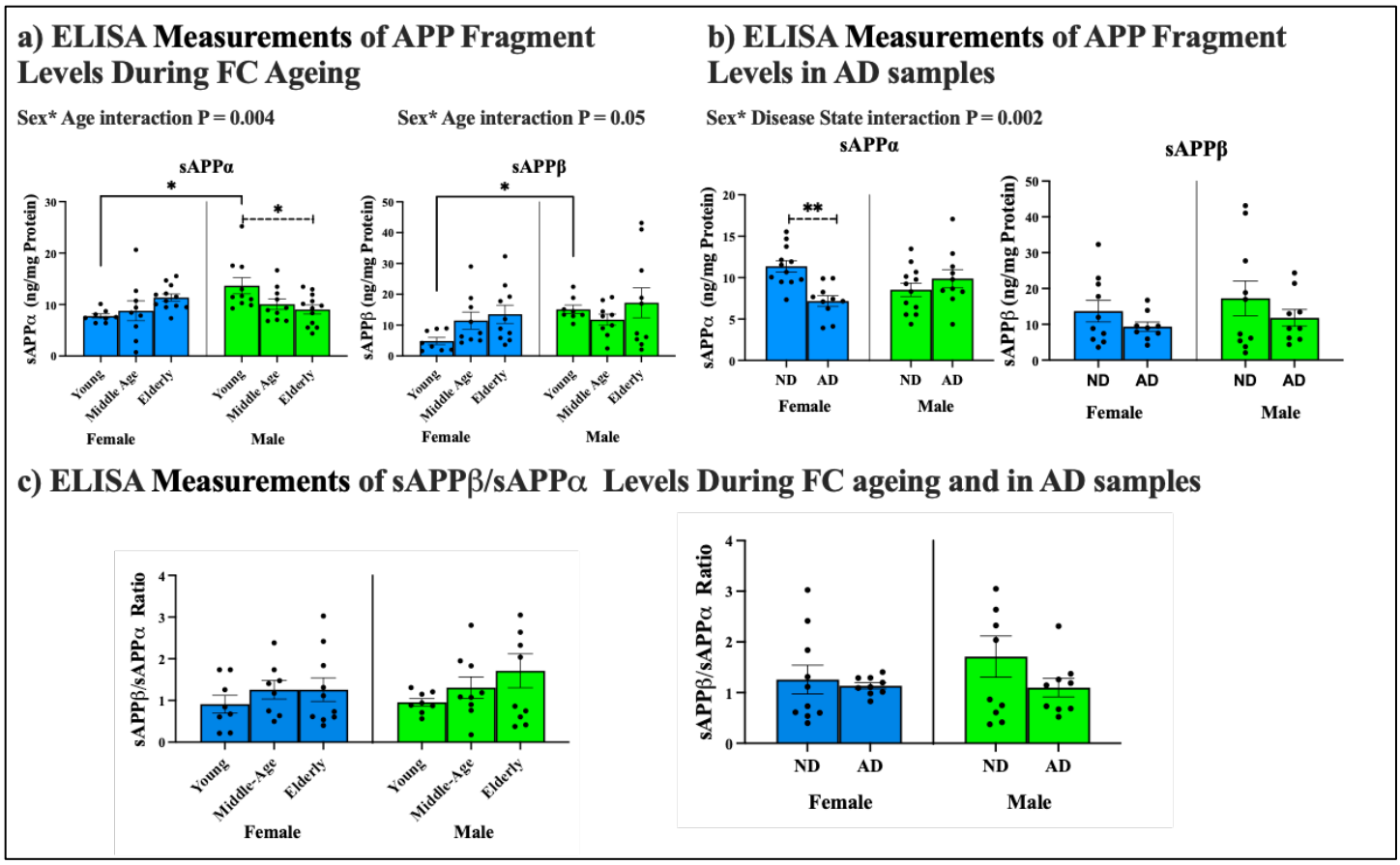


Figure 3.5 ELISA quantification of sAPPα and sAPPβ expression in human female and male frontal cortex between **(a)** different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=12) subjects, with two-way ANOVA revealed young male had a higher levels of sAPPα and sAPPβ than young female samples, along with decrease in the expression of sAPPα in male subjects with age (Sex*Age $F(2, 55)= 6.27$, $P = 0.004$ and $F(2, 55)=3.81$, $P=0.05$ for sAPPα and sAPPβ respectively). **(b)** Significant two-way ANOVA show a drop down in sAPPα in AD female samples (Sex*Disease State $F(1, 40)= 11.43$, $P = 0.002$), ND (male n=12, female n=12) and AD (male n=10, female n=10) subjects. **(c)** A comparison between the relative levels of sAPPα & sAPPβ within each age group, no significant effect of sex and age or sex and disease state on the investigated protein. Data are presented as mean ± S.E.M. *p<0.05,

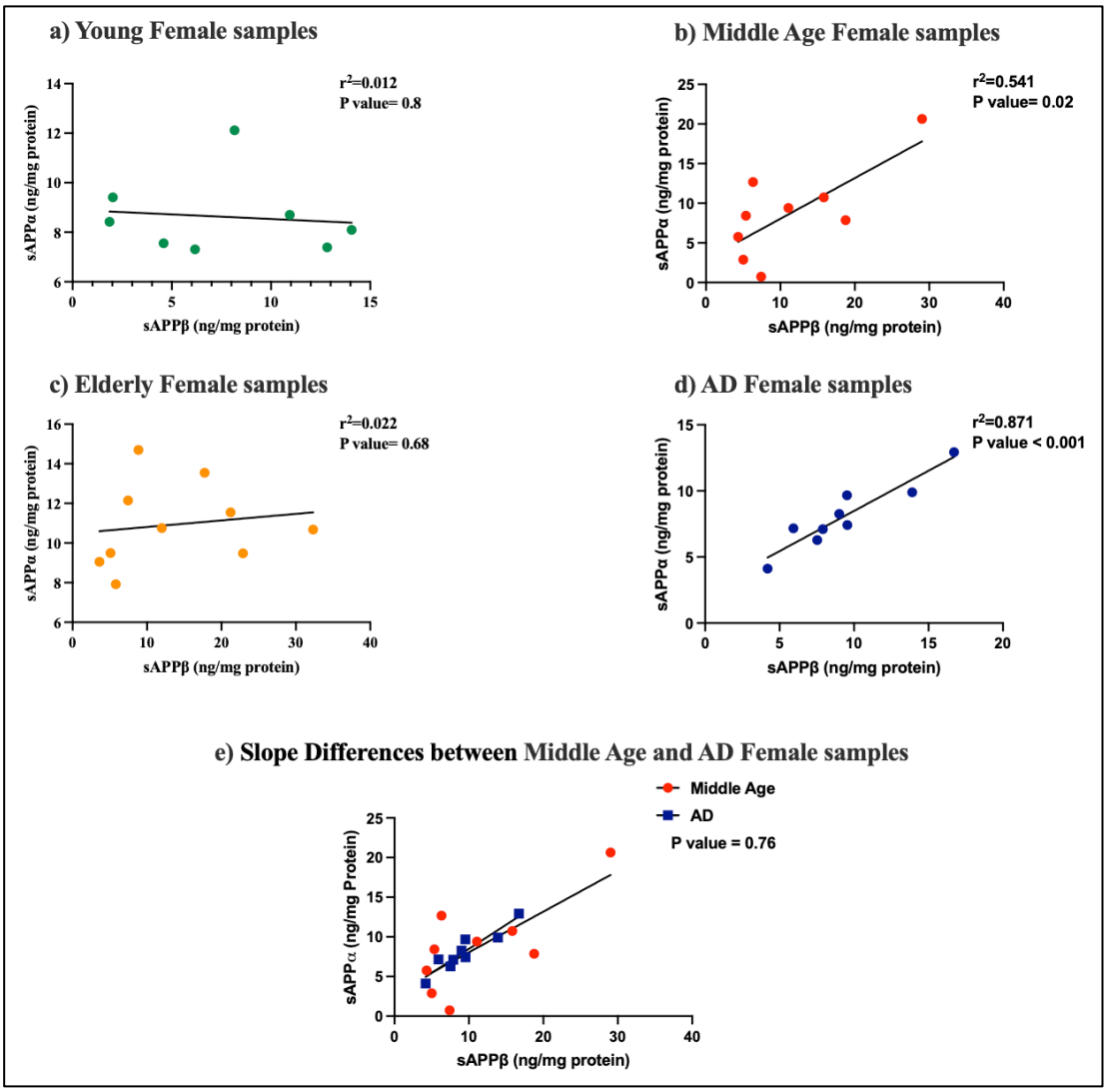


Figure 3.6 Linear regression analysis of sAPPα and sAPPβ levels in females: young (n=8) **(a)**, middle-age (n=9) **(b)**, elderly (n=12) **(c)** and AD (n=10) **(d)** samples. The level of sAPPα was significantly and positively correlated with that of sAPPβ in the middle-age and AD groups, and no slope differences was observed between them, $p<0.05$ (Pearson correlation analysis).

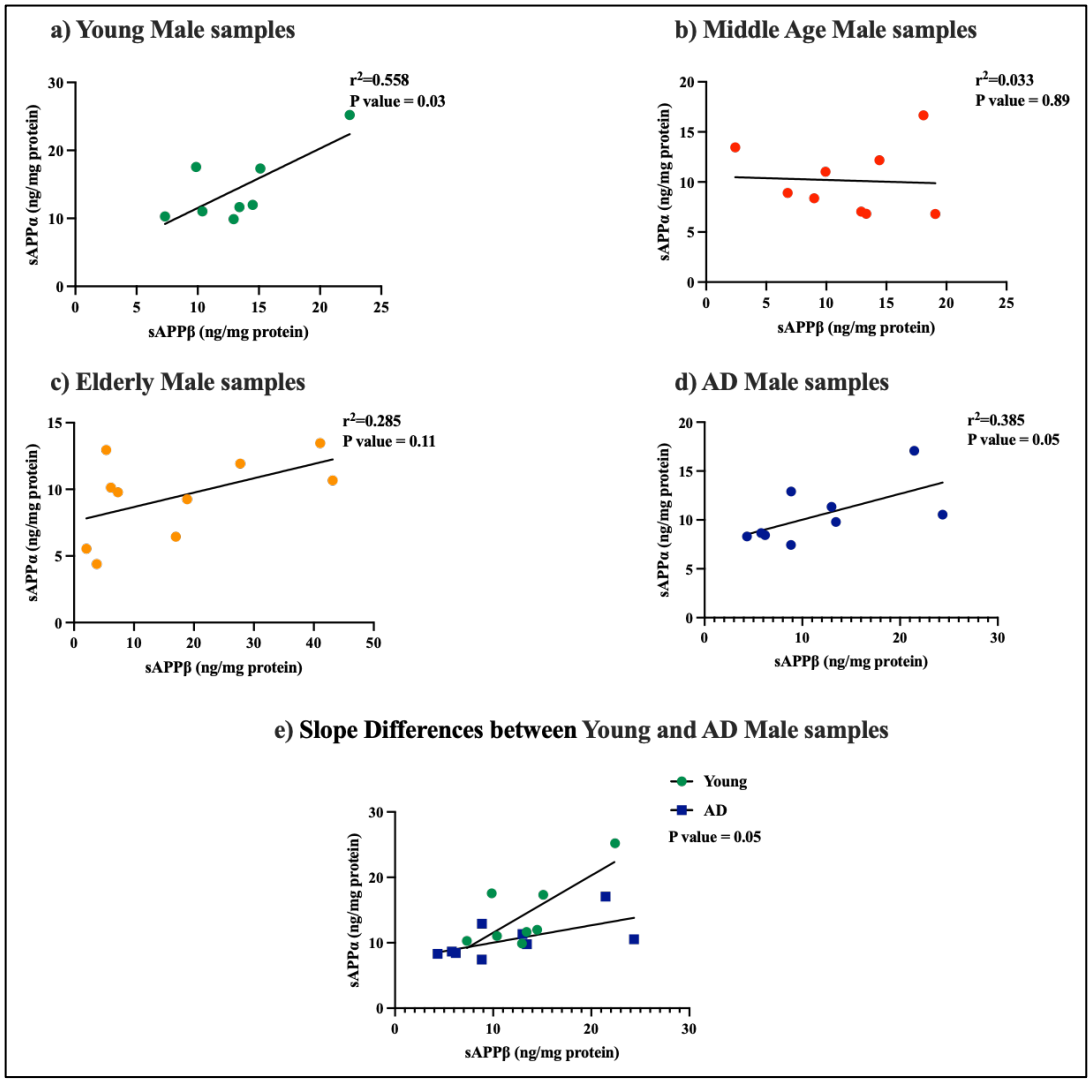


Figure 3. 7 Linear regression analysis of sAPPα and sAPPβ levels in males: young (n=10) **(a)**, middle-age (n=10) **(b)**, elderly (n=12) **(c)** and AD (n=10) **(d)** samples. The level of sAPPα was significantly and positively correlated with that of sAPPβ in the young and AD groups, and a positive slope difference was calculated between them, $p<0.05$ (Pearson correlation analysis).

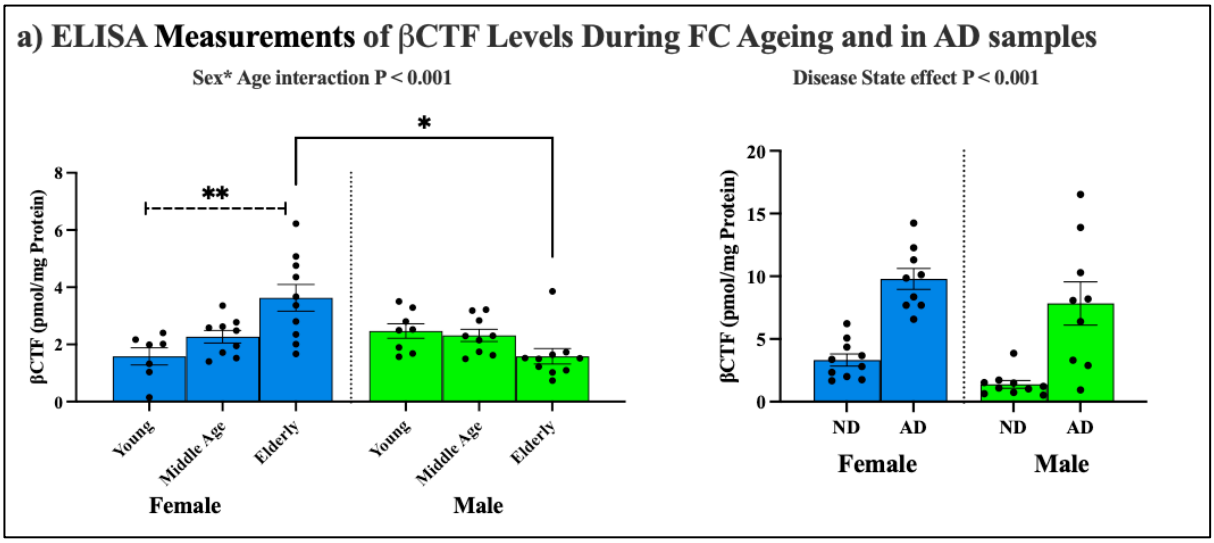


Figure 3.8 ELISA quantification of βCTF expression in human female and male frontal cortex between **(a)** different age groups of young (male n=8, female n=8), middle-aged (male n=9, female n=9) and elderly (male n=10, female n=10) subjects and between ND (male n=10, female n=10) and AD (male n=10, female n=10) subjects. A factorial two-way ANOVA revealed a significant increase of βCTF with age in female samples and a higher level of it was presented in elderly female compared to elderly male subjects (Sex*Age $F(2, 47)= 11.92$, $P<0.001$). Also, two-way ANOVA show a significant increases of βCTF levels in AD sample relative to ND sample (Disease State $F(1, 34)= 46.02$, $P<0.001$). Data are presented as mean ± S.E.M. *p<0.05, **p<0.002.

3.4.3 Aβ peptides were altered and showed sex differences with ageing and in AD

3.4.3.1 Female Samples:

Aβ was also investigated during ageing by ELISA. While levels of soluble $A\beta_{1-40}$ did not change significantly with age, soluble $A\beta_{1-42}$ was at a significant higher level in elderly subjects than in young and middle-aged subjects ($p<0.05$, Figure 3.9a). In addition, the soluble $A\beta_{1-42}/A\beta_{1-40}$ ratio was significantly increased with age ($p<0.05$, Figure 3.9c). Finally, a strong positive correlation between βCTF and soluble $A\beta_{1-42}$ was observed only in elderly subjects ($r^2 = 0.677$, $p = 0.003$, Figure 3.12c). However, although a significant correlation between βCTF and soluble $A\beta_{1-40}$ was seen in elderly individuals, it was only driven by one sample so it was considered as a false result ($r^2 = 0.532$, $p<0.05$, Figure 3.11c).

The insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides were tested here in the younger age groups, given that, when Alsaqati et al. (Alsaqati et al., 2018) investigated them in male brains, they were not detectable in young and middle-aged individuals. Both soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides were significantly higher in AD compared to ND subjects ($p<0.05$ and $p<0.001$ respectively, Figure 3.9a and 3.10a). Further analysis identified that both the soluble $A\beta_{1-42}/A\beta_{1-40}$ and insoluble $A\beta_{1-42}/A\beta_{1-40}$ ratios did not change in AD compared to ND subjects (Figure 3.9d & Figure 3.10b). However, the insoluble / soluble ratio was strongly significantly higher in AD compared to ND subjects ($p<0.001$, Figure 3.10c).

The correlation between βCTF and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in elderly (ND) subjects was similar to that reported above for the soluble Aβ fragments, a positive correlation was identified ($r^2 = 0.599$, $p=0.009$ and $r^2 = 0.609$, $p = 0.008$, Figure 3.13a & 3.13c, respectively).

3.4.3.2 Male Samples:

Soluble Aβ peptides showed a different pattern to females, as an age-related increase was seen with both soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ ($p<0.05$, Figure 3.9a). Also, a significant increase of soluble $A\beta_{1-42}$ was calculated during age advancement from middle age to elderly, this statistically significant increase was higher than the one calculated during age advancement from young to elderly ($p<0.002$, $p<0.05$ respectively, Figure 3.9a). The ratio of soluble $A\beta_{1-42}/A\beta_{1-40}$ was not significantly altered with ageing (Figure 3.9c). However, a positive correlation

between βCTF and soluble $A\beta_{1-40}$ ($r^2 = 0.563$, $p<0.05$, Figure 3.14b) and with soluble $A\beta_{1-42}$ ($r^2 = 0.826$, $p<0.001$, Figure 3.15c) was identified in middle-age and elderly subjects, respectively.

In AD, in contrast to females, males samples had a higher soluble $A\beta_{1-42}$/ $A\beta_{1-40}$ ratio compared to ND subjects ($p<0.05$, Figure 3.9d), in addition to higher levels of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ fragments ($p<0.002$, $p<0.001$, respectively, Figure 3.9b). For the insoluble fragments, there was a similar pattern to that observed in female subjects where $A\beta_{1-40}$ and $A\beta_{1-42}$ were significantly higher in AD compared to ND ($p<0.001$, 3.10a), whereas the soluble $A\beta_{1-42}$/ $A\beta_{1-40}$ ratio did not change (Figure 3.10b),

The ratio of insoluble/soluble of each Aβ variant was similar to female findings where a significant increase was seen ($p<0.05$, $p<0.001$, Figure 3.10c). A positive correlation in AD subjects' brains was observed between βCTF and soluble $A\beta_{1-42}$ ($r^2 = 0.725$, $p< 0.05$, Figure 3.15d). In addition, there was a positive correlation between βCTF and insoluble $A\beta_{1-42}$ in elderly (ND) subjects ($r^2 = 0.598$, $p< 0.05$, Figure 3.16c).

3.4.3.3 Sex Disparities:

Elderly females had higher levels of soluble $A\beta_{1-42}$ and insoluble $A\beta_{1-40}$ (mean= 14.87±18.75 and mean= 64.74±46.97, respectively) than males (mean= 3.433±3.55, $p<0.05$), and mean= 28.83±9.838, respectively $p<0.002$, Figure 3.9a & Figure 3.10a). However, neither soluble $A\beta_{1-40}$ nor insoluble $A\beta_{1-42}$ levels showed any significant sex differences among any of the different age groups (Figure 3.9b & 3.10a). There were also no significant differences in the ratios of soluble and insoluble $A\beta_{1-42}$ / $A\beta_{1-40}$ when examined between elderly male and female subjects (Figure 3.9c, 3.10b and 3.10c).

For AD samples, no changes between sexes were seen in soluble $A\beta_{1-40}$, and soluble $A\beta_{1-42}$ (Figure 3.9b). On the other hand, insoluble $A\beta_{1-42}$ was significantly higher in female (mean= 33872±2344) than in male AD subjects (mean= 6200±1477 $p<0.001$, Figure 3.10a). No sex differences were found in the insoluble/soluble Aβ ratios (Figure 3.10b,c).

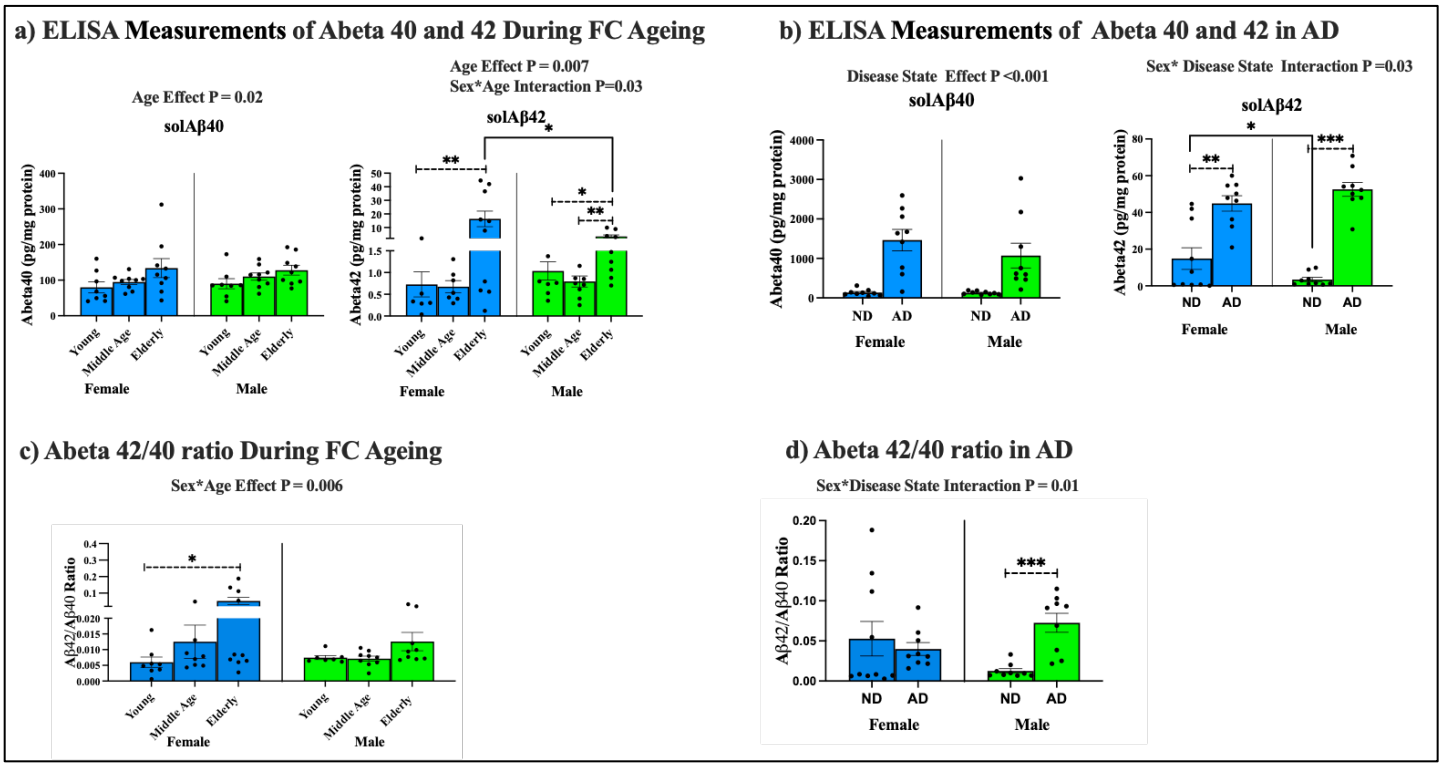


Figure 3.9 ELISA quantification of soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ expression in human female and male frontal cortex between **(a)** different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=12) subjects , with two-way ANOVA revealed an age related increase in soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in both male and female samples. In addition, level of soluble $A\beta_{1\text{-}42}$ was significantly higher in elderly female compared to elderly male samples (Age $F(2,46)=4.22$, $P=0.02$ and $F(2,44)=5.49$, $P=0.007$ for soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ respectively) additional to (Sex*Age $F(2,44)=3.65$, $P=0.03$ for soluble $A\beta_{1\text{-}42}$). **(b)** ELISA quantification between ND (male n=12, female n=12) and AD (male n=10, female n=10) subjects, with two-way ANOVA showing a significant increase in the expression of soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in AD samples relative to ND sample (Disease State $F(1.32)=29.92$, $P<0.001$ and Sex*Disease State $F(1,33)=5$, $P=0.03$ for soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ respectively) **(c)** soluble $A\beta_{1\text{-}42}$ / $A\beta_{1\text{-}40}$ ratio quantification during brain ageing with female samples showing an age-related increase in the ratio of soluble $A\beta_{1\text{-}42}$ / $A\beta_{1\text{-}40}$ (Sex*Age $F(2,45)=5.75$, $P=0.006$) **(d)** soluble $A\beta_{1\text{-}42}$ / $A\beta_{1\text{-}40}$ ratio quantification between ND and AD subjects, with AD male subjects showing a higher level of soluble $A\beta_{1\text{-}42}$ / $A\beta_{1\text{-}40}$ ratio (Sex*Disease $F(1,33)=7.14$, $P=0.01$). Data are presented as mean ± S.E.M. *p<0.05, **p<0.002 and *** p<0.001.

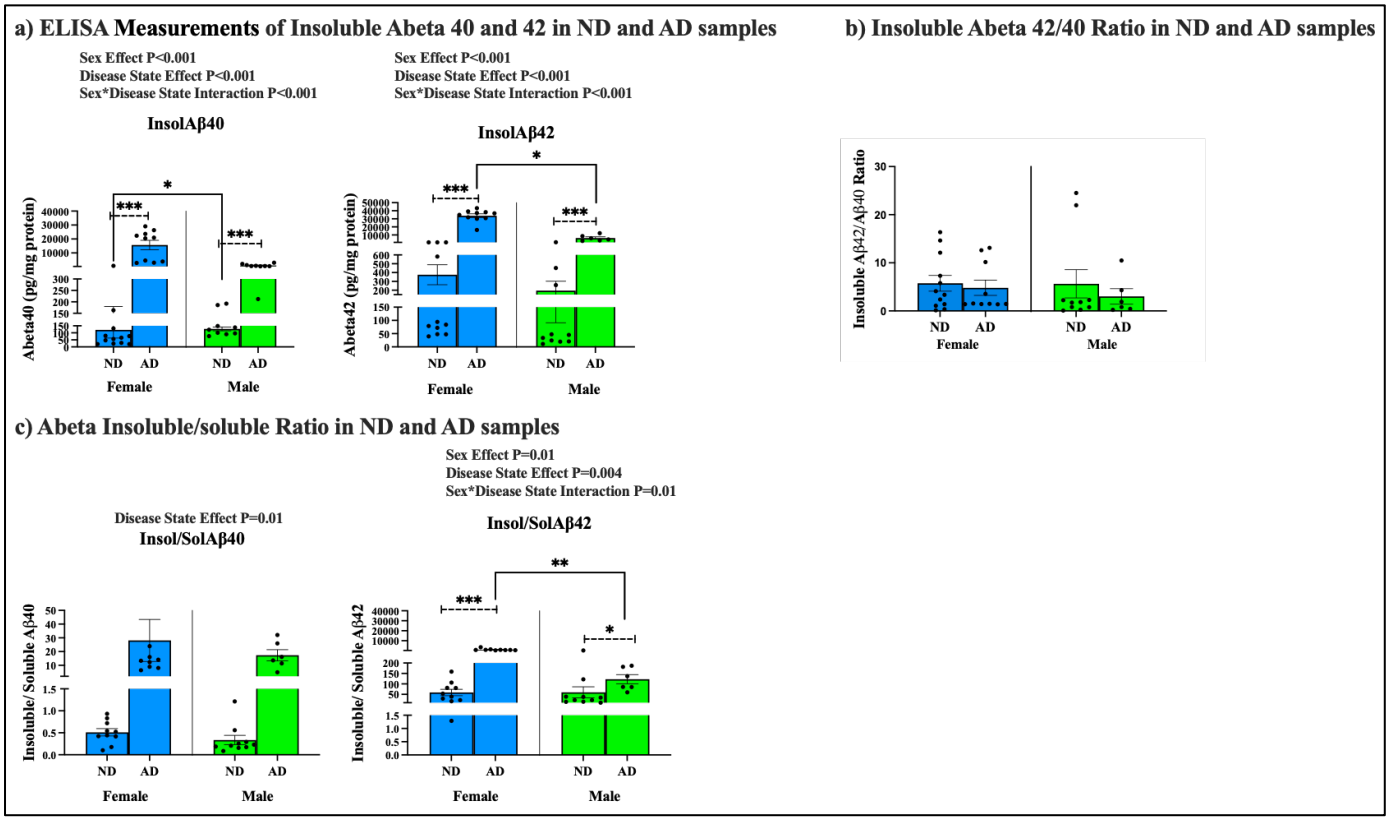


Figure 3.10 ELISA quantification of insoluble $A\beta_{1-40}$ & $A\beta_{1-42}$ expression in human female and male frontal cortex compared between **(a)** ND (male n=9, female n=12) and AD subjects (male n=6, female n=10), with two-way ANOVA revealed a higher level of insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in both male and female AD samples (Sex*Disease State $F(1,36)=18.17$, $P<0.001$ and $F(1,34)=101.5$, $P<0.001$ for insoluble $A\beta_{1-40}$ & $A\beta_{1-42}$ respectively) and (Disease State $F(1,36)=23.15$, $P>0.001$ and $F(1,34)=209.5$, $P<0.001$ for insoluble $A\beta_{1-40}$ & $A\beta_{1-42}$ respectively), also elderly female presented with higher level of insoluble $A\beta_{1-40}$ compared to age-match male subjects and AD female samples presented with significant higher levels of insoluble $A\beta_{1-42}$ compared to AD male subjects (Sex $F(1,36)=18.14$, $P<0.001$ and $F(1,34)=104.2$, $P<0.001$ for insoluble $A\beta_{1-40}$ & $A\beta_{1-42}$ respectively). **(b)** The ratio of insoluble $A\beta_{1-42}$/ $A\beta_{1-40}$ between ND and AD subjects, no significant effect of sex and disease state on the investigated protein. **(c)** Insoluble $A\beta_{1-42}$ / $A\beta_{1-40}$ ratio quantification investigated between ND and AD subjects, AD samples show a significant increases in the ratio of Insoluble/Soluble Aβ relative to ND sample (Disease State $F(1,31)=7.44$, $P=0.01$ and $F(1,31)=9.52$, $P=0.004$ for Insoluble/Soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively) additional to (Sex*Disease State $F(1,31)=7.37$, $P=0.01$ for Insoluble/Soluble $A\beta_{1-42}$). Furthermore, levels of Insoluble/Soluble $A\beta_{1-42}$ was significantly higher in AD female compared to AD male samples (Sex $F(1,31)=7.35$, $P=0.01$ for Insoluble/Soluble $A\beta_{1-42}$). Data are presented as mean ± S.E.M. *p<0.05, **p<0.002 and *** p<0.001.

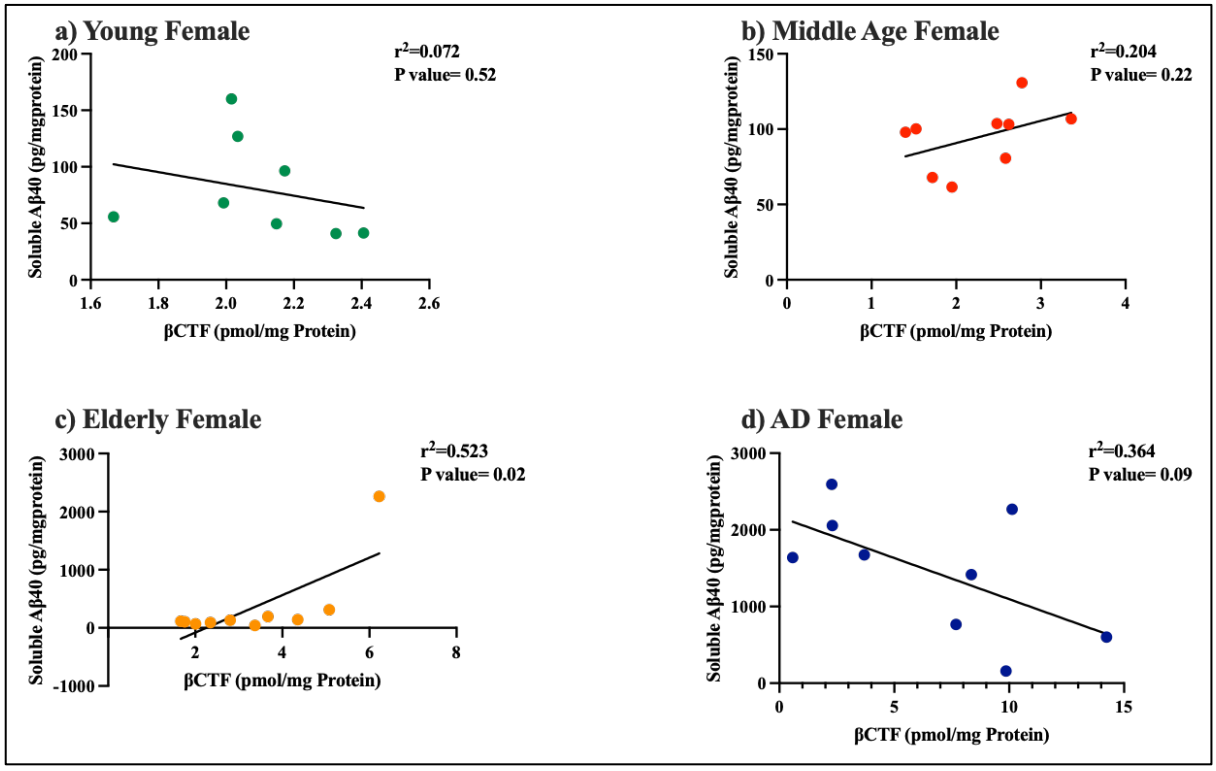


Figure 3.11 Linear regression analysis between βCTF and soluble $A\beta_{1\text{-}40}$ levels in females: young (n=8) **(a)**, middle-age (n=9) **(b)**, elderly (n=10) **(c)** and AD (n=9) **(d)** samples. The level of βCTF was significantly and positively correlated with that of soluble $A\beta_{1\text{-}40}$ in the elderly group but this was driven by only one sample. $p<0.05$ (Pearson correlation analysis).

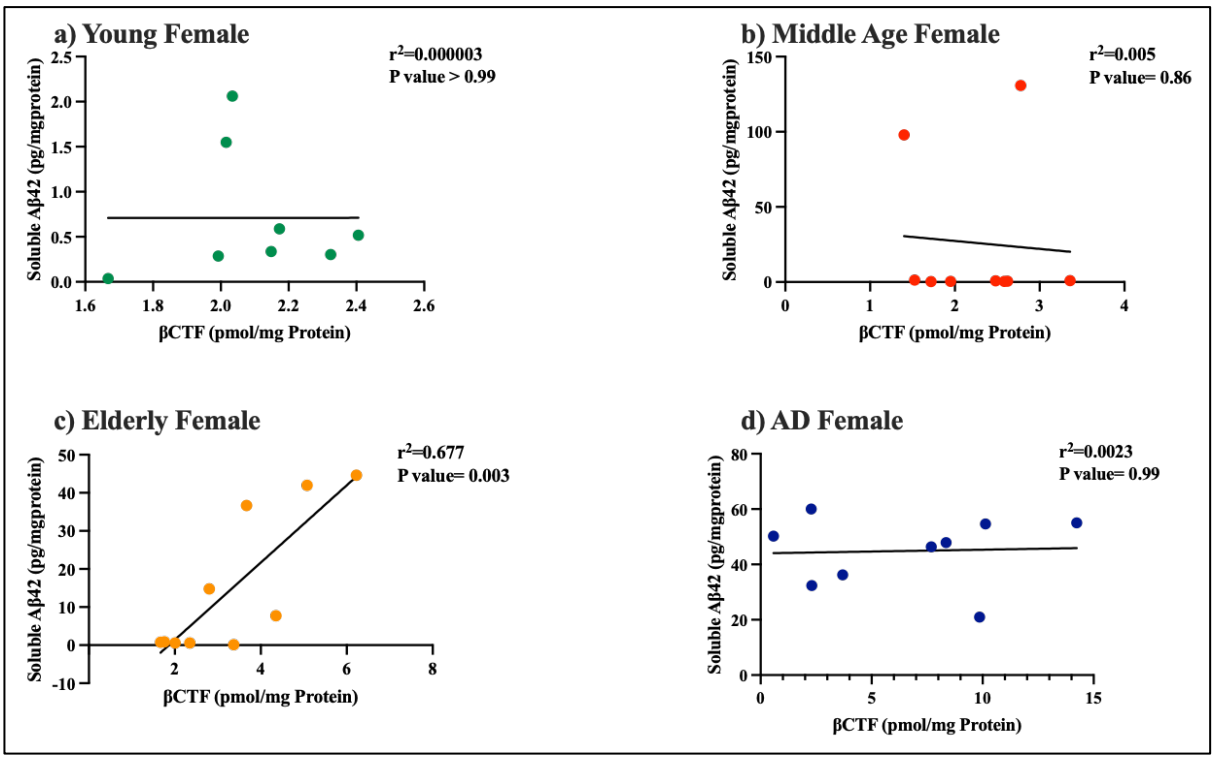


Figure 3.12 Linear regression analysis between βCTF and soluble $A\beta_{1-42}$ levels in females: young (n=8) **(a)**, middle-age (n=9) **(b)**, elderly (n=10) **(c)** and AD (n=9) **(d)** samples. The level of βCTF was significantly and positively correlated with that of soluble $A\beta_{1-42}$ in the elderly group. $p<0.05$ (Pearson correlation analysis).

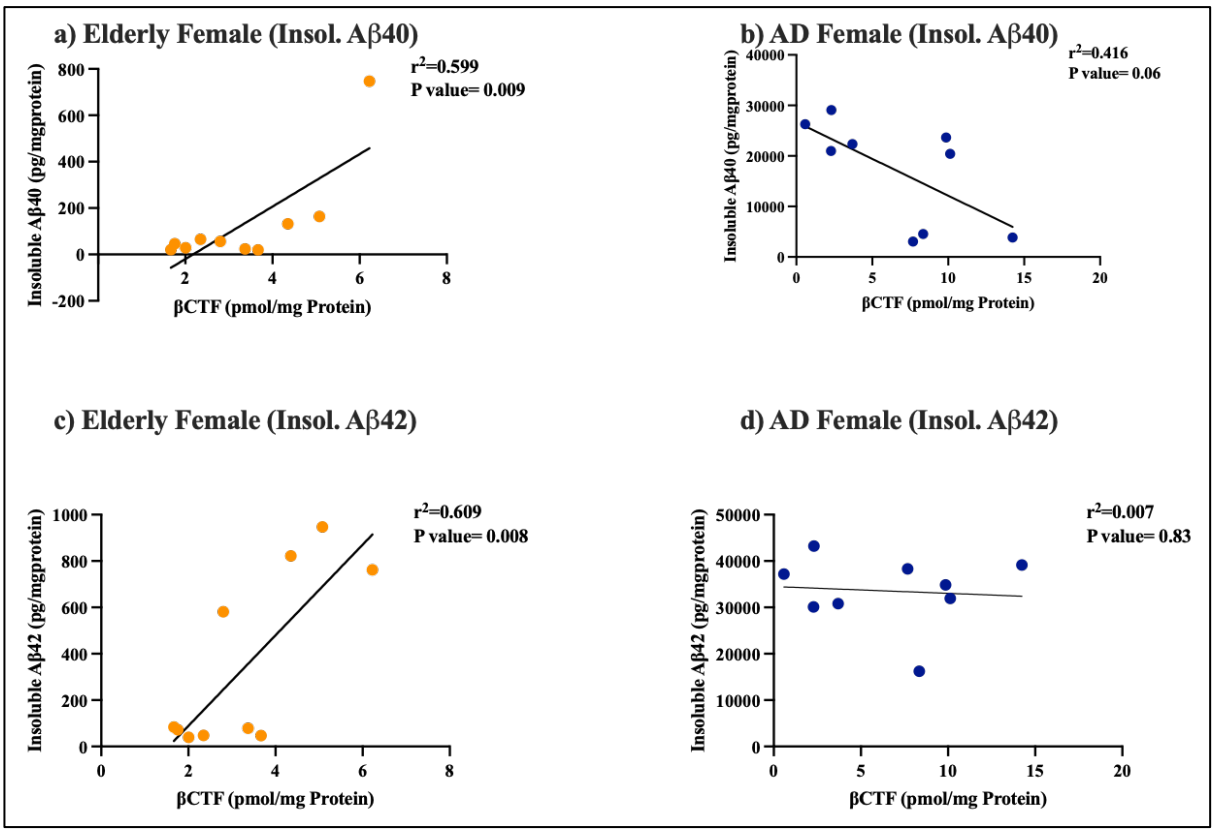


Figure 3.13 Linear regression analysis between βCTF and insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in females: elderly (n=10) **(a&c)** and AD (n=9) **(b&d)** samples, respectively. The level of βCTF was significantly and positively correlated with that of insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in the elderly group. The negative corelation with insoluble $A\beta_{1\text{-}40}$ in the AD group was not significant. $p<0.05$ (Pearson correlation analysis).

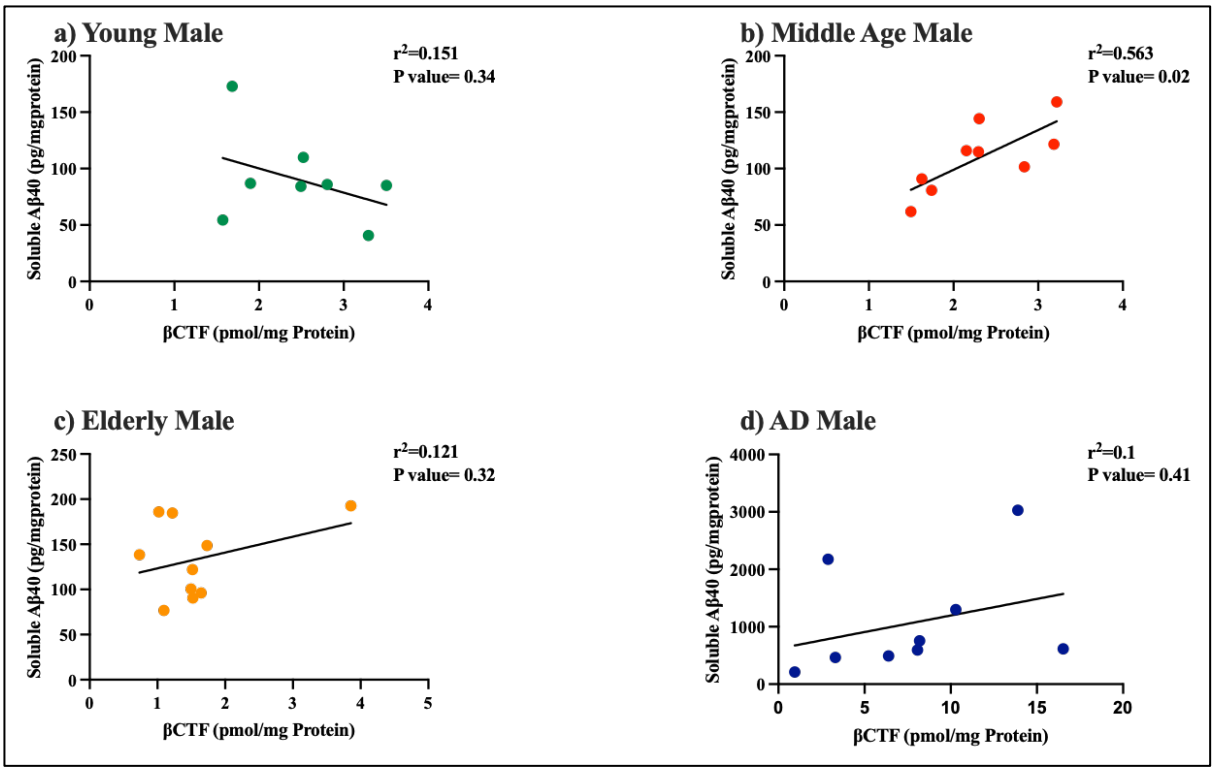


Figure 3.14 Linear regression analysis between βCTF and soluble $A\beta_{1\text{-}40}$ levels in males: young (n=8) **(a)**, middle-age (n=9) **(b)**, elderly (n=10) **(c)** and AD (n=9) **(d)** samples. The level of βCTF was significantly and positively correlated with that of soluble $A\beta_{1\text{-}40}$ in the middle-age group. $p<0.05$ (Pearson correlation analysis).

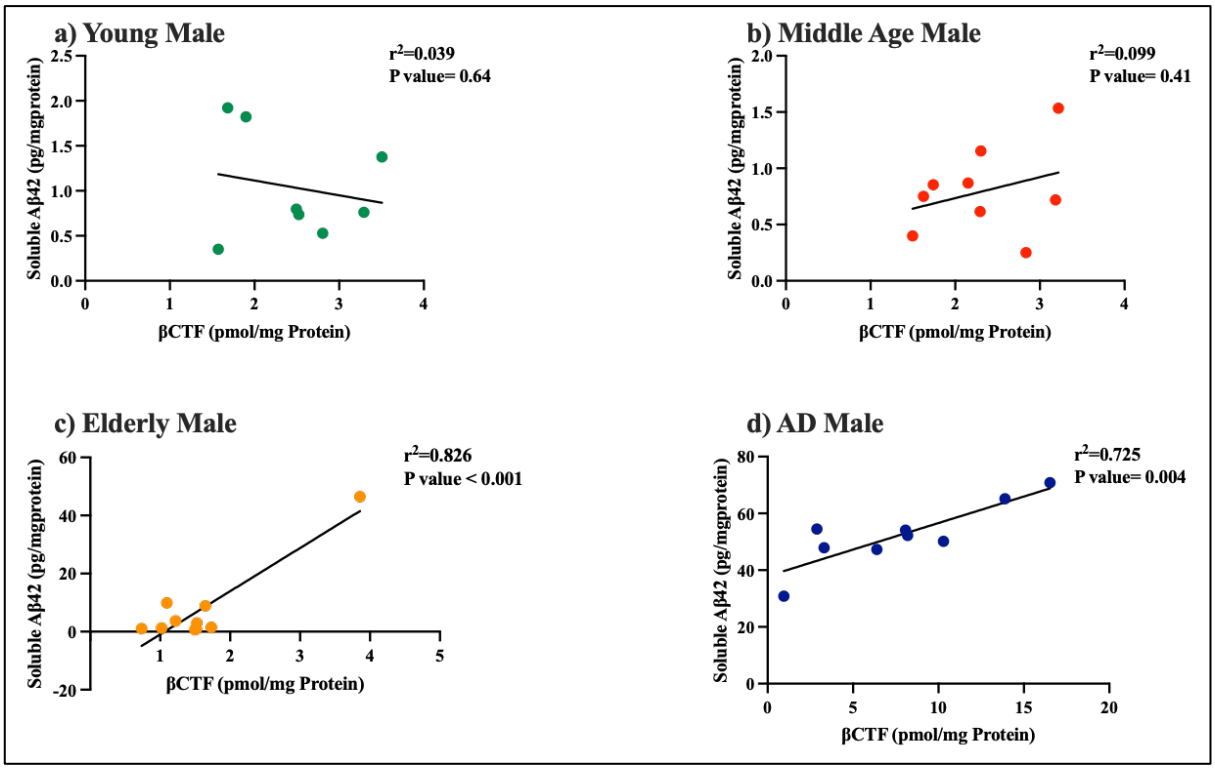


Figure 3.15 Linear regression analysis between βCTF and soluble $A\beta_{1-42}$ levels in males: young (n=8) **(a)**, middle-age (n=9) **(b)**, elderly (n=10) **(c)** and AD (n=9) **(d)** samples. The level of βCTF was significantly and positively correlated with that of soluble $A\beta_{1-42}$ in the elderly and AD groups. $p<0.05$, $p<0.002$ (Pearson correlation analysis).

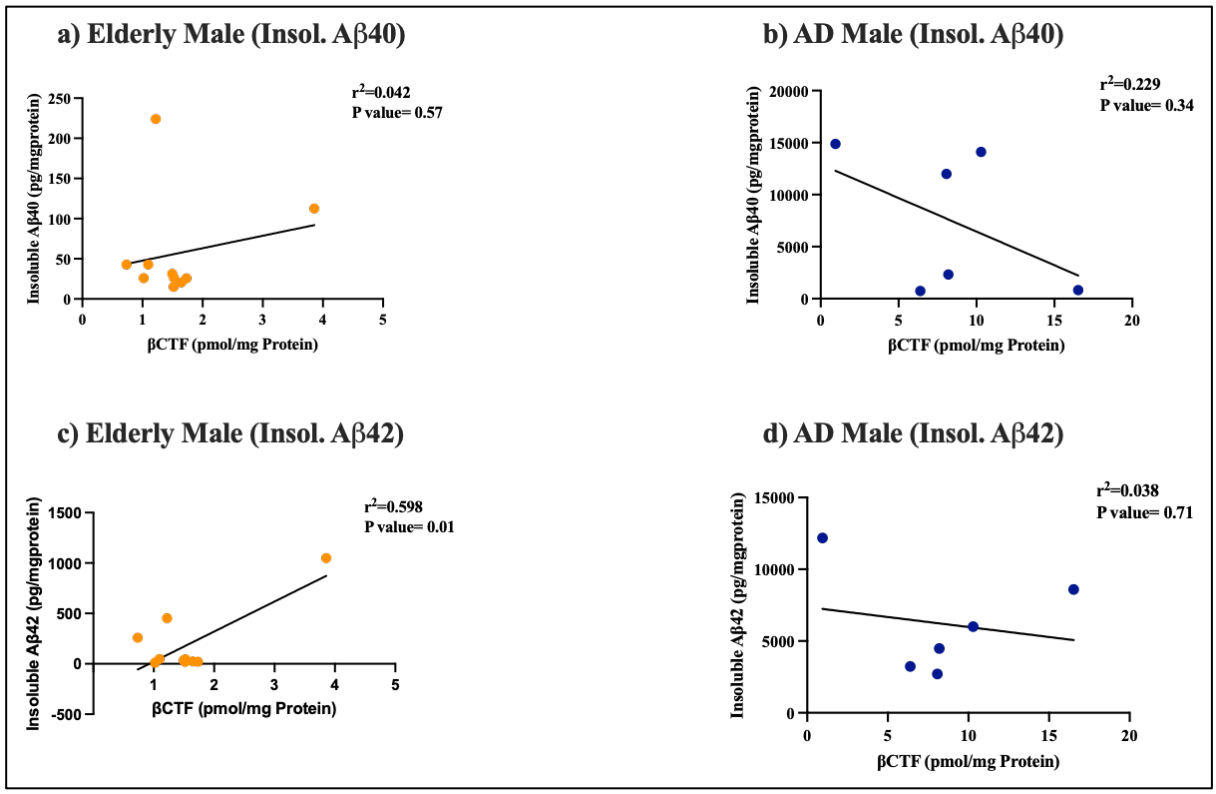


Figure 3.16 Linear regression analysis between βCTF and insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in males: elderly (n=10) **(a&c)** and AD (n=6) **(b&d)** samples, respectively. The level of βCTF was significantly and positively correlated only with insoluble $A\beta_{1\text{-}42}$ in the elderly group. $p<0.05$ (Pearson correlation analysis).

3.5 Discussion

This study demonstrates for the first time a sex difference in the expression of AD biomarkers in human frontal cortex with ageing and in AD. APP and some of its metabolites showed an age-related increase in female individuals but not in male subjects. An age-related increase in the absolute levels of soluble $A\beta_{1-42}$and the much higher levels of insoluble $A\beta_{1-42}$ in female brains, could be evidence for a different trajectory in APP cleavage pathways in males and females with APP metabolism in aged females switching more to the amyloidogenic pathway.

These results all together also suggest that the sexes follow different patterns in the onset of Aβ burden in the ageing brain with possible sex differences in the neurobiological processes. However, both the proposed different APP cleavage patterns in males and females would eventually lead to the same clinical AD phenotype. This suggestion is not unexpected, given the reported sex differences in Aβ burden and behaviour in AD transgenic mice models (Turner, 2001).

Since the expression of the proteins of interest was analysed during ageing followed by comparing ND to AD, the discussion below will focus first on the impact of age on the expression of these proteins and then consider the proteins in AD. Tables 3.2 and 3.3 summarise the findings of this chapter.

3.5.1 APP and its metabolic processing during ageing in the frontal cortex

3.5.1.1 Age as a risk factor

It has been well documented that brain ageing is associated with an increase in Aβ burden in the human brain (Fukumoto et al., 2004). These findings were replicated here where levels of soluble $A\beta_{1-42}$ were dramatically higher in the brains of aged female and male individuals in relation to younger subjects. A wide expression range of soluble Aβ was seen among individuals which is thought to be representative of the natural variation in $A\beta_{1-40}$ and $A\beta_{1-42}$ (Alsaqati et al., 2018). This was compatible with another study reporting that levels of Aβ increase in parallel with age in both APP transgenic mice and ND humans (Kumar-Singh et al., 2006). From the data above, it can be seen that levels of insoluble $A\beta_{1-42}$ were ~ 5 times

Table 3. 2 Summary of Chapter 3 Age-Related Findings.

	APP	ADAM-10			BACE-1	PS1	PS2	sAPP Fragments					Soluble. Aβ			Insoluble. Aβ				βCTF				
		proADAM-10	matADAM-10	Pro vs. Mat				sAPPα	sAPPβ	sAPPα/sAPPβ Ratio	sAPPα vs sAPPβ	Correlation of sAPPα vs sAPPβ	Solu.Aβ $_{1-40}$	Solu.Aβ $_{1-42}$	Solu.Aβ$_{1-42}$/Solu.Aβ$_{1-40}$ Ratio	Insolu.Aβ $_{1-40}$	Insolu.Aβ $_{1-42}$	Insolu.Aβ$_{1-42}$/Insolu.Aβ$_{1-40}$ Ratio	Insolu.Aβ/solu.Aβ Ratio	βCTF	Correlation to Solu.Aβ $_{1-40}$	Correlation to Solu.Aβ $_{1-42}$	Correlation to Insolu.Aβ $_{1-40}$	Correlation to Insolu.Aβ $_{1-42}$
During Frontal Cortex Ageing																								
Female	↑	-	-	-	↑	↑	↑	-	-	-	-	↑(m)	-	↑	↑	n/d	n/d	n/d	n/d	↑	↑(e)	↑(e)	↑(e)	↑(e)
Male	-	-	-	-	↑	-	-	↓	-	-	↑(e)	↑(y)	↑	↑	-	n/d	n/d	n/d	n/d	-	↑(m)	↑(e)	-	↑(e)
ND vs AD																								
Female	-	-	-	-	-	-	-	↓	-	-	-	↑(AD)	↑	↑	-	↑	↑	-	↑	↑	-	-	-	-
Male	-	-	-	-	-	-	↑	-	-	-	-	↑(AD)	↑	↑	↑	↑	↑	-	↑	↑	-	↑(AD)	-	-

y= young. m = middle-Age. n/d = non detectable.
e = elderly. AD= Alzheimer's Disease.

Table 3. 3 Summary of Chapter 3 Sex-Related Findings.

	Age Groups	APP	proADAM-10	matADAM-10	BACE-1	PS1	PS2	sAPPα	sAPPβ	Soluble. Aβ			Insoluble Aβ				βCTF
										Solu.Aβ $_{1-40}$	Solu.Aβ $_{1-42}$	Solu.Aβ$_{1-42}$/Solu.Aβ$_{1-40}$ Ratio	Insolu.Aβ $_{1-40}$	Insolu.Aβ $_{1-42}$	insolu.Aβ$_{1-42}$/insolu.Aβ$_{1-40}$ Ratio	Insolu.Aβ/solu.Aβ Ratio	
Female compared to Male	**Young**	-	-	-	-	-	-	↓	-	-	-	-	n/d	n/d	n/d	n/d	-
	Middle Age	-	-	-	-	-	-	-	-	-	-	-	n/d	n/d	n/d	n/d	-
	Elderly	-	-	-	↓	↑	↑	-	-	-	↑	↑	↑	-	-	-	↑
	AD	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	-	-

n/d = non detectable

higher than insoluble $A\beta_{1-40}$ in the elderly group of both sexes. These findings are compatible with earlier studies showing that $A\beta_{1-42}$ is hydrophobic and more prone to aggregation than $A\beta_{1-40}$, thus $A\beta_{1-42}$ is considered as the initial main component of plaque deposition (Welander et al., 2009). The $A\beta_{1-42}/A\beta_{1-40}$ ratio is a useful marker in the brain for the diagnosis of EOAD, since the age of onset of EOAD was inversely correlated with the $A\beta_{1-42}/A\beta_{1-40}$ ratio and the level of $A\beta_{1-42}$ but was directly correlated with $A\beta_{1-40}$ levels (Kumar-Singh et al., 2006). In AD brains, the $A\beta_{1-42}/A\beta_{1-40}$ ratio is predominantly shifted to a higher $A\beta_{1-42}$ percentage and associated with synaptic toxicity (Pauwels et al., 2012). Since similar alterations were identified in elderly female individuals here, this finding indicates a higher probability for aged female subjects to develop AD than age-matched males.

Surprisingly, the increase in Aβ levels with age was associated with corresponding increases in the levels of APP only in female subjects. This finding is novel to the best of my knowledg, and is in contrast with other studies where no change in APP was seen *in vivo* (Alsaqati et al., 2018, Fukumoto et al., 2004) but agrees with findings in human male subjects in this chapter. However, the major APP metabolites, sAPPα and sAPPβ, were unchanged with age in both sexes, while a significant positive correlation was seen between sAPPα and sAPPβ in young male and middle-aged female subjects. These data suggest that APP metabolism is controlled differently with age in males and females, indicating a physiological difference in the brain between sexes. APP metabolism appears to be regulated in the early life of male subjects through coordination between α- and β-secretases. While in female brains this coordination occurs in middle age. Interestingly, this period is representative of the menopause transition phase. However, this coordination appears to be lost with ageing and shifts to Aβ overproduction as both sexes get older.

βCTF was another APP by-product investigated as representative of amyloidogenic pathway activity in the brain. βCTF increased with ageing only in female subjects. Thus, the upregulation of βCTF in females was compatible with increases in soluble and insoluble Aβ in elderly subjects supported by the strong positive correlation between βCTF and Aβ in elderly female frontal cortex. In contrast, males did not show any alterations in βCTF with age regardless of the increase in Aβ levels with brain ageing, in agreement with a previous study (Alsaqati et al., 2018). It was surprising that βCTF did not increase in male aged frontal cortex giving the significant increase seen in $A\beta_{1-40}$ and $A\beta_{1-42}$ production. In elderly male subjects, βCTF did correlate positively with soluble and insoluble $A\beta_{1-42}$, agreeing with the findings of

the Alsaqati et al study. This might indicate that the levels of βCTF are indeed associated with Aβ overproduction, but that the absolute alteration in βCTF levels was too small to be detected. The accumulation of βCTF is initiated early in the pathogenesis of AD and may lead to neurotoxicity through synaptic loss and cell death and hence, might contribute to early AD pathology (Kim et al., 2016). In addition, βCTF is also thought to contribute to the endosomal formation and enlargement reported in both AD and DS brains (Kim et al., 2016) which might involve internalization of APP in both sexes and increase amyloidogenic cleavage. Thus, even if no change in APP levels was observed in male subjects in this and other studies (Alsaqati et al., 2018, Fukumoto et al., 2004), stimulation of APP internalization through βCTF might potentiate APP amyloidogenic cleavage and explain the increased Aβ production seen in aged male individuals.

The increase in APP levels seen here in female individuals was associated with changes in some APP catabolic enzymes in contrast to male subjects where no alteration was visible. ADAM-10 results in the production of the sAPPα fragment which is neuroprotective against Aβ deposition (Manzine et al., 2019). In this study, there was no alteration in ADAM-10 levels during brain ageing in both sexes. The lack of change in ADAM-10 is backed up by no alterations seen in sAPPα in both sexes. The no alternation of ADAM-10 during brain ageing could suggest that ADAM-10 might sustain an age-related neuroprotection action. However, the lateral suggested proposal, that ADAM-10 sustains a neuroprotective effect in ageing frontal cortex, could be contradicted by the significant rise in Aβ production in elderly subjects compared to the other age groups. The cognitive function trait of the elderly subjects which used in the thesis was healthy, even with the significant build-up of Aβ in their brains reported in this chapter, thus ADAM-10 might somehow sustain a neuroprotection function during brain ageing in both sexes. It has been reported that ADAM-10 can metabolise many membrane proteins including Notch during development (Reiss and Saftig, 2009). Notch signalling is crucial for several cellular process such as cell-cell interaction, angiogenesis, cell permeability and survival (Alberi et al., 2013, Ramasamy et al., 2016, Mack and Iruela-Arispe, 2018, Sainson and Harris, 2006, Gustafsson et al., 2005, Brou et al., 2000). Thus, here, the sustained levels of ADAM-10 with ageing might correlate with its major shedding role for other membrane proteins, e.g Notch, rather than acting only on mediating non-amyloidogenic APP processing.

The increase in βCTF fragment levels with ageing in female subjects might indicate an increase in BACE1 activity. An age-related significant increase in BACE1 levels was seen in both sexes. This could indicate higher BACE1 activity contributing to higher levels of βCTF in aged male and females' brains. This finding is opposite to previous work by the lab reporting no alteration of BACE1 expression with male brain ageing (Alsaqati et al., 2018). This difference in the outcomes could be due to the use of a different anti-BACE1 antibody in the previous study. Also, it has been documented previously that BACE1 activity was significantly increased with ageing (Fukumoto et al., 2004). This indicates that the increase in βCTF in the female ageing brain is not only a result of the increase in APP levels and its metabolism.

PS1, a catalytic subunit of γ-secretase (Wolfe, 2008), was investigated as a marker of γ-secretase expression in female brains. When its expression showed an age-related increase, PS2 expression was investigated too since the expression patterns of PS1 and PS2 were reported to be similar in the brain (Lee et al., 1996). As expected, PS2 expression was also associated with an age-related increase in female brains. Thus, this increase in γ-secretase expression suggests increased activity and, according to the Aβ hypothesis, this would cause further cleavage of βCTF and increased production of Aβ. Also, the upregulation of PS1/ γ-secretase activity is considered a major risk factor for the development of LOAD (Tong et al., 2011). It is interesting that both PS1 and PS2 presented with age-related increases in female frontal cortex as, in an earlier study, it was shown that PS1 and PS2 mRNA levels were highest in embryonic brains and then declined to remain constant with increasing age (Fukumoto et al., 2004). That study only investigated PS1 and PS2 mRNA in male samples, ignoring the sex-disparity factor. In contrast to female findings, here male subjects had no alteration in the levels of PS1 or PS2. However, with an increase in $A\beta_{1-40}$ and $A\beta_{1-42}$ production in aged male frontal cortex, it could be hypothesised that the increase of Aβ in the ageing brains could be caused by increase in γ-secretase activity without an increase in expression.

Intriguingly, while BACE1 generates βCTF, PS1 function favours the generation of the hydrophobic isoform, $A\beta_{1-42}$ (Herzig et al., 2007). Thus, in elderly male subjects, several hypothesized mechanisms might explain the increase in Aβ production independent of γ-secretase levels. The first hypothesis is the ability of Aβ to induce a feed-forward mechanism on APP. *In vivo*, it was reported that Aβ can stimulate the phosphorylation of APP at Thr_{688}, leading to enhanced APP proteolysis and Aβ production (Kirouac et al., 2017). Another possible process is related to APP internalisation which occurs either mediated by βCTF, as

mentioned previously (Kim et al., 2016), or by an alteration in endocytosis during male brain ageing (Alsaqati et al., 2018), thus APP would then come into contact with more enzymes, for instance γ-secretase.

Based on the age-related data in this chapter, both sexes process APP through non-amyloidogenic and amyloidogenic pathways during ageing until they reach an advanced age where the amyloidogenic pathway is favoured, with Aβ overproduction data here as evidence. However, it is possible that male and female subjects process APP via distinct pathways during ageing and the net result is increased production of Aβ with frontal cortex ageing. In detail, for females, the amyloidogenic processing is carried out by an increase in the catabolic enzymes, BACE1 and γ-secretase. While in males, potentiation of the amyloidogenic pathway could be carried out by the stimulation of endosomal mechanisms as seen with the increase in the endocytosis markers, PICALM and Rab5 (Alsaqati et al., 2018) in the same male samples used in this project. However, Alsaqati et al didn't investigate the state of endosomes in females with brain ageing (Alsaqati et al., 2018).

3.5.1.2 Sex as a risk Factor

When levels of APP, its metabolites and enzymes were compared between male and female subjects, PS1 and PS2 levels were found to be significantly higher in old female subjects than in males. This could increase the risk to develop LOAD in females more than in males (Tong et al., 2011). However, the expression of BACE1 was shown to be significantly higher in aged male subjects than in females. Even given this finding, the activity of BACE1 could be higher in ageing female brains; this suggestion is backed up by the higher soluble $A\beta_{1-42}$/ $A\beta_{1-40}$ ratio and the higher levels of soluble and insoluble $A\beta_{1-42}$ and βCTF in aged females compared to male subjects. Additionally, the positive correlation of βCTF to soluble and insoluble $A\beta_{1-42}$ and $A\beta_{1-40}$ in aged female brains could indicate a more critical contribution of Aβ in their brain function. Elderly males only had a correlation between βCTF and $A\beta_{1-42}$, the more hydrophobic peptide which leads to plaque formation (Welander et al., 2009). It has been argued that this insoluble peptide is not the main cause of toxicity seen in AD, the soluble oligomers are generally thought to be responsible for neuronal toxicity in AD (Deshpande et al., 2006), refer to Chapter 1, section 1.4.2.

Moreover, in female subjects, even if ADAM-10 didn't show sex disparities, alterations in PS1 and PS2 could be related to another AD pathology hypothesis, the "amyloid-notch

hypothesis". Notch signalling cascades depend on the cell-cell microenvironment condition and ageing, and stress, inflammation, hypoxemia and hyperglycaemia also activate Notch downstream signalling (Alberi et al., 2013, Ramasamy et al., 2016, Mack and Iruela-Arispe, 2018, Sainson and Harris, 2006, Gustafsson et al., 2005, Brou et al., 2000). The interaction of Notch with its ligand leads to a conformational change in Notch followed by proteolytic processing. Firstly, the ectodomain of Notch is processed by ADAM-10 leading to formation of Notch extracellular truncation (NEXT) (Brou et al., 2000, Mumm et al., 2000). The remaining fragment is then cleaved by γ-secretase releasing the Notch intracellular domain (NICD) that translocates to the nucleus and acts as a transcription factor (Reiss and Saftig, 2009). It has been reported recently that Notch protein expression is significantly increased in AD brains, and is deposited in $A\beta_{1-42}$ plaques (Brai et al., 2016). A recent *in vivo* study reported that chronic expression of NICD in the hippocampus induced vascular thickening with the aggregation of Aβ causing an exacerbation in spatial memory deficits. Additionally, chronic activation of the Notch cascade caused impaired blood flow and a reduction in nutrient delivery which led to brain dysfunction in AD-model transgenic mice (Galeano et al., 2018). Consequently, chronic activation of Notch could accelerate Aβ aggregation and deposition leading to defects in spatial memory (Welander et al., 2009). Therefore, the higher levels of soluble and insoluble $A\beta_{1-42}$ in the old female individuals compared to their peer males could be due to the processing of both APP and Notch. APP is processed by BACE and PS1/ γ-secretase leading to the production of Aβ, and Aβ aggregation and deposition in the brain might be accelerated by Notch which is processed by ADAM-10 and PS-1/ γ-secretase (Welander et al., 2009, Cho et al., 2019).

3.5.2 APP and its metabolic processing in the AD brain

The expression of APP and its metabolic enzymes has been studied in depth in AD. Transgenic mice models with overproduction of APP and its metabolic enzymes; PS1 and BACE1 (Drummond and Wisniewski, 2017), develop pathology that is similar to that found in the human AD brain; particularly, an age-dependent occurrence of Aβ accumulation, plaque deposition and tau hyperphosphorylation.

A variety of detection methods and types of samples obtained from AD patients, either CSF or brain lysates, showed distinct expression patterns of APP secretases. For instance, ADAM-10 activity was reported to be increased in brain lysates (Bernstein et al., 2003) and

decreased in both CSF (Colciaghi et al., 2002) and brain lysates (Nyarko et al., 2018). Also, BACE1 levels were either unchanged (Tong et al., 2011, Nyarko et al., 2018) or decreased in AD brain lysates (Boris et al., 2013). γ-secretase shows the same conflicting results with either no changes in brain lysates (Nyarko et al., 2018) or an increase in CSF (Aitana et al., 2016). These studies pooled male and female data for analysis and, even when data were separated according to the sex of donors, little attention has been paid to comparing male and female data.

Here in this experiment, APP and its metabolites showed sex differences in protein expression in AD patients. In AD females no alteration in APP was observed, which is not surprising given this was reported previously (Johnston et al., 1996), also no alteration in the secretase enzymes was observed in AD females compared to ND subjects. However, surprisingly, here AD male subjects showed a significant increase in PS2 compared to ND subjects. A similar pattern was reported previously for the levels of PS1 and PS2 (Lee et al., 1996), but here in male AD brains only PS2 was increased. However, *in vitro* knockout of PS genes has revealed that PS1 is essential for γ-secretase activity and this function is accelerated by PS2. Also, PS1 knockout reduces γ-secretase cleavage function (Sisodia and St George-Hyslop, 2002). Thus, the increase in PS2 levels in males might be associated with an increase in PS1 activity. This explanation is supported here by the increase in soluble and insoluble fragments of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42,}$ γ-secretase by-products, in AD males. This suggestion could also be true in the female AD samples as a dramatic increase in the levels of soluble and insoluble fragments of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ was seen. Also, the levels of these peptides were significantly higher in AD females compared to males, which could be associated with increased activity of amyloidogenic enzymes even if no change in PS1 and PS2 levels were observed, or due to a decline in Aβ clearance/metabolism. The latter suggestion is supported by *in vivo* studies reporting a fall in neprilysin, an enzyme which plays an essential role in the breakdown of Aβ, in the 3xTg-AD mice model and it was also more marked in females (Hirata-Fukae et al., 2008, Iwata et al., 2005). Furthermore, ovariectomy markedly decreased neprilysin detected in the brain and levels were returned to normal by E2 supplementation (Huang et al., 2004). In addition, Hirata-Fukae et al. determined that, prior to plaque formation in mice, Aβ quantities were equivalent between the sexes, and female mice with plaques had notably elevated Aβ levels associated with augmented BACE activity and a reduced level of neprilysin (Hirata-Fukae et al., 2008). These data imply that the enhanced synthesis and decreased breakdown of Aβ may augment the risk of AD in female rodents. However, no clinical studies

have investigated neprilysin levels in both sexes during normal brain ageing. This could be looked at in future works.

The ratio of soluble $A\beta_{1-42}/A\beta_{1-40}$ increased in male but not in female AD brains relative to ND subjects. Since increased $A\beta_{1-42}/A\beta_{1-40}$ ratios were reported previously in AD and associated with synaptic toxicity (Kumar-Singh et al., 2006, Pauwels et al., 2012, Nyarko et al., 2018), and the brain samples used here are from patients diagnosed with AD (Braak V-VI), the ratio of soluble $A\beta_{1-42}/A\beta_{1-40}$ was expected to be higher in female patients than in ND subjects (Marttinen et al., 2019). However, the lack of significant increase could be due to the wide variation of $A\beta_{1-42}/A\beta_{1-40}$ levels in the female ND subjects.

sAPPα and sAPPβ did not change in male AD brains, while in female AD brains only sAPPα declined which was in contrast to earlier findings (Nyarko et al., 2018, Lewczuk et al., 2010), where they reported respectively no alteration and an increase in sAPPα levels in AD patients. Thus, the neuroprotective function of ADAM-10 is likely to be decreased here in females (Manzine et al., 2019). Also, a significant positive correlation between sAPPα and sAPPβ in female AD brains was detected, parallel to what reported in AD cerebral spinal fluid (Lewczuk et al., 2010). Interestingly, the correlation plot slopes of sAPPα to sAPPβ between the AD and middle-aged female subjects was not different which could highlight a similar APP processing environment in the two groups. This alteration was absent in male AD brains, correlation slope was significantly different from the young male correlation slope which could suggest that different aspects of APP processing in earlier life in males are essential for brain health and development. One explanation of this sex-disparity is the biological differences in males and females including the menopause transition phase females face in middle-age which could trigger switching APP metabolism towards the pathological disease trajectory.

βCTF was significantly higher in AD patients of both sexes compared to ND subjects and, along with the increase in $A\beta_{1-42}$ levels, this would indicate that APP metabolism favours the amyloidogenic pathway in the Alzheimer's disease state (Zhang et al., 2011b).

The data presented in this chapter suggest that females may start to develop AD earlier than males based on the increase in APP and its secretase enzymes during female brain ageing and the dramatically higher levels of soluble and insoluble $A\beta_{1-42}$ compared to males for the ND and AD groups (Altmann et al., 2014, Liu et al., 2019a). However, females may remain cognitively healthy compared to age-matched males, even with the earlier increase in Aβ levels

seen during female brain ageing (Goyal et al., 2019). Evidence in favour of female resilience to age-related changes and AD pathology compared to males, is that female brains are reported to be younger and healthier than age-matched males (Goyal et al., 2019). In adddition, Cavedo et al. looked at sex disparities in glucose metabolism using FDG-PET imaging. They noted significant hypometabolism of glucose in a number of brain regions, such as the precuneus, posterior cingulate and inferior parietal cortices, in males compared to females in a pre-clinical group (Cavedo et al., 2018). Sex variations in brain metabolism, as ranked employing a composite imaging scale, have also been proposed (Goyal et al., 2019) and imply that females have a more youthful brain metabolic age than males. This work needs further confirmation and expanding to include patients with AD. Additional support for this idea comes from a clinical study on males and females aged 45 to 55 for the assessment of episodic memory and estimating verbal IQ, which found that females outperformed males in these tests. Furthermore, pre-menopausal and peri-menopausal females scored higher than postmenopausal females (Rentz et al., 2017, Kakimoto et al., 2016).

3.6 Conclusion

In conclusion, the data presented here support the possibility that APP processing follows different pathways in male and female brains during ageing and in AD. In females there is an earlier switch to the amyloidogenic pathway (BACE1, PS1 and PS2 in aged female brains), increasing the production of soluble and insoluble Aβ which might contribute to their susceptibility to develop early preclinical AD. Even so, this switch alone is not enough to result in neuronal toxicity in the brain. Several studies have presented Aβ deposition as an early event of AD pathology, while the co-association of NFTs is the cause of synaptic toxicity and cell death (Perrin et al., 2009). The next chapter will therefore investigate how tau and its hyperphosphorylation, leading to NFT deposition in the brain, are affected by ageing and sex.

CHAPTER 4 _Exploring the Levels of TAU, its main Kinase (GSK3) and Phosphatase (PP2A) enzymes, and Anti-apoptotic (Bcl2) and Pro-Apoptotic (Caspase-3) Proteins in Female and Male Frontal Cortex during Ageing and in Alzheimer Disease

4.1 Introduction

4.1.1 *The influence of age and sex on Tau*

The velocity of tau spread in the brain is age-dependent as, when human-tau was injected in the entorhinal cortex (EC) region in WT mice of different ages, young (3-months) versus old (22-24 months), tau spread faster to other brain regions, hippocampus and adjacent cortical areas, in the old brains and initiated misfolding tau in EC neurons (Wegmann et al., 2019). Regarding clinical sex disparities, brain tauopathy can be determined utilising PET or by assaying phosphorylated tau in the CSF. Sex variations in total tau burden have been documented in large cross-sectional imaging studies in cognitively healthy individuals with Aβ accumulation (Buckley et al., 2019, Jack Jr et al., 2018). In that study it was reported that females show higher levels of tau in their entorhinal cortices compared to males (Buckley et al., 2019, Jack Jr et al., 2018). Also, CSF concentrations of tau have also revealed sex disparities in healthy subjects and in patients with MCI and AD, with females having higher levels of tau in all conditions (Smith et al., 2020). It is possible that sex- ApoE genotype associations may influence tau levels; a more robust correlation between ApoE ε4 and total and phosphorylated CSF tau concentrations has been demonstrated in females (Altmann et al., 2014, Mattsson et al., 2017).

4.1.2 *Bcl2 and Caspase-3 as indicators of neuronal death or survival*

The viability of neurons in the adult human brain is limited during the ageing process, and, particularly, when it is affected by disease. In AD, neuronal impairment antecedent to cell demise is associated with histopathological and biochemical anomalies and abnormal amyloid management (LeBlanc, 1994). Nevertheless, there is a dearth of information regarding the apoptotic mechanisms that underpin neuronal death in neurodegenerative conditions (Kerr et al., 1972). A major controlling factor in the brain apoptotic process is the protooncogene product, Bcl2, an anti-apoptotic protein (Hengartner and Horvitz, 1994a, Hockenbery et al., 1991, LeBrun et al., 1993, Merry et al., 1994). In Bcl2 transgenic mice, there is improved protection of cortical neurons in the brain and spinal motoneurons against experimentally induced ischemia (Dubois-Dauphin et al., 1994, Martinou et al., 1994). On the other hand, caspase3, an apoptotic stimulator, was reported to be highly activated in the early stages of AD (Gastard et al., 2003). Furthermore, tau phosphorylation is reported to be triggered by caspase3 cleavage after apoptosis stimulation at early AD stages preceding tau hyperphosphorylation (Canu et al., 1998, Yang et al., 1998, Gamblin et al., 2003, Rissman et al., 2004). In AD, soluble

tau truncated fragments are reported to be elevated in AD brains and used as a hallmark of cognitive impairments (Liu et al., 2020).

4.2 Hypothesis and Aims

In the context of AD, while aggregation of Aβ fibril plaques in the brain is considered an early event in the disease process, formation of NFTs is thought to be a more proximate cause of later neuronal malfunction and death (Hardy and Higgins, 1992, GÛmez-Isla et al., 1997, Bennett et al., 2004). According to the findings of Chapter 3, the high upregulation of Aβ burden in the female brain could be associated with tau dysfunction.

Hypothesis: alterations in tau are likely to occur with advances in brain age in both males and females associated with Aβ overproduction, with no change in apoptosis. While in AD individuals, tau phosphorylation will increase along with stimulation of apoptosis. In addition, elderly and AD female will harbour more tau levels in their frontal cortex than age match males individuals.

Therefore, this chapter will consider changes in the enzymes associated with tau phosphorylation. Also, Bcl2 and caspase-3 were used as markers of apoptotic toxicity during brain ageing and in AD. Thus, the aims of this chapter were to:

1) Determine the brain levels of tau phosphorylation and its phosphorylation status through investigating GSK3 and PP2A from 'healthy' individuals without neurodegeneration aged 20 to 90 years old and ND vs AD age-matched samples.
2) Determine tau neurotoxicity through investigating the levels of the cell death markers Bcl2 (anti-apoptotic) and caspase3 (apoptotic) in the same individuals.
3) Investigate the sex differences in tau-associated proteins and apoptosis regulator proteins.

4.3 Experimental Methods

All the methods used in this Chapter have previously been detailed in Chapter 2.

4.3.1 Samples:

Samples used in this chapter were prepared and grouped as described in Chapter 2.

4.3.2 Experiments and targeted proteins

Western blotting (WB) was used for all the sample groups to explore the expression of tau, GSK3, PP2A, Bcl2 and caspase3 (refer to Table 4.1 for band molecular masses and phosphorylation sites). It is important to highlight that the antibody used for caspase3 could identify only the immature form of the protein, ProCaspase3. Thus, in this Chapter, caspase3 will be called ProCaspase3.

The method used for WB to load samples to investigate age and sex disparities was descried in Chapter 3, Section 3.3.2. The expression of proteins was analysed across more than one gel. Therefore, all protein bands were expressed as the relative density of a standard human brain sample, a healthy young female sample, for exploring protein levels during ageing and for the ND vs AD experiments. The exception was p.tau, since it was undetectable in healthy brains and could only be measured in AD brains, when running samples in more than one gel, the p.tau/t.tau ratio in AD brains was calculated (Figure 4.1d) with the protein ratio expressed as the relative density to the same AD samples.

During WB analysis of Tau and GSK3, some samples were identified as outliers and removed from the analysis (described in Chapter2, Section 2.9.2). Also, when measuring the band intensities of GSK3α and GSK3β proteins, images were enlarged by 75% to aid in selecting bands of interest without overlap between the two GSK3 isoforms.

Table 4.1 Expected WB band molecular masses and the phosphorylated residues for all proteins examined in Chapter 4.

Proteins	**Phosphorylation Site**	**Expected Size (kDa)**	**References**
Total tau (t.tau)	-	70-50	(Alsaqati et al., 2018)
Phospho tau (p.tau)	Ser396/Ser404	70-50	(Alsaqati et al., 2018)
Total GAK3 (tGSK3)	-	GSK3α (51) GSK3β (47)	(Alsaqati et al., 2018
Phospho GSK3 (pGSK3)	GSK3α (Tyr279) GSK3β (Tyr216)	GSK3α (51) GSK3β (47)	(Alsaqati et al., 2018
PP2A	-	51	(Ye et al., 2020)
ProCaspase3	-	32	(Park et al., 2021)
Bcl2	-	26	(Xu et al., 2020)

4.3.3 Statistical analysis

WB data were normalized then analyzed as described in Chapter 2, Section 2.9.2. All data in this chapter were parametric and were analysed using a factorial two-way ANOVA to compare the main effects of age and biological sex (IV), and the disease state and biological sex (IV), as well as the interaction effects of those factors against each other [Sex*Age or Sex*Disease state Interaction]. If the lateral interaction [Sex*Age or Sex*Disease state Interaction] is significant it indicates that male and female presented with different patterns in protein expression throughout ageing or in the disease state, thus, to investigate that further, a one-way ANOVA would be conducted for each sex separately. The statistical results of two-way ANOVA will be included in the figures legends, while the post-hoc test results will be discussed more deeply in the results section.

4.4 Results

4.4.1 Levels of pGSK3β are increased but total Tau is not altered with ageing in both sexes

4.4.1.1 Female samples:

The expression of phosphorylated tau was investigated using the widely recognised PHF-1 antibody (p.Ser$_{396}$/p.Ser$_{404}$) (Petry et al., 2014) but no phosphorylated tau was detected in ageing brain samples even in elderly subjects (Figure 4.1a). However, total tau was detected as multiple tau isoforms (Figure 4.1a). When all detected total tau isoform bands were quantified together, the levels did not alter with ageing (Figure 4.1b). Next, the expression of GSK3α and GSK3β during brain ageing was investigated as GSK3 is one of the most important enzymes involved in the phosphorylation of tau. GSK3α and GSK3β were detected at 49 kDa and 45 kDa, respectively (Figure 4.2a). The expression of tGSK3α and pGSK3α, along with the ratio of pGSK3α/tGSK3α, did not change with ageing (Figure 4.2b and 4.3a). However, pGSK3β, tGSK3β along with the ratio of pGSK3β/tGSK3β, all had significant age-related increases ($p<0.05$, Figures 4.2d and 4.3a). The significant age-related increases in both pGSK3β and tGSK3β could be seen between middle-age and ND elderly individuals while pGSK3β levels decreased significantly from the young to the middle-age group ($p<0.05$, Figure 4.2 d). Also, the pGSK3β/tGSK3β ratio was significantly higher in aged brains than the pGSK3α/tGSK3α ratio ($p<0.05$, Figure 4.3a).

The levels of PP2A, a phosphatase enzyme of tau, was also studied in ageing brain samples and no alterations in its levels were seen (Figure 4.4b).

4.4.1.2 Male samples:

No alteration in Total tau during male ageing was found here (Figure 4.1b). The proteins regulating tau phosphorylation had broadly similar findings in males as those described above for females during ageing. While tGSK3α, pGSK3 α and the pGSK3α/tGSK3α ratio were not altered with ageing (Figures 4.2b and 4.3a), pGSK3β and the pGSK3β/tGSK3β ratio showed a significant age-related increase ($p<0.05$, Figures 4.2d and 4.3a). Also, the pGSK3β/tGSK3β ratio was significantly higher in aged brains than the pGSK3α/tGSK3α ratio ($p<0.05$, Figure 4.3a). As with female samples, PP2A expression did not change during ageing (Figure 4.4b).

4.4.1.3 Sex Disparities:

Sex disparities were identified in the levels of tau and GSK3α. Aged females showed a significant increase in t.tau (female mean=0.741±0.11, male mean=0.428±0.07) and pGSK3α (female mean=0.771±0.09, male mean=0.431±0.05) compared to the male samples ($p<0.05$, Figure 4.1b and 4.2b). However, no sex disparities were found for GSK3β (Figure 4.2d and 4.3a) or PP2A (Figure 4.4b).

4.4.2 Tau and GSK3 were altered in AD for both female and male subjects.

4.4.2.1 Female samples:

The level of t.tau was not altered in AD frontal cortex, however p.tau was detectable in AD samples but not in ND samples (Figure 4.1a), indicating an observational significant increase in Tau phosphorylation in AD. importantly, p.tau was detected as multiple bands ranging from 250 kDa to 37 kDa (Figure 4.1 a).

However, the alteration in the expression of tau regulatory enzymes was opposite to the p.tau data, as GSK3 significantly decreased in AD compared to ND subjects. This was seen for pGSK3β and the ratios of both pGSK3α/tGSK3α and pGSK3β/tGSK3β ($p<0.05$, Figures 4.2e and 4.3b) but there was no difference between the ratios for pGSK3α/tGSK3α and pGSK3β/tGSK3β (Figure 4.3b). Finally, PP2A expression did not change in AD compared to ND (Figure 4.4c).

4.4.2.2 Male samples:

In AD males, tau data had the same pattern seen in female samples with no alteration in the level of t.tau compared to ND subjects, but an observational significant increase in p.tau was seen with similar multiple bands in AD samples (Figure 4.1a). For GSK3, only pGSK3β along with its pGSK3β/tGSK3β ratio, had a significant increase in AD, while GSK3α and its pGSK3α/tGSK3α ratio were not altered ($p<0.05$, Figures 4.2c, 4.2e and 4.3b). However, the pGSK3β/tGSK3β ratio was significantly higher than the pGSK3α/tGSK3α ratio in the AD samples ($p<0.05$, Figure 4.3b). Finally, PP2A expression did not change in AD compared to ND (Figure 4.4c).

4.4.2.3 Sex Disparities:

While p.tau presented with multiple bands from 250 to 37 kDa, the band density was only carried out for the band at 50kDa, since the t.tau band was detected at 50kDa.

Tau expression was associated with sex disparities; both t.tau (female mean=4.128±0.71, male mean=1.775±0.52) and p.tau (female mean=2.86±0.67, male mean=0.761±0.24) were significantly higher in AD female brains then male ($p<0.05$, Figures 4.1c and 4.1d). However, the ratio of p.tau/t.tau was not different between males and females (Figure 4.1d). In contrast, male AD samples had significantly higher levels of both pGSK3α (female mean=0.313±0.12, male mean=0.594±0.16) and pGSK3β (female mean=0.35±0.09, male mean=1.02±0.17), ($p<0.05$ and $p <0.002$, Figures 4.2c and 4.2e, respectively). The other proteins, tGSK3α, tGSK3β, their phosphorylated/total ratios and PP2A, had similar expression levels in both sexes (Figures 4.2c, 4.2e, 4.3b and 4.4c).

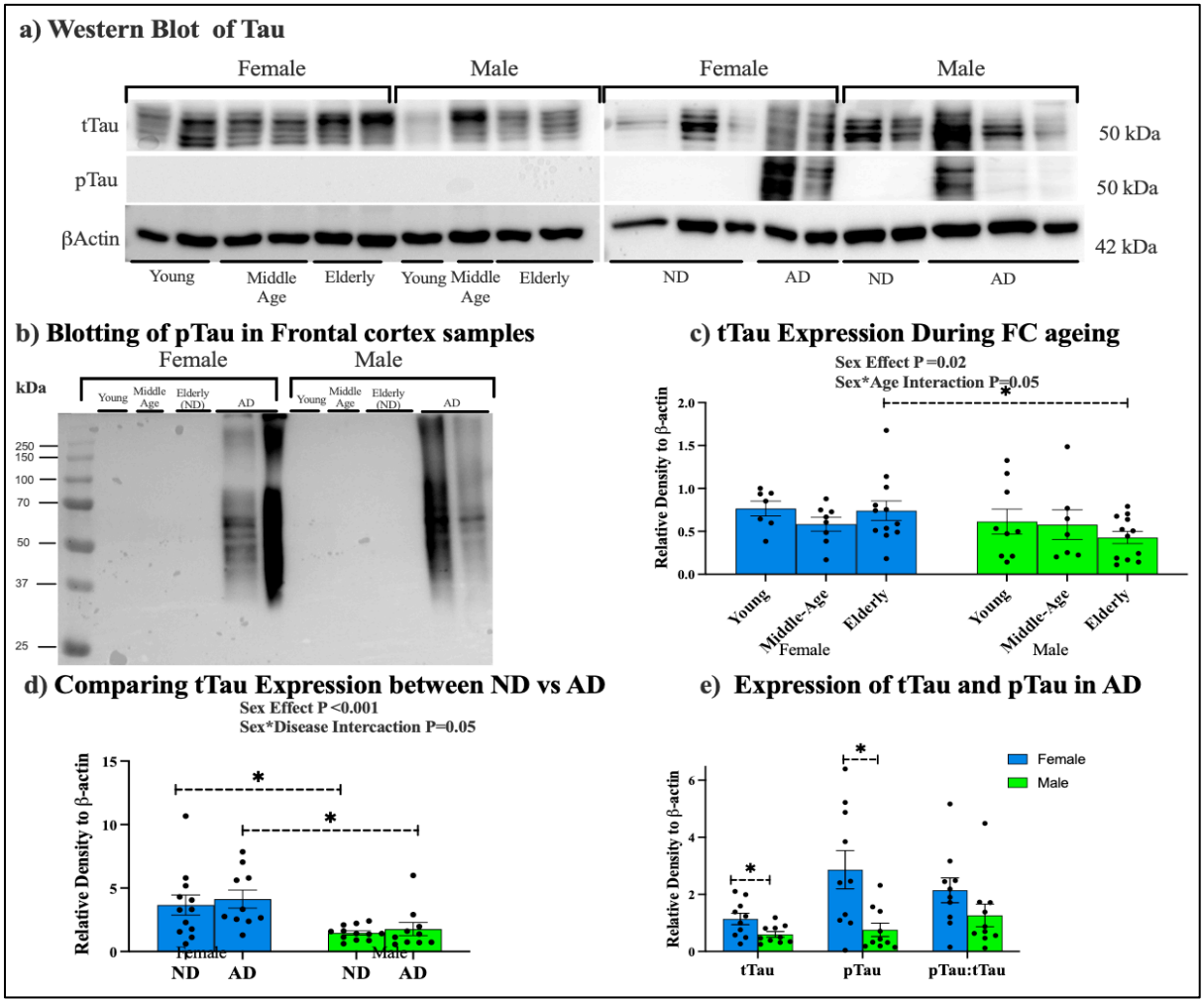


Figure 4.1 Comparison of the expression of tau in human female and male frontal cortex between different age groups of young (male n=9, female n=7), middle-aged (male n=7, female n=8) and elderly (male n=12, female n=12) subjects and between ND (male n=12, female n=11) and AD (male n=10, female n=10) subjects. (**a)** representative blotting data for t.tau and p.tau and the house keeping protein βactin. **(b)** Immunoblot for p.tau in ND and AD presenting a long, smeared band from 250kDa to 37 kDa in AD samples while no bands were detected in cognitive healthy samples. (**c)** Densitometric analysis of t.tau immunoblots during brain ageing, two-way ANOVA presented a significant higher level of t.tau in elderly female samples compared to elderly male samples (Sex*Age $F(2,49)=3.23$, $P=0.05$) and (Sex F $(1,49)=5.86$, $P=0.02$). (**d)** Densitometric analysis of t.tau immunoblots between ND vs AD, a significant higher level of t.tau was calculated in female samples compared to age-match male samples (Sex*Disease State $F(1,40)=3.86$, $P=0.05$) and (Sex F $(1,49)=56.49$, $P<0.001$). **(e)** Comparison between t.tau and p.tau expression in AD samples using two-tail student t-test, the standard to normalize samples run in multiple gels used here was an AD sample since p.tau was undetectable in ND brains. Data are presented as mean ± S.E.M. *$p<0.05$.

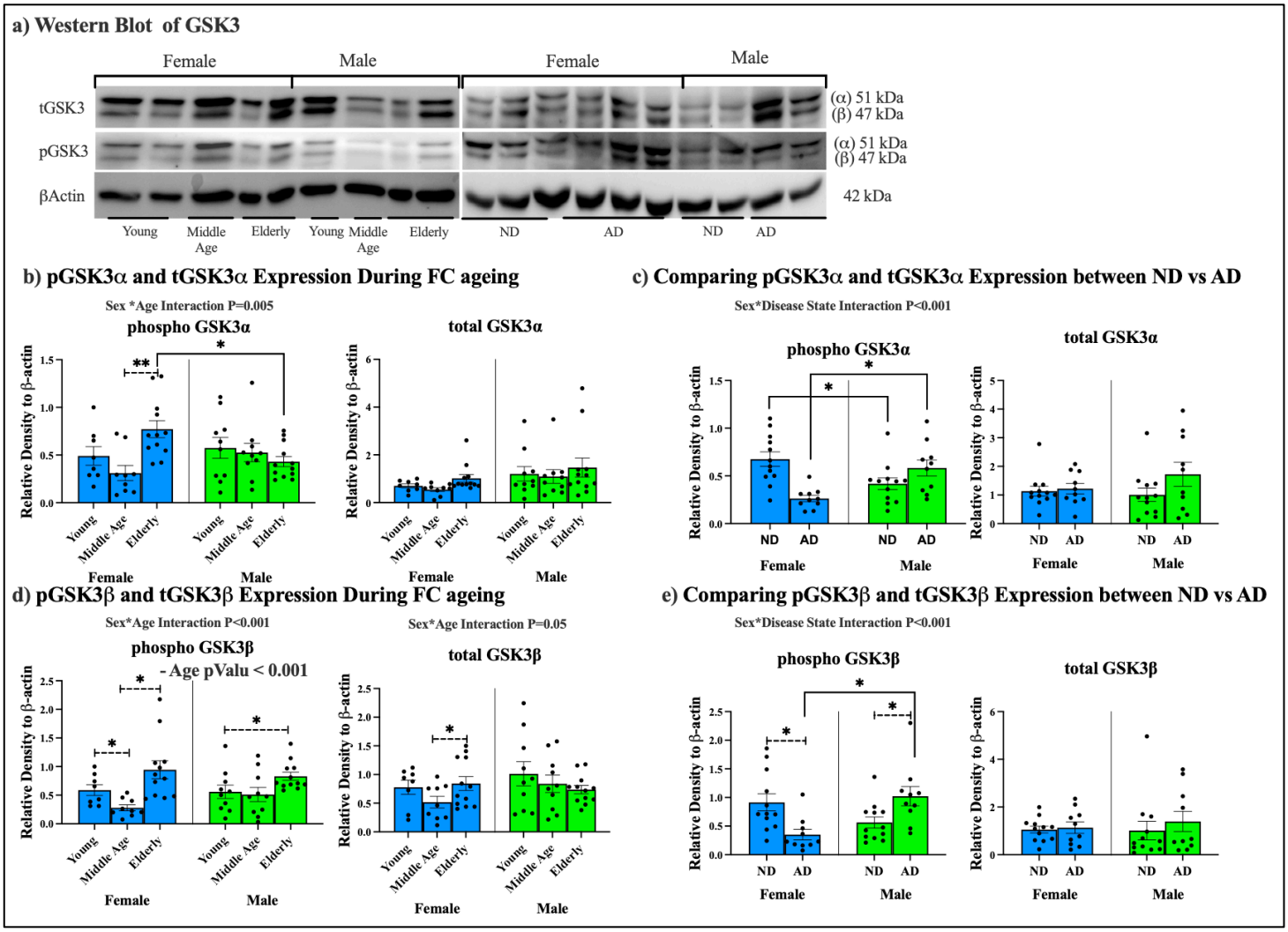


Figure 4.2 Comparison of the expression of GSK3 in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. (**a)** representative blotting data for tGSK3 and pGSK3 and the house keeping protein βactin. (**b&d)** Densitometric analysis of GSK3α and GSK3β immunoblots during brain ageing, with two-way ANOVA showing a significant increase in pGSK3α expression with age in female samples, along with higher level of it presenting in elderly female compared to elderly male samples (Sex*Age $F(2,55)=5.84$, $P=0.005$ for pGSK3α). While GSK3β showing an age related increase in its levels in both male and female samples (Sex*Age $F(2,55)=3.78$, $p=0.05$ and Sex*Age $F(1,55)=10.2$, $P<0.001$ for tGSK3β and pGSK3β respectively). **(c&e)** Densitometric analysis of GSK3α and GSK3β immunoblots between ND vs AD, with significant lower level of pGSK3α and pGSK3β presenting in AD female subjects compared to AD male. In addition, levels of pGSK3β decrease in AD female while in AD male samples it increases significantly (Sex*Disease State $F(1,40)=18.36$, $P<0.001$ and Sex*Disease State $F(1,40)=15.18$, $P<0.001$ for pGSK3α and pGSK3β respectively) .Data are presented as mean ± S.E.M. *$p<0.05$.

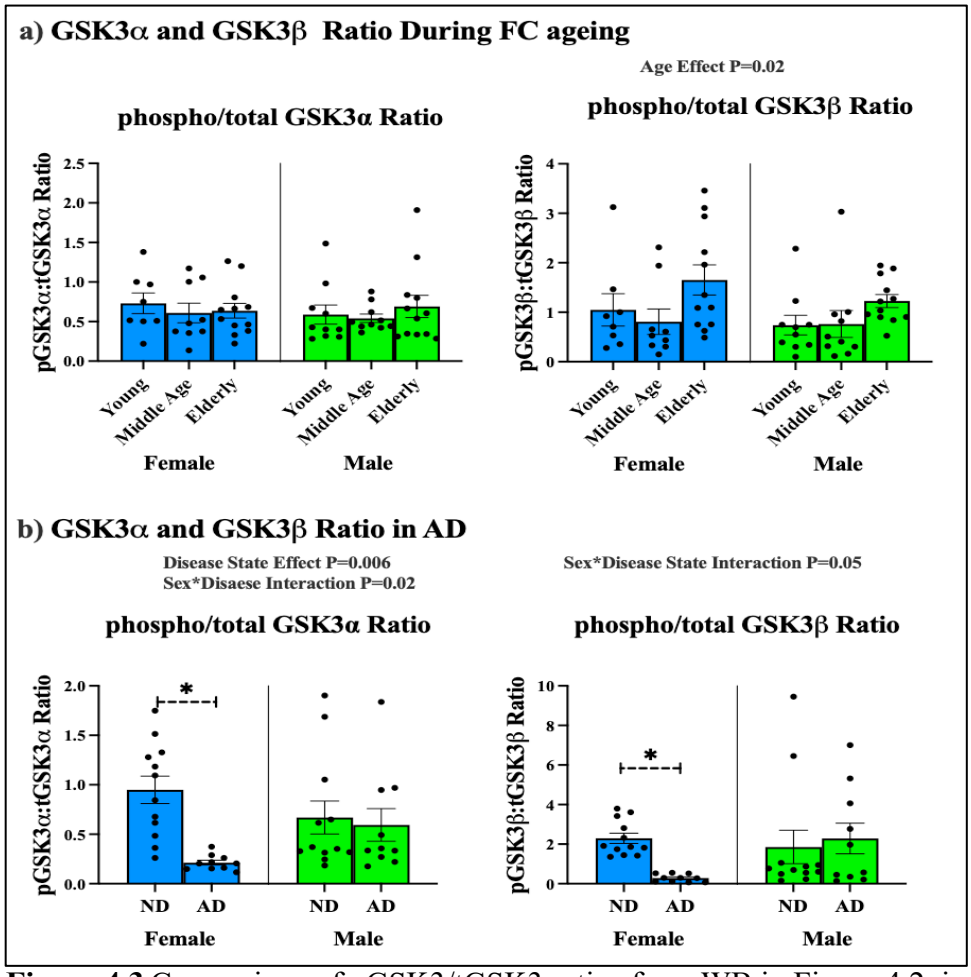


Figure 4.3 Comparison of pGSK3/tGSK3 ratios from WB in Figure 4.2, in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. **(a)** Densitometric analysis of pGSK3/tGSK3α= immunoblots during brain ageing, with two-way ANOVA revealing a significant age-related increase in the ratio of pGSK3β/tGSK3β in both male and female samples (Age $F(2, 55)=4.18$, $P=0.02$) **(b)** Densitometric analysis of pGSK3/tGSK3 immunoblots between ND vs AD, with a significant decline in the level of pGSK3/tGSK3 ratio in only AD female samples (Disease State $F(1,40)=8.27$, $P=0.006$ and Sex*Disease State $F(1,40)=5.503$, $P=0.02$ for pGSK3α/tGSK3α) and (Sex*Disease State $F(1,40)=4.12$, $P=0.05$ for pGSK3β/tGSK3β). Data are presented as mean ± S.E.M. *$p<0.05$.

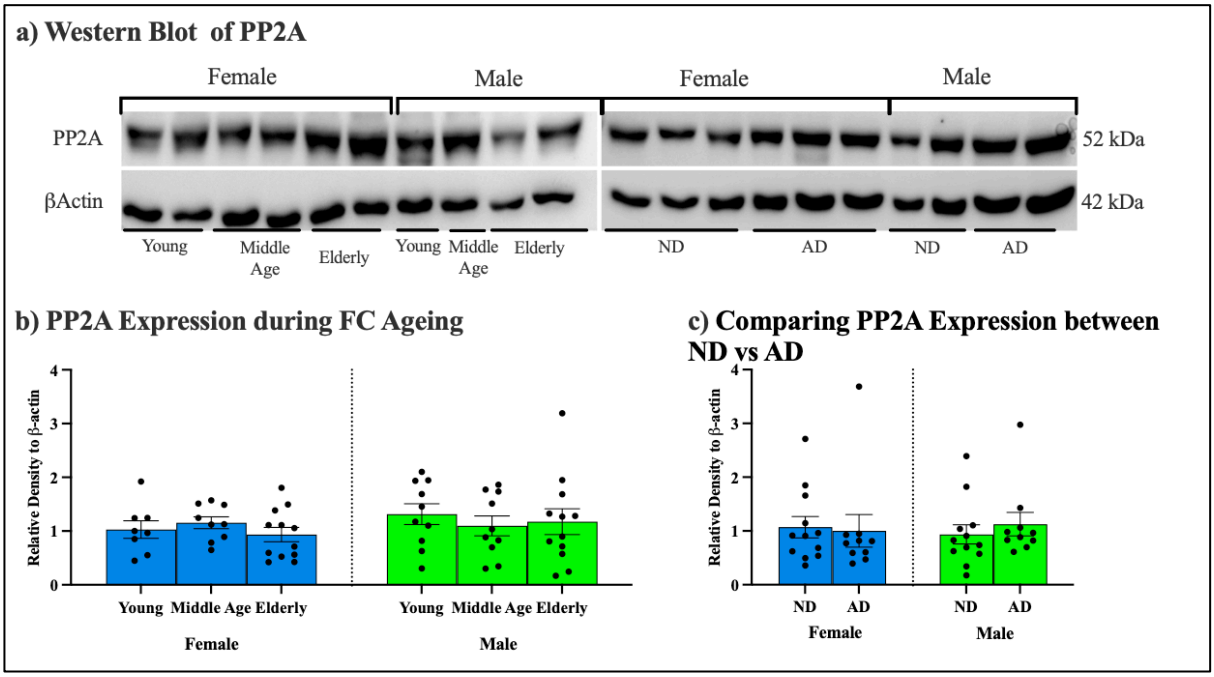


Figure 4.4 Comparison of the expression of PP2A in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. (**a)** representative blotting data for PP2A and the house keeping protein βactin, (**b&c)** Densitometric analysis of PP2A immunoblots during brain ageing and between ND vs AD, respectively. No significant effect of sex and age or sex and disease state on PP2A levels. Data are presented as mean ± S.E.M.

4.4.3 ProCaspase3 was not altered during brain ageing in both sexes and was upregulated in female AD brains.

The levels of ProCaspase3 were not altered with ageing in male or female brains (Figure 4.5b). However, in AD a significant increase compared to ND subjects was seen only in the female samples ($p<0.05$, Figure 4.5c). No sex disparities were identified in any group (Figure 4.5).

4.4.4 Bcl2 expression was associated with age-related increases in both sexes, while no alteration was identified in AD brains.

Bcl2 was associated with significant increases in expression in both male and female ageing brains ($p<0.05$ and $p<0.002$, Figure 4.6b). However, no changes were seen in AD brains compared to ND samples for males and females (Figure 4.6c) and there were also no sex disparities seen in ageing brains or in AD samples (Figure 4.6b and 4.6c).

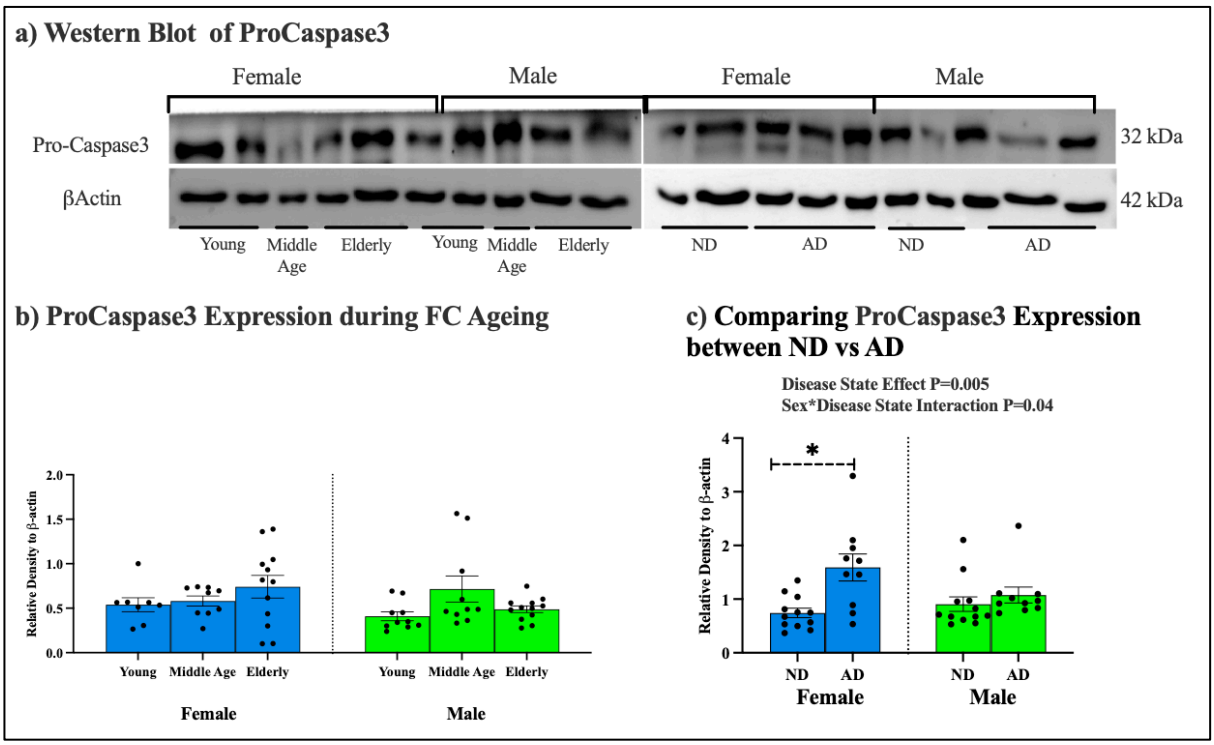


Figure 4.5 Comparison of the expression of ProCaspase3 in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. (**a)** representative blotting data for ProCaspase3 and the house keeping protein βactin. (**b)** Densitometric analysis of ProCaspase3 immunoblots during brain ageing, with two-way ANOVA revealing no significant effect of Sex nor Age on ProCaspase3 level(**c)** Densitometric analysis of ProCaspase3 immunoblots between ND vs AD, a significant higher level of ProCaspase3 was calculated in AD female samples (Disease State $F(1,40)=10.21$, $P=0.005$ and Sex*Disease Interaction $F(1,40)=4.5$, $P=0.04$). Data are presented as mean ± S.E.M. *$p<0.05$.

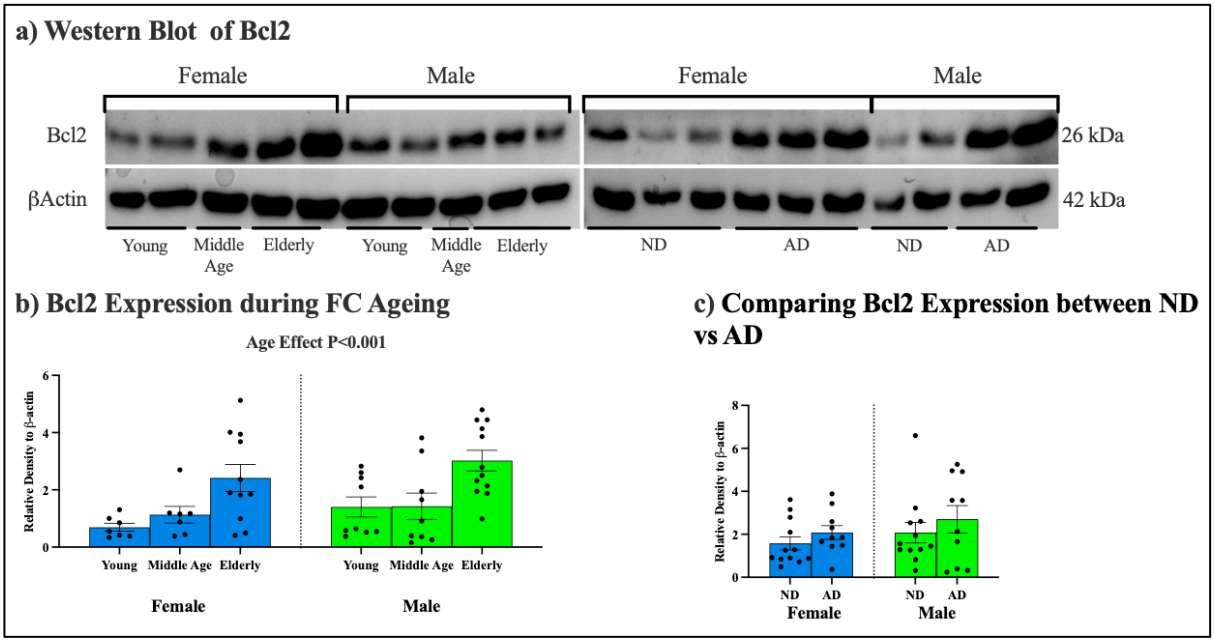


Figure 4.6 Comparison of the expression of Bcl2 in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. (**a)** representative blotting data for Bcl2 and the house keeping protein βactin. (**b)** Densitometric analysis of Bcl2 immunoblots during brain ageing, with two-way ANOVA revealing an age-related increases in the expression of Bcl2 in both male and female samples (Age $F(2,49)=11.34$, $P<0.001$). (**c)** Densitometric analysis of Bcl2 immunoblots between ND vs AD, with two-way ANOVA revealing no significant effect of Sex nor Disease State on Bcl2 level. Data are presented as mean ± S.E.M. *$p<0.05$, **$p<0.002$.

4.5 Discussion

Despite the lack of any tau hyperphosphorylation in the elderly brains of both sexes, the increase in GSK3β phosphorylation, phosphorylated forms are the active form of the target protein, could be a precursor for NFT development. In addition, Bcl2 upregulation with advanced brain ageing could overcome the synaptic damage of tau hyperphosphorylation. Nonetheless, AD was associated with high levels of tau hyperphosphorylation and Caspase3, the former agreeing with a previous reported study (Alsaqati et al., 2018).

4.5.1 Tau and its regulatory enzymes, GSK-3 and PP2A, during ageing and in AD

4.5.1.1 Age as a risk factor

Abnormal tau hyperphosphorylation is widespread in AD pathogenesis (Citron, 2004). When phosphorylated tau was investigated here in the brain samples, it was undetectable in the ageing samples despite using a high concentration (1:10) of a well-characterized, anti-phosphorylated tau antibody (PHF-1) (Figure 4.1 b) (Ide et al., 2016). Post mortem interval is reported to rapidly alter tau phosphorylation, 50% of tau was dephosphorylated in the mouse brain within 40sec of death (Wang et al., 2015). Brain samples used in this project had long PMIs which could cause dephosphorylation of tau and this might be the reason of it being undetectable here. However, the most likely explanation is that a very low level of phosphorylated tau is present in healthy human brains (Alsaqati et al., 2018, Hefti et al., 2019). Also, several clinical studies have reported the same results for tau phosphorylated at Ser396/Ser404 and detected with PHF-1, where the protein was undetectable in healthy adult brain, while it was expressed during infant development and highly expressed in AD brains (Alsaqati et al., 2018, Hefti et al., 2019, Goedert et al., 1993). Aβ levels were very high in the elderly individuals compared to the other age groups (see results in Chapter 3) in parallel with "the amyloid cascade hypothesis" where Aβ accumulation and deposition develop before tau tangle pathology (Perrin et al., 2009). Thus, since none of the ND elderly subjects used here were diagnosed with AD before death, it is unlikely that they would have developed NFTs. Nonetheless, the observation that memory and synaptic alterations are not mediated by NFT formation, and that insoluble tau deposition might be a compensatory neuroprotective mechanism, suggests that soluble oligomeric molecules of tau might be the key player in

synaptic failure, resembling the "oligomeric hypothesis" (Ward et al., 2012) described previously in Chapter 1 for Aβ.

Table 4. 2 Summary of Chapter 4 age-related findings.

	tau			GSK3									
	t.tau	p.tau	p.tau/t.tau	tGSK3α	pGSK3α	pGSK3α/tGSK3α Ratio	tGSK3β	pGSK3β	pGSK3β/tGSK3β Ratio	GSK3α Ratio vs GSK3β Ratio	PP2A	ProCaspase3	Bcl2
During Frontal Cortex Ageing													
Female	-	n/d	n/d	-	-	-	↑	↓ (m)	↑	↓ (e)	-	-	↑
Male	-	n/d	n/d	-	-	-	-	↑	↑	↓ (e)	-	-	↑
ND vs AD													
Female	-	↑	↑	-	-	↓	-	↓	↓	-	-	↑	-
Male	-	↑	↑	-	-	-	-	↑	-	↓ (AD)	-	-	-

m = middle-Age. e = Elderly. AD= Alzheimer Disease. n/d = non detectable

Table 4. 3 Summary of Chapter 4 sex-related findings.

	Age Groups	tau			GSK3								
		t.tau	p.tau	p.tau/t.tau	tGSK3α	pGSK3α	pGSK3α/tGSK3α Ratio	tGSK3β	pGSK3β	pGSKβ/tGSK3β Ratio	PP2A	ProCaspase3	Bcl2
Female compared to Male	***Young***	-	n/d	n/d	-	-	-	-	-	-	-	-	-
	Middle-age	-	n/d	n/d	-	-	-	-	-	-	-	-	-
	Old age	↑	n/d	n/d	-	↑	-	-	-	-	-	-	-
	AD	↑	↑	-	-	↓	-	-	↓	↓	-	-	-

n/d = non detectable

GSK3 plays an important role in AD pathogenesis (Hooper et al., 2008) since it is reported to be capable of phosphorylating tau at multiple residues, about 42 , of which 29 are linked to AD (Jiang et al., 2015b, Martin et al., 2013b). Interestingly, for both sexes, a significant increase in the level of the active form of GSK3β, phosphorylated GSK3β ($Tyr_{279/216}$), was detected in the aged groups compared to the other groups, whereas the ratio of active to total GSK3α were not altered with brain ageing, consistent with earlier studies (Alsaqati et al., 2018, Lee et al., 2006). This finding supports the different functions proposed for these two isoforms. The increase in GSK3β activity could be a precursor for NFT development. An *in vivo* study reported the capability of $A\beta_{1-42}$ to activate GSK3β (Kirouac et al., 2017), and, since $A\beta_{1-42}$ levels were higher in elderly subjects in both sexes (Chapter 3), this could contribute to the higher levels of possible GSK3β activity seen here. Thus, it might be expected that more PP2A, an important tau phosphatase (Liu et al., 2005), would be required to regulate GSK3 activity. However, when the levels of PP2A was measured, no age-related alterations were found in either male or female frontal cortex. These results suggest a sustained tau dephosphorylation during brain ageing although it is possible that other enzymes may also dephosphorylate tau, e.g. PP1, PP5, and PP2B (Martin et al., 2013a, Sandra et al., 2014, Liu et al., 2005). It is also possible that PP2A is present in excess of the amount required normally so no increase in its levels is required to cope with extra phosphorylation of tau. In addition, tau phosphorylation is also regulated by other kinase enzymes, such as mitogen-activated protein kinase (MAPK), Erk1/2, p38, JNK, and Cyclin-dependent kinase 5 (cdk-5). Also, tau is one of the most complicated phosphorylated proteins. This protein consist of 85 possible phosphorylation sites; 5 tyrosine, 35 threonine, and 45 serine residues (Kimura et al., 2018). Thus, in future lab work, it would be useful to investigate other tau phosphatase and kinase enzymes along with other common phosphorylated sites of tau to understand more about its activity status in the brain during advancing age.

4.5.1.2 Sex as a risk factor

Alzheimer's disease is more common in females than in males (Chêne et al., 2015), neuropathological studies did indicated that females AD had higher scores in CERAD and Braak scores (Hohman et al., 2018) indicating female might had higher levels of Aβ and tau in their brains at death. In this chapter, t.tau and pGSK3α expression were dramatically higher in the aged female group than the males. The increase in levels of tau granular oligomers starts prior to NFT formation and before the manifestation of clinical symptoms of AD (Braak-stage

I), suggesting that the increase in tau oligomer levels may illustrate a very early sign of brain aging and AD development (Maeda et al., 2006). In *in vivo* studies, tau oligomer levels were reported to: correlate with cognitive deficits (Berger et al., 2007), impair synaptic and memory function (Lasagna-Reeves et al., 2011) and accelerate the pathology of AD (Gerson et al., 2016). Thus, the higher level of t.tau in elderly female subjects could be due to granular oligomers involved in early AD development. In addition, GSK3α was reported to stimulate APP metabolism through the amyloidogenic pathway, promoting Aβ production and promoting cell death (Phiel et al., 2003). Also, there is a high direct affinity between GSK3α and $A\beta_{1-42}$ resulting in the stimulation of tau hyperphosphorylation (Dunning et al., 2016). While p.tau was undetectable in aged brains, the high levels of t.tau, pGSK-3α and $A\beta_{1-42}$ seen in the female samples could highlight more crucial, and possibly earlier, tau hyperphosphorylation in their aged brains than their male peers.

Other sex disparities were reported above in GSK3β. GSK3β has the ability to modify the function and cellular localization of PS1 (Uemura et al., 2007). Even though both sexes were associated with upregulation in pGSK3β, its cellular function might be more critical in female brains. In Chapter 3, PS1 was shown to have an age-related increase in female samples along with a significantly higher level in their aged brains compared to males. Thus, an increase in pGSK3β could result in significantly more PS1 activation, a suggestion supported by the Aβ overproduction reported in aged female brains in Chapter 3.

In AD, phosphorylated tau was detectable unlike the ND subjects mentioned above, thus it is clearly much higher in AD (Kopke et al., 1993). As mentioned previously, significant tau hyperphosphorylation has been connected to protein misfolding and NFT development (Smith, 2002). The higher levels of phosphorylated and total tau in AD females reported here were highlighted by Buckley et al. (Buckley et al., 2020) where they found through PET imaging that different brain regions of females with MCI were highly enriched with t.tau compared to age-matched male patients.

Surprisingly, in female samples, the ratios of pGSK3α and pGSK3β to their total forms were lower in AD samples compared to age-matched ND female controls. In contrast, male AD subjects showed an increase in pGSK3β expression compared to ND subjects, as expected from previous studies (Perrin et al., 2009, Martiskainen et al., 2015). Furthermore, male brains had higher levels of pGSK3α and pGSK3β than female AD brains. The female finding is

surprising, since some clinical studies reported that more tau accumulates in female brains (Altmann et al., 2014, Liu et al., 2019a), and here tau hyperphosphorylation was clearly apparent in female AD brains. Also, increased Aβ in AD was reported with an increase in GSK3β activity (Kirouac et al., 2017). Thus, the reason for the reduction in the GSK3α and GSK3β ratios in female AD samples is unclear. It is important to highlight that the tau isoforms measured here were the soluble fragments as the insoluble NFTs were not extracted. Wang et.al. showed that the extraction of insoluble NFTs from brain tissue was associated with oestrogen receptor attachment (Wang et al., 2016a). This finding could apply to GSK3 as it may be that not all the GSK3 was extracted with the soluble fragments, some might still be attached to the insoluble NFTs.

4.1.3 Bcl2 and ProCaspase3 during brain ageing and in AD

4.1.3.1 Age as a risk factor

While Aβ and tau hyperphosphorylation are reported to interfere with synaptic function and cause cognitive impairments, it is not clear if these disease markers seen in the female samples could really cause preclinical AD environment. Apoptosis is one mechanism which is responsible for significant neuronal cell loss. Thus, two apoptotic regulatory proteins were investigated here, Bcl2 and Casapse3. In an *in vivo* experiment using rats, an increase in Bcl2 and a decrease in Caspase3 were corelated with an improvement in cognitive function (Zhang et al., 2018b).

Age-related increased expression of Bcl2 was observed in both sexes, in parallel with other findings (Jarskog and Gilmore, 2000). In view of its anti-apoptotic characteristics (Ferrer et al., 1994, Merry et al., 1994, Shimohama et al., 1998, Vyas et al., 1997), a neuroprotective function for Bcl2 has been postulated. Castré et al have hypothesised that abundant quantities of Bcl2 mRNA are a requisite for post-mitotic neuron cell longevity (Castre et al., 1994). The upregulation seen here may therefore reflect a compensatory reaction to neurodegeneration. Normal ageing has been linked in a comparable manner with free radical-generated oxidative injury, as determined by raised oxidised protein levels, lipofuscin, DNA damage and glycosylated proteins (Mecocci et al., 1997, Mrak et al., 1997). Long-term oxidative stress may precipitate an increase in the cortical Bcl2 protein concentration during ageing to preserve neuron cell integrity.

For Caspase3, the antibody used in this Chapter was a cocktail mAb against apoptotic biomarkers (ProCaspase3, PARP and the cleaved form of Capsase3 (p17-caspase3)). However, only the inactive ProCaspase3 band was identified in the brain samples, similarly to previous reported in monkey brains *in vivo* (Zhang et al., 2006), thus it was analysed here. Preclinical studies reported no alteration of caspase3 with ageing in three brain regions of the monkey; cerebellum, motor cortex and hippocampus (Zhang et al., 2006), in parallel to what was observed here in this Chapter. Here, no age-related alteration in ProCaspase3 was seen in either sex. *In vivo* studies showed an increase in Bcl2 and reduction of Casapse3 in mouse brains containing Aβ, suggesting that Aβ deposition could be the stimulus for higher Bcl2 expression (Zhang et al., 2018b, Chen et al., 2013). In addition, Bcl2 was shown to have a negative relationship with tau hyperphosphorylation (Chen et al., 2005). In Chapter 3 and here, an increase in Aβ along with increased expression of pGSK3β during ageing was observed in both sexes, which could explain the increased expression of Bcl2 seen here in the elderly subjects, even if ProCaspase3 expression was not altered.

4.1.3.2 Sex as a risk factor

No sex disparity was seen in Bcl2 and ProCaspase3 levels during frontal cortex ageing. The next part will focus on the AD results.

In AD, neuronal impairment antecedent to cell death is associated with histopathological and biochemical abnormalities ~ Aβ processing (LeBlanc, 1994). Nevertheless, there is a dearth of information regarding the mechanisms that underpin neuronal death in neurodegenerative conditions; apoptosis, or programmed cell death (Kerr et al., 1972). A major controlling factor in the cerebral apoptotic process is the protooncogene product, Bcl2 (Hengartner and Horvitz, 1994a, LeBrun et al., 1993). Here in this Chapter, the expression of Bcl2 was unchanged in AD for both sexes inferring that, although there is a reduced level of a number of cellular activities, there is ongoing production of the Bcl2 protein which may assist in neuronal preservation (Vyas et al., 1997). In contrast, ProCaspase3 was significantly increased in female AD samples compared to ND subjects and there was also an increase, although not significant, in male AD samples compared to ND subjects. This finding was reported previously, where Caspase 3 activity was enriched at the synapse, particularly the postsynaptic density, of the cortex and hippocampus in AD samples (Louneva et al., 2008). Thus, while Bcl2 might sustain an anti-apoptotic function in AD, caspase3 probably

participates in crucial effects on neuronal loss in the brain in AD through apoptotic cellular damage activity. Also, Caspase3 was reported to be associated with non-apoptotic cellular damage in AD. For instance, it was reported to cleave APP and stimulate Aβ production and induce synapse loss (Park et al., 2020). Also, it can stimulate tau hyperphosphorylation via GSK3β (Chu et al., 2017). Thus, since female AD samples possessed significantly higher levels of Aβ (Chapter 3) and phosphorylated tau in their frontal cortex compared to age-matched AD males, the significant increase in ProCaspase3 might indicate a potentiation of neurodegenerative processes in female AD brains.

4.6 Conclusion

To summarise, both sexes were associated with age-related upregulations in pGSK3β and Bcl2, which could translate as a neuroprotective effect of Bcl2 during the disturbance of cellular processes associated with the possible tau phosphorylation by pGSK3β. Furthermore, the higher levels of tau in aged female frontal cortex could highlighted an important negative effect on pathogenic processes in their brains compared to age-matched male samples. This theory was also seen in AD where female subjects possessed higher level of ProCaspase3, which might indicate crucial increased apoptotic and non-apoptotic cellular damage activity.

Thus, an obvious question arising from the findings so far is why do women have more amyloid deposition and tau than men? Hormonal changes in brain ageing are one possible explanation for these results. Reproductive ageing in females presents a serious, yet understudied, factor which may be essential for understanding the early processes during brain ageing that participate in cognitive decline and dementia risk. In fact, *in vivo* studies indicate that steroid hormones including oestradiol, progesterone and testosterone play a significant role in supporting brain function (Jacobs and Goldstein, 2018, Taylor et al., 2019). Female subjects, in this study, showed a significant increase in APP levels with age and also had significant increases in PS1 & PS2 with age, reported in Chapter 3. In addition, they had higher levels of tau and pGSK3α in their aged brains, reported in this Chapter. It could be that both APP processing and tau phosphorylation are under careful regulation until females hit the menopause transition phase when this regulation is lost and levels then increase. The next Chapter will focus on investigating the expression of reproductive hormone-associated proteins during ageing and in AD in all the age groups to determine the relevance of reproductive hormone on these processes.

CHAPTER 5 _ Exploring the Level of Oestrogen Receptors (ER), Progesterone Receptor (PR), G-Protein ER (GPER), and ER Downstream Signalling (MAPK and Akt) in Female and Male Frontal Cortex during Ageing and in AD

5.1 Introduction

Oestrogens (E2), predominantly as 17β-oestradiol, are members of the neuroactive steroid hormone class and are associated with improving memory and cognitive ability (Hojo et al., 2008, Wharton et al., 2011); cell longevity and plasticity are reliant on E2 signalling (Frick et al., 2018). Numerous studies have described the role of E2 as enabling the development of synapses, defending against oxidative stress and governing neuromodulators, e.g. serotonin, noradrenaline, dopamine and acetylcholine (Galvin and Ninan, 2014, Almey et al., 2015, Hara et al., 2015, Rossetti et al., 2016). In advancing age, the cognitive-promoting influence of E2 is less effective, a phenomenon likely to be associated with age-related changes in ER expression and signalling (Foster, 2012). Ovarian hormone reduction diminishes prefrontal cortex (PFC) neuron spine density (Hao et al., 2006), and causes working memory ability to deteriorate (Rapp et al., 2003a). In the murine hippocampus, CA1 neuron dendritic spine density differs throughout the oestrous phases (Woolley et al., 1990, Woolley and McEwen, 1993) as does the sex-hormone-governed hippocampal volume (Galea et al., 1999, Qiu et al., 2013). These empirical observations indicate that E2 concentration changes cause structural and operational alternations in the brain regions responsible for memory. It is therefore likely that female menopausal E2 reduction is closely associated with AD (Barnes et al., 2005). The protective influence of reproductive hormones in relation to AD has been described in Chapter 1 (Section 1.10). To summarise, the neuroprotective impact of E2 on AD development arises through the reduction of Aβ (Xu et al., 1998), synapse plasticity enhancement, neurotrophic constituent preservation, cerebral inflammation suppression (Pompili et al., 2012, Correia et al., 2010), and reduction in tau hyperphosphorylation (Lee et al., 2014, Goodenough et al., 2005, Zhang et al., 2008).

It is well known that the brain acts as an extra-gonadal site for E2 synthesis (Do Rego et al., 2009), supported by the presence of all the enzymes responsible for E2 synthesis, P450scc and aromatase (Azcoitia et al., 2011, Naftolin et al., 1971, Do Rego et al., 2009). Aromatase is expressed in neurones and astrocyte indicating a specific role for E2 synthesis in these cells (Gatson et al., 2011, Honda et al., 2011). In addition to synthesising its own E2, the brain utilises circulating E2 to provide an essential supply to the brain (Kancheva et al., 2011). The sudden and major loss of reproductive hormones in females which occurs during the menopause suggests a loss of their neuroprotective effects, thus increasing female susceptibility to develop AD earlier than males. While no changes in E2 levels were observed

in AD brain (Twist et al., 2000), to date no one has looked at sex disparities of brain steroid hormones during human ageing, especially during the menopause transition phase, and in AD. Assaying the levels of brain reproductive steroids is fraught with several practical difficulties. These difficulties include: relatively large cerebral lipid volumes, which necessitate careful specimen preparation in order to prevent interference on assays (Rash et al., 1980); extensive cerebral tissue spatial inconsistencies, which give rise to tiny sample quantities (Charlier et al., 2010); occasional low steroid titres (Gomez-Sanchez et al., 2005); ease of transmembrane steroid diffusion owing to their hydrophobic properties (Toran-Allerand et al., 2005); and swift changes in steroid manufacture and metabolism (Balthazart and Ball, 2006). With respect to the latter, E2 activity can be explored via its downstream target proteins. Nuclear receptors, i.e. ERα and ERβ (Perlmann and Evans, 1997, Katzenellenbogen, 1996, Hirahara et al., 2013), together with the de novo recognised G protein-bound oestrogen receptor-1 (GPER1 or GPER30), mediate the activity of E2 (Filardo et al., 2000, Kanda and Watanabe, 2004).

Both ERα and ERβ are widely distributed throughout the brain and exhibit variations in tissue expression (Pérez et al., 2003, Kruijver et al., 2003). The greatest ERα expression occurs in the hypothalamus, forebrain nuclei and amygdala, whereas ERβ expression is higher in the hippocampus and neocortex. Furthermore, ERα exhibits agonist properties, but ERβ may behave as an antagonist of E2-mediated responses in certain cases (Paech et al., 1997). ERβ demonstrates a lower efficacy than ERα for the induction of transcription associated with the hormone response element (McInerney et al., 1998, Cowley and Parker, 1999, Yi et al., 2002). Chronic ovariectomy is characterised by a notable diminution of ERα in the murine hippocampus; with no effect on ERβ levels (Zhang et al., 2011a, Zhang et al., 2009, Qu et al., 2013). The two receptors govern gene transcription via traditional genomic cascades (Prossnitz and Barton, 2009, Prossnitz and Barton, 2014, Schultz-Norton et al., 2011) or by influencing non-genome-related cellular signalling pathways, e.g. MAPK and ERK (Wade et al., 2001) (Figure 5.1).

GPER1 are extensively enriched in forebrain neuronal and non-neuronal cells, i.e. in the cortex, hypothalamus, hippocampus, hypothalamic-pituitary axis and striatum (Brailoiu et al., 2007, Hammond and Gibbs, 2011, Hazell et al., 2009). Furthermore, GPER1 mRNA levels are more concentrated in the hippocampus and frontal cortex compared with the septum and striatum (Hammond et al., 2011). GPER1 activities stimulated by E2 can arise through-

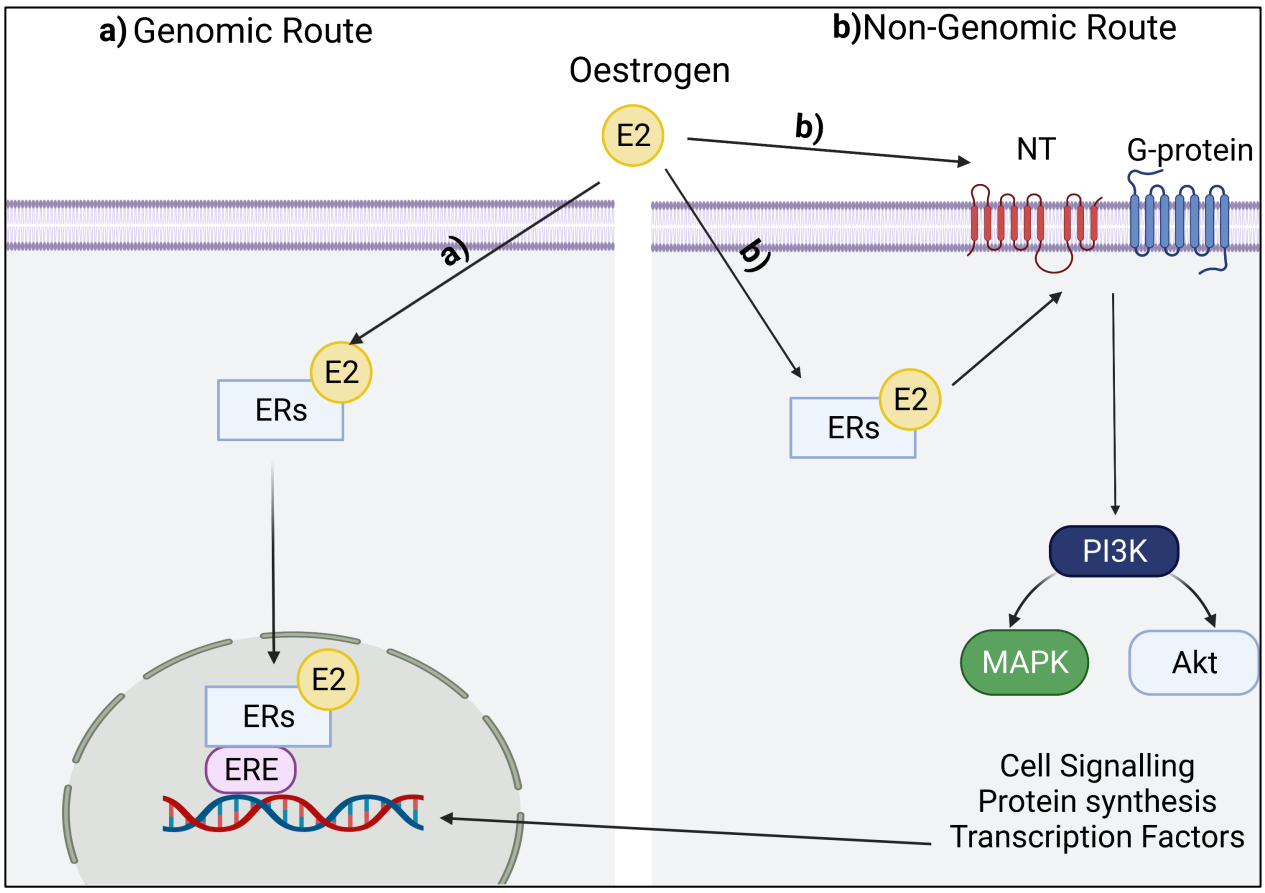


Figure 5.1 Scheme illustrating the mechanism of 17β-oestradiol in modulating cellular functions. a) the genomic/classical pathway, where oestradiol (E2) diffuses through the cell membrane and binds to ERs either in the cytoplasm or the nucleus. The ER-oestradiol complex is then bound to the oestrogen response element (ERE) on the DNA, leading to stimulation of gene transcription. **b)** In the non-genomic/non-classical pathway, E2 activates cell receptors (growth factor (GF)/G-protein-coupled receptors) and/or L-glutamate receptors (neurotransmitter (NT)) by either directly binding to them or by indirect activation through ERs. For the latter indirect activation of cell surface receptors, E2 activates ERs causing it to translocate to the cell membrane and activate G-protein and/or NT receptors causing stimulation of cell signalling cascades. The cell signalling cascades have several cytoplasmic and nuclear functions such as protein synthesis and modification of post-translation proteins in the cytoplasm in addition to stimulation of an epigenetic process and transcription factors through modulating gene transcription in the nucleus.

-genomic and non-genomic cascades, the latter via the MAPK/ERK pathway (Filardo et al., 2000, Kanda and Watanabe, 2004). GPER1 expression occurs in the plasma membrane (Filardo et al., 2000, Funakoshi et al., 2006, Hammond et al., 2011, Almey et al., 2012), and in the cytoplasm, especially in intracellular compartment membranes, e.g. the endoplasmic reticulum (Matsuda et al., 2008, Otto et al., 2008, Revankar et al., 2005) and Golgi apparatus (Matsuda et al., 2008). Stimulation of GPER1 within the brain promotes cognitive functions, e.g. learning and memory (Alexander et al., 2017). The neuroprotection arising via GPER1-selective stimulation involves a wide range of pathways which vary from apoptosis inhibition, triggering of neurotrophic factor expression, ion channel modification, suppression of neuroinflammation, governance of gliosis, preservation of BBB and blood vessel function (Brailoiu et al., 2007, Dun et al., 2009, Hazell et al., 2009).

Progesterone, another steroid hormone, is manufactured in females within the ovaries and placenta, and in both sexes, in the adrenal glands. It is a small lipid-soluble molecule, and so serum progesterone is effortlessly able to traverse the BBB via free transmembrane transport and diffuse into cerebral tissues (Pardridge, 2012, Pardridge and Mietus, 1979, Witt and Sandoval, 2014, Banks, 2012). Progesterone is a neurosteroid, which can be manufactured in situ within the brain by non-neurones and neurones (Baulieu and Robel, 1990, Mensah-Nyagan et al., 1999, Schumacher et al., 2014, Baulieu et al., 2001, Mellon and Vaudry, 2001). Thus, the cerebral progesterone reservoir relies on its peripheral production, cerebral absorption, and its accretion along with local manufacture and breakdown. The brain expression of the enzymes implicated in progesterone production and breakdown is well-documented (Mensah-Nyagan et al., 1999, Compagnone and Mellon, 2000, Mellon and Griffin, 2002, Schumacher et al., 2014). Progesterone exhibits a neuroprotective influence with respect to AD (see Chapter 1, Section 1.11). In summary, physiologically relevant concentrations ameliorate oxidative damage (Kaur et al., 2007, Nilsen and Brinton, 2002) and hypoglycaemia-induced toxicity (Goodman et al., 1996), and also protect against Aβ-triggered toxicity in primary hippocampal cultures (Goodman et al., 1996). Progesterone acts via its receptors, PR-A and PR-B (Schumacher et al., 2014, Guennoun et al., 2015, Brinton et al., 2008, Singh et al., 2013, González et al., 2020), which, as with E2 receptors, exert their effects via control of gene transcription and stimulating extranuclear signalling pathways, e.g. Akt and MAPK (Hagan et al., 2012, Singh, 2001). PR-A has a negative influence on transcription arising through PRs and ERs (Vegeto et al., 1993). the latter phenomenon may to some extent be responsible for the way in which progestins oppose the effects of E2 at a functional level (Bikle et al., 1992).

5.2 Hypothesis and Aims

Hypothesis: female frontal cortex samples will show a decline in ERs, GPER and PR expression during brain ageing, parallel to the serum E2 age-related diminished levels. This might partially be the cause of females being associated with a higher Aβ burden and tau hyperphosphorylation in aged brains, as observed in Chapters 3 & 4.

The aim of this chapter is to investigate the expression of reproductive steroid-associated proteins in the ageing human brain and in AD along with looking for any sex disparities. Thus, the overall aims of this chapter are:

1) Determine the distribution of ERs, GPER1, PR and their downstream signalling targets (MAPK, Akt) in healthy brain samples from people aged 20 to 90 years and in ND vs AD age-matched samples.
2) Investigate sex disparities in the expression of reproductive steroid-associated proteins in each age group and in AD.

5.3 Experimental Methods

All the methods used in this Chapter have previously been detailed in Chapter 2.

5.3.1 Samples

Samples used in this chapter were prepared and grouped as described in Chapters 2 & 3.

Table 5.1 Expected WB band molecular masses and the phosphorylated residues for all proteins examined in Chapter 5.

Proteins	Phosphorylation Site	Expected Size (kDa)	References
Total ERα (tERα)	-	60	(Ma et al., 2020)
Phospho ERα (pERα)	Ser118	60	
Total ERβ (tERβ)	-	59	(Lin et al., 2020)
Phospho ERβ (pERβ)	Ser105	59	(Hamilton-Burke et al., 2010)
PR	-	90	(Zhang et al., 2018a)
GPER1	-	42	(Lin et al., 2020)
Total Akt (tAkt)	-	60	(Colin et al., 2005)
Phospho Akt (pAkt)	Ser473	60	(Hou et al., 2022)
Total MAPK (tMAPK)	-	ERK1=42 ERK2=40	(Wang et al., 2021)
Phospho MAPK (pMAPK)	ERK1=Thr202 ERK2=Tyr204	ERK1=42 ERK2=40	(Yu et al., 2021)

The number of samples used in this chapter was, for female: young group (n=8), middle-aged group (n=9), elderly group (n=12) and AD group (n=10), and for male: young group (n= 10), middle-aged group (n=10), elderly group (n= 12) and AD group (n= 10). Western blotting (WB) was used to explore total and phosphorylated ERα, Erβ, MAPK and Akt expression and the total expression of GPER1 and Progesterone (refer to Table 5.1 for the molecular masses of bands and phosphorylation sites). To explore the impact of ageing on protein expression with WB, male and female samples were run separately, while for investigating the impact of sex, both age groups were run together on the same gel.

The expression of proteins was analysed across more than one gel. Therefore, all protein bands were expressed as the relative density of a standard human brain sample, a young female sample for exploring proteins levels during ageing and for the ND vs AD experiments. Also, for measuring the band intensity of ERK1 & ERK2 for MAPK, images were enlarged to 75% to aid in selecting bands of interest without overlapping between ERK1 and ERK2.

5.3.2 Statistical analysis

WB data were normalized then analyzed as described in Chapter 2, Section 2.9.2. All data in this chapter were parametric and were analysed using a factorial two-way ANOVA to compare the main effects of age and biological sex (IV), and the disease state and biological sex (IV), as well as the interaction effects of those factors against each other [Sex*Age or Sex*Disease state Interaction]. If the lateral interaction [Sex*Age or Sex*Disease state Interaction] is significant it indicates that male and female presented with different patterns in protein expression throughout ageing or in the disease state, thus, to investigate that further, a one-way ANOVA would be conducted for each sex separately. The statistical results of two-way ANOVA will be included in the figures legends, while the post-hoc test results will be discussed more deeply in the results section.

5.4 Results

5.4.1 Phosphorylation of ERβ and the ratio of pERβ/tERβ were increased in aged male brain samples, but not female.

5.4.1.1 Female samples:

When the expression of tERα and pERα were investigated separately, no differences in their expression during frontal cortex aging were seen (Figure 5.2b). Next, when the ratio of

pERα/tERα was measured during brain ageing, again no significant differences were found (Figure 5.2d). For ERβ, the expression of tERβ, pERβ (Figure 5.3b) and the pERβ/tERβ ratio (Figure 5.3d) did not change during frontal cortex ageing. Finally, GPER1 protein showed no changes in its expression during frontal cortex ageing (Figure 5.4b).

5.4.1.2 Male samples:

The male samples presented with the same ERα findings as female samples (Figure 5.2b & 5.2d), with no alterations in the levels of tERα, pERα, and the ratio of pERα/tERα. For ERβ, in contrast to female samples, male samples had changes in ERβ expression during brain ageing. Two-way ANOVA revealed a significant age-related increase in the pERβ/tERβ ratio in male frontal cortex between the young and elderly groups ($p<0.05$, Figure 5.3d). This increase was as a result of increases in pERβ levels ($p<0.05$, Figure 5.3b), while no change was seen in total expression (Figure 5.2b). Finally, GPER1 expression was not altered during frontal cortex ageing (Figure 5.4b).

5.4.1.3 Sex Disparities:

When considering sex as an influencer factor on ERα, ERβ, and GPER1 expression, no sex disparities in the levels of these proteins were found within any age group (Figure 5.2b & 5.2d, Figure 5.3b & 5.3d, Figure 5.4b).

5.4.2 Expression of oestrogen-associated proteins are altered in AD

5.4.2.1 Female samples:

Interestingly, female AD samples, in contrast to the ageing series, had alterations in ERα expression. The ratio between pERα/tERα was upregulated significantly in AD female frontal cortex compared to ND subjects ($p<0.05$, Figure 5.2e). However, this upregulation in female AD subjects was not associated with any changes in the levels of either pERα and tERα, separately (Figure 5.2c). When the level of pERα was compared to the level of tERα, AD subjects had significantly higher levels of pERα than tERα ($p<0.05$, Figure 5.2c). For ERβ, female AD subjects did not have any changes in pERβ, tERβ, or their ratio compared to ND subjects (Figure 5.3c and e). Also, GPER1 expression did not change in AD compared to ND subjects (Figure 5.4c).

5.4.2.2 Male samples:

Male AD subjects did show increases in the level of the pERα/tERα ratio compared to ND subjects ($P<0.05$, Figure 5.2e). The male AD subjects had significant increases in the levels of both pERα and tERα, separately ($p<0.05$, Figure 5.2c). In addition, level of pERα were significantly higher than those of tERα, the same as the female findings ($p<0.05$, Figure 5.2c). For ERβ, male AD subjects had a significant downregulation in tERβ levels ($p<0.001$, Figure 5.3c) compared to ND subjects, with no changes in pERβ or the ratio (Figure 5.3c and 5.3e). Finally, no alterations in GPER1 expression were identified in AD subjects (Figure 5.4c).

5.4.2.3 Sex Disparities:

The same results found during frontal cortex ageing were observed in AD. No sex disparities in pERα, tERα or their ratio, pERβ, tERβ, or their ratio or GPER1 were seen between male and female AD subjects (Figure 5.2c & 5.3 e, and Figure 5.4c).

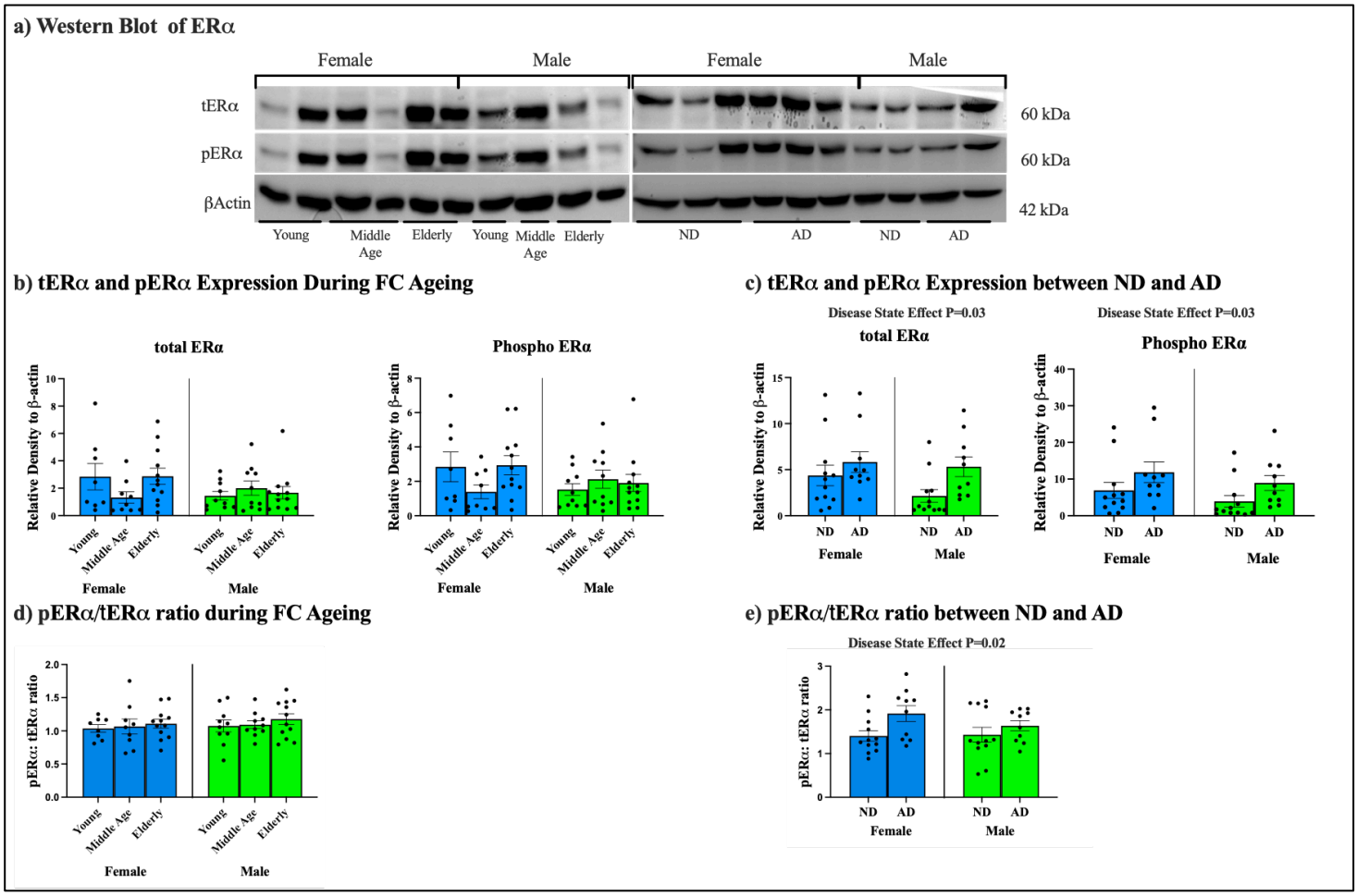


Figure 5.2 Comparison of the expression of ERα in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. **(a)** representative blotting data for ERα and the house keeping protein βactin. **(b)** Densitometric analysis of ERα immunoblots during brain ageing, with two-way ANOVA revealing no significant effect of Sex nor Age on ERα level **(c)** Densitometric analysis of ERα immunoblots between ND vs AD, with two-way ANOVA revealing a significant increase in ERα level in AD sample relative to ND sample (Disease State $F(1,40)=5.32$, $P=0.03$ and $F(1,40)=5.27$, $P=0.03$ for tERα and pERα respectively). **(d&e)** ratio of pERα/tERα during brain ageing and between ND vs AD, with two-way ANOVA showing a significant higher level of this ratio in AD samples relative to ND sample (Disease State $F(1,40)=5.75$, $P=0.02$). Data are presented as mean ± S.E.M.*$p<0.05$.

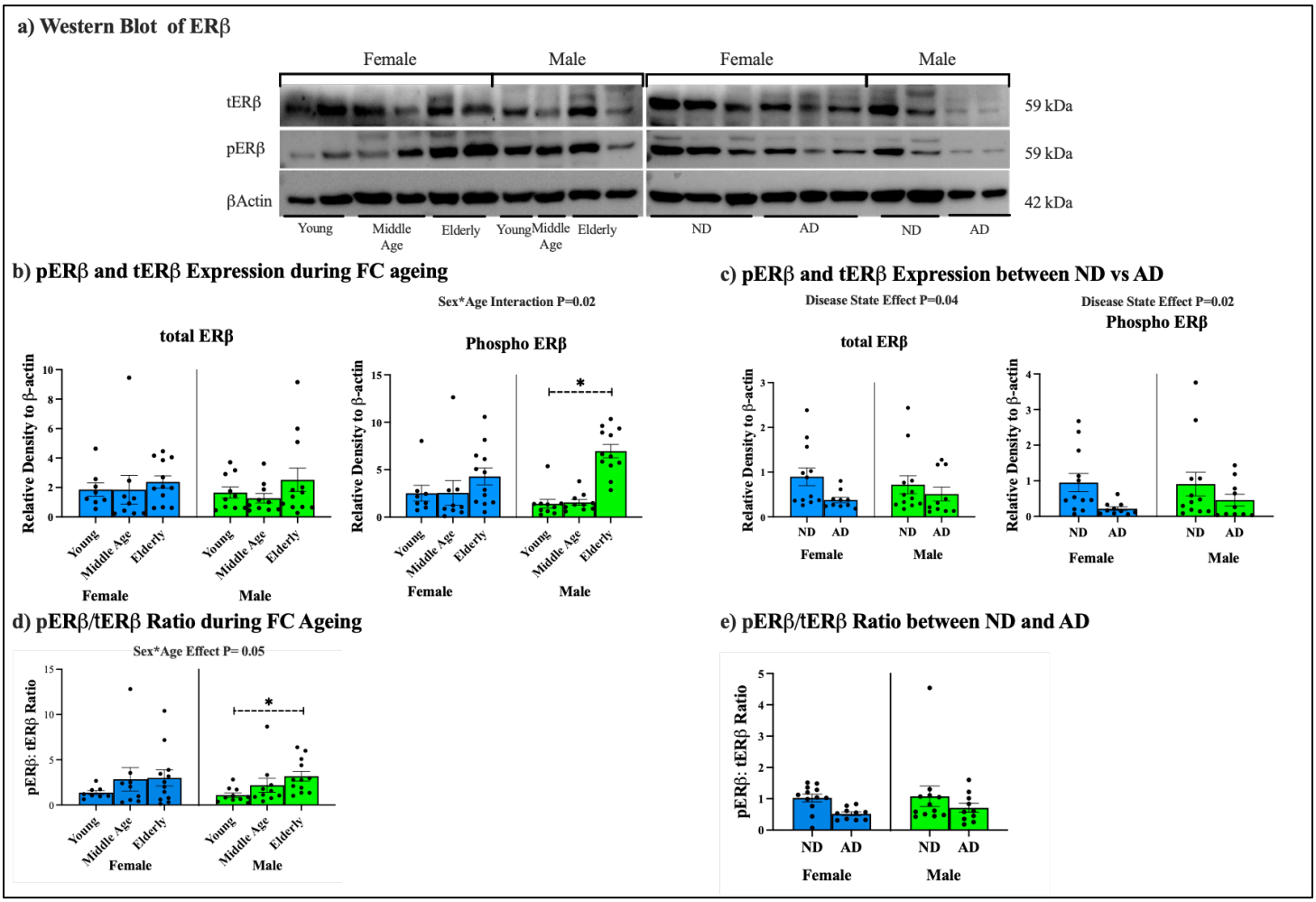


Figure 5.3 Comparison of the expression of ERβ in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. **(a)** representative blotting data for ERα and the house keeping protein βactin, **(b)** Densitometric analysis of ERβ immunoblots during brain ageing, two-way ANOVA reveal a significant age-related increase in pERβ expression only in male samples (Sex*Age $F(2,55)=3.85$, $P=0.02$). **(c)** Densitometric analysis of ERβ immunoblots between ND vs AD, two-way ANOVA reveal a significant lower levels of ERβ only in AD samples relative to ND sample (Disease State $F(1,40)=4.45$, $P=0.04$ and $F(1,40)=5..85$, $P=0.02$ for tERβ and pERβ respectively). **(d&e)** Ratio of pERβ/tERβ during brain ageing and between ND vs AD, with two-way ANOVA showing an age-related increases of this ratio only in male samples (Sex*Age $F(2,55)=3.49$, $P=0.05$). Data are presented as mean ± S.E.M. *$p<0.05$.

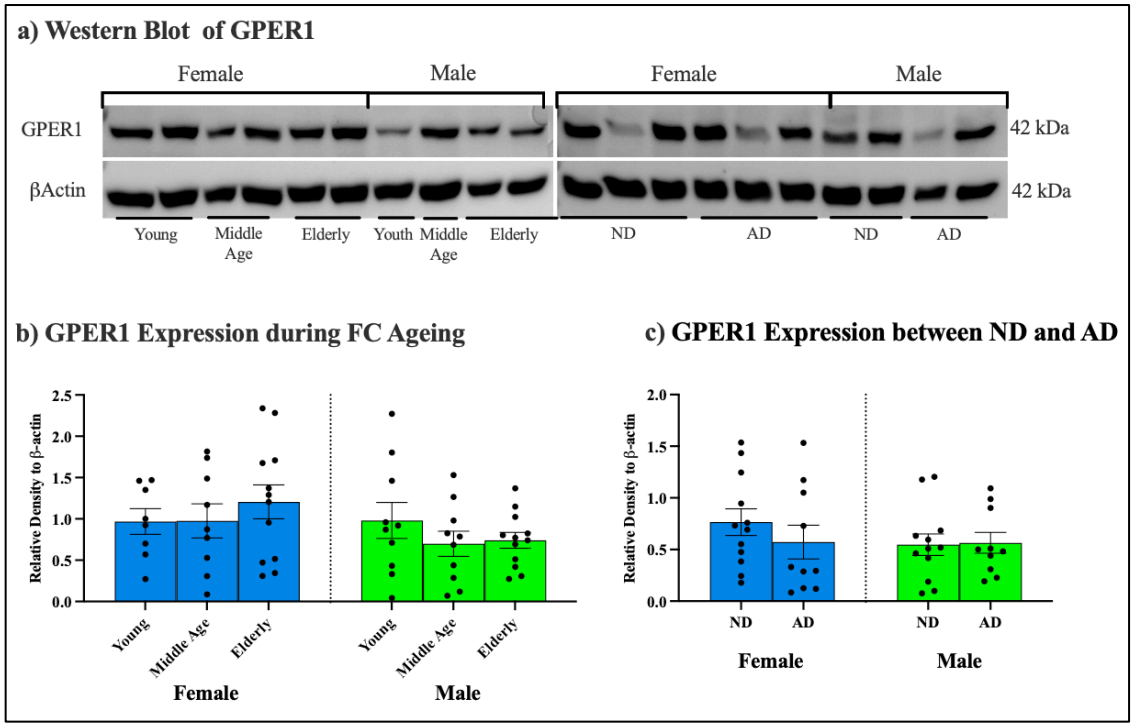


Figure 5.4 Comparison of the expression of GPER1 in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. **(a)** representative blotting data for ERα and the house keeping protein βactin, **(b&c)** Densitometric analysis of GPER1 immunoblots during brain ageing and between ND vs AD, respectively. With two-way ANOVA revealing no significant effect of sex and age or sex and disease state on the investigated protein. Data are presented as mean ± S.E.M.

5.4.3 *Expression of PRs was not altered during frontal cortex ageing or in AD in both sexes*

The PR antibody used in this project gave a band at 114kDa, larger than the expected size according to the manufacturer (Table 5.1). Nonetheless, on the manufacturer's website they provided data showing that their antibody could identified PR-A (99kDa) and PR-B (114kDa) (https://www.abcam.com/progesterone-receptor-antibody-alpha-pr6-ab2765.html). Therefore, it is likely that the PR seen here is the PR-B isoform. In both male and female frontal cortex samples, no alterations in PR-B levels were found during frontal cortex ageing (Figure 5.5b). Furthermore, no changes in its levels were seen in AD male and female samples (Figure 5.5c). Finally, no sex disparities were found in any age group (Figure 5.5b and 5.5c).

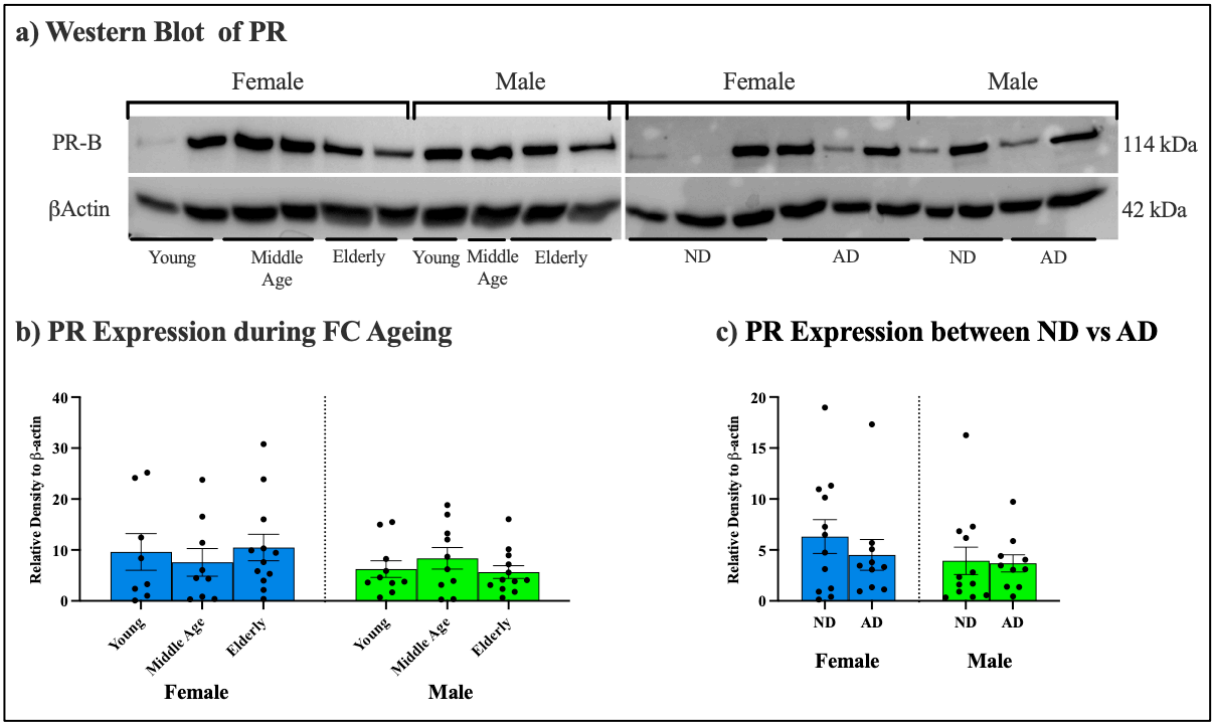


Figure 5.5 Comparison of the expression of PR in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. **(a)** representative blotting data for PR and the house keeping protein βactin, **(b&c)** Densitometric analysis of PR immunoblots during brain ageing and between ND vs AD, respectively, with two-way ANOVA revealing no significant effect of sex and age or sex and disease state on the investigated protein. Data are presented as mean ± S.E.M.

5.4.4 Akt and ERK2 showed an age-related increase in male brains

The expression of two proteins involved in downstream signalling from oestrogen receptors was measured, Akt and MAPK (ERK1&2).

5.4.4.1 Female samples:

Females did not have any alterations in tAkt, pAkt or their ratio during brain ageing (Figure 5.6b and 5.6d). The MAPK antibody used here detected ERK1 and ERK2 which presented as two bands. When the two bands were analyzed together as a total representation of MAPK, no alterations in MAPK in the total or phosphorylated forms or their phospho/total ratio were observed in female frontal cortex ageing (Figure 5.7b and 5.7d). When ERK1 and ERK2 were analyzed independently, no alterations in their total and phosphorylated forms or in their phospho/total ratios were seen during female frontal cortex ageing (Figure 5.8a and 5.9a).

5.4.4.2 Male samples:

The pAkt/tAkt ratio had a significant increase in male ageing in the frontal cortex between young and elderly male samples ($p<0.05$, Figure 5.6d) while no changes were observed in the levels of total and phosphorylated Akt, separately (Figure 5.6b). For MAPK, when both ERK1 and ERK2 bands was analysed together, no alterations in MAPK in the total or phospho forms or their ratio were observed during frontal cortex ageing (Figure 5.7b and 5.7d). However, when ERK1 and 2 were measured independently, pERK2/tERK2 ratio had an age-related increase in its expression in elderly males compared to young males ($p<0.05$, Figure 5.9c). ERK1 expression did not change (Figure 5.8a and 5.8c)..

5.4.4.3 Sex Disparities:

Despite the age-related differences in Akt observed here, no sex disparities were seen between males and females in any group (Figure 5.6b and 5.6d). Nor were any significant differences in sex found for total MAPK and ERK1 and 2 (Figure 5.7b and 5.7d, Figure 5.8a, 5.8c, 5.9a and 5.9c).

5.1.1 No changes in MAPK and Akt were seen in AD patients

In AD patients, Akt (Figure 5.6c and 5.6e), total MAPK (Figure5.7c and 5.7e) and ERK1 and ERK2 (Figure 5.8b, 5.9b, 5.8d and 5.9d) phosphorylated and total proteins and the

corresponding ratios were not altered in both male and female AD frontal cortex. Nor were any sex disparities found between male and female AD samples for Akt (Figure 5.6c and 5.6e), total MAPK (Figure 5.7c and 5.7e) or ERK1 and 2 (Figure 5.8b, 5.9b, 5.8d and 5.9d).

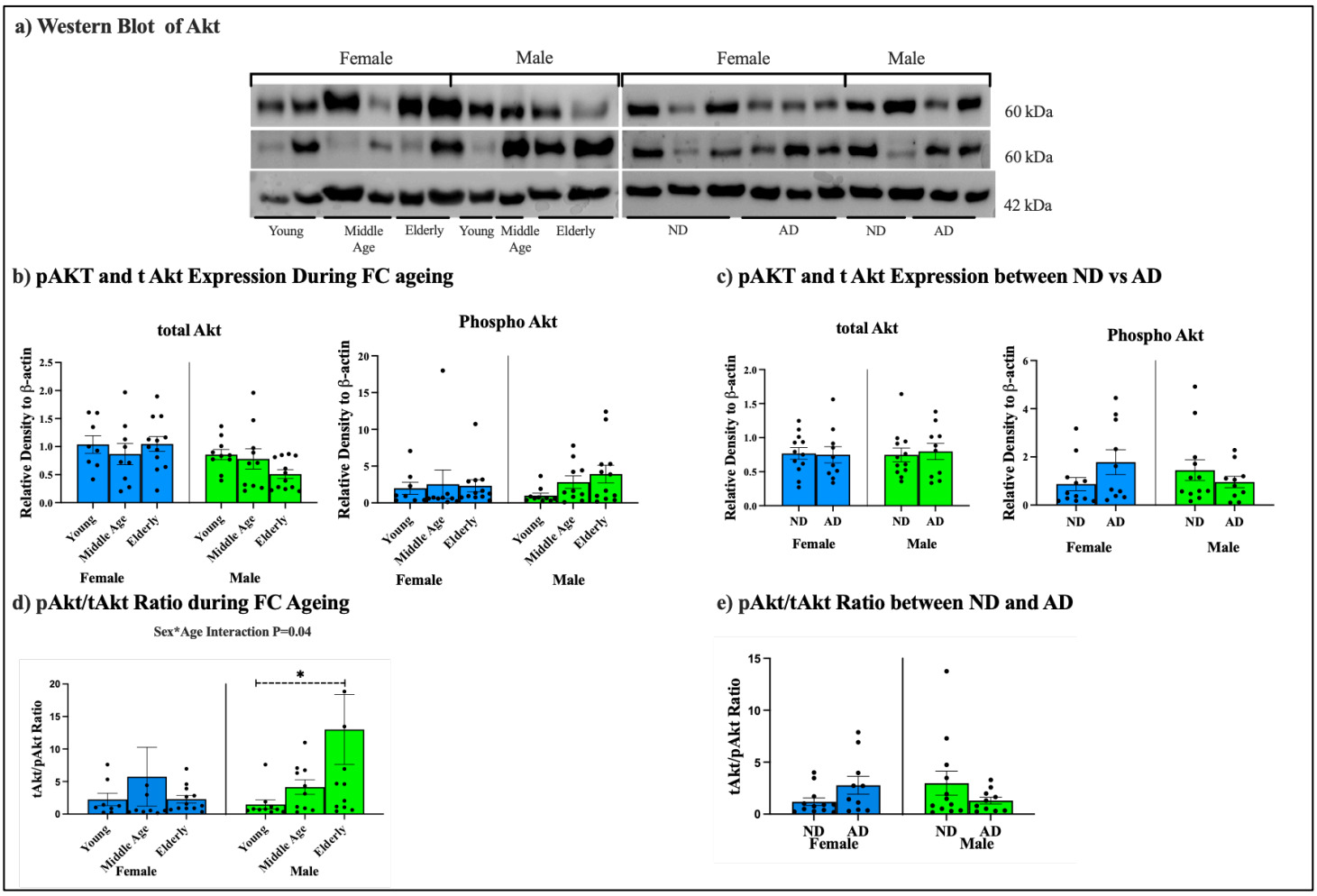


Figure 5.6 Comparison of the expression of Akt in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. **(a)** representative blotting data for Akt and the house keeping protein βactin, **(b)** Densitometric analysis of Akt immunoblots during brain ageing and between ND vs AD, respectively, with two-way ANOVA revealing no significant effect of sex and age or sex and disease state on the investigated protein. **(d&e)** Ratio of pAkt/tAkt during brain ageing and between ND vs AD, with a significant age-related increase in this ratio only in male samples (Sex*Age $F(2,55)=3.55$, $P=0.04$). Data are presented as mean ± S.E.M. *$p<0.05$.

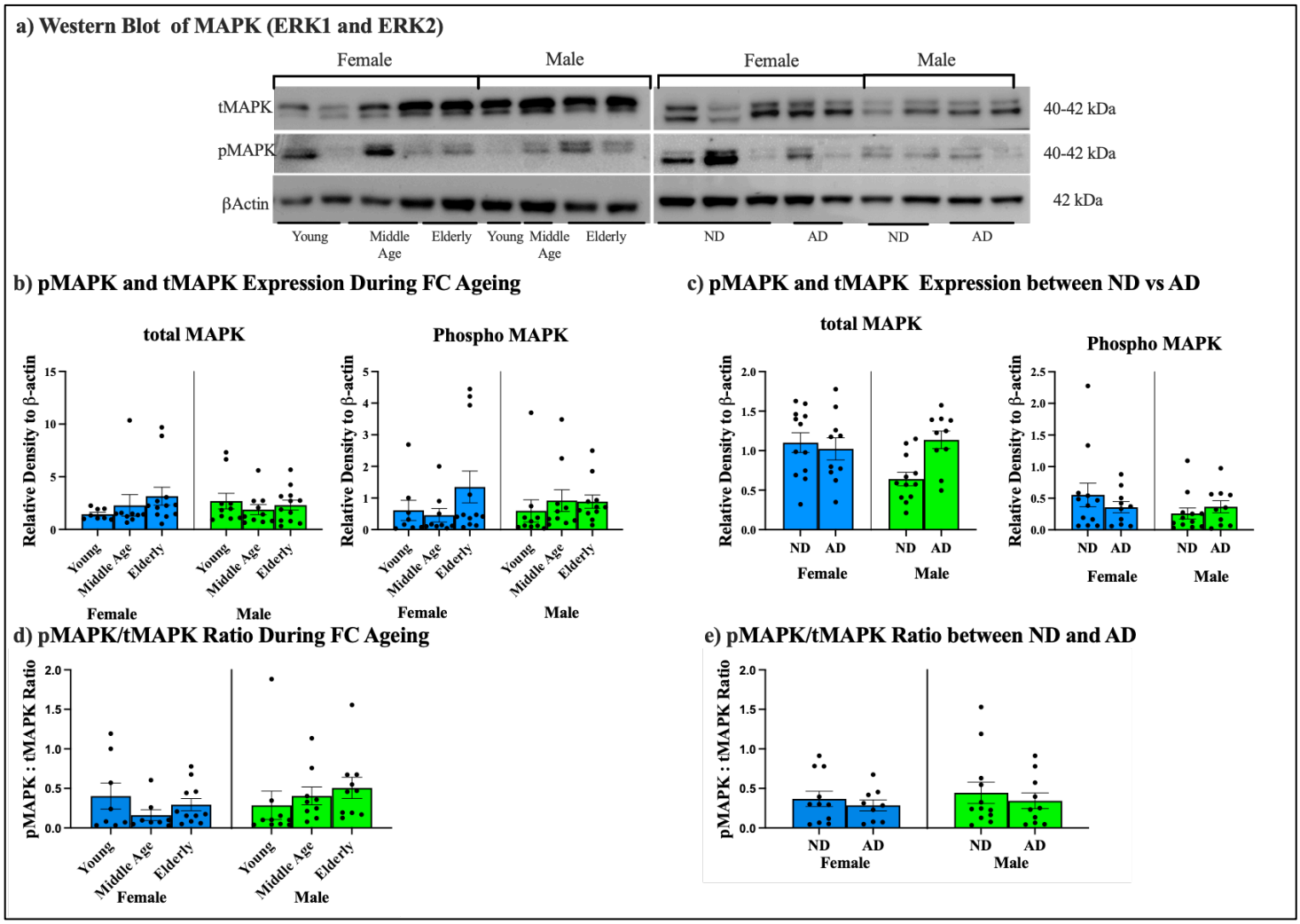


Figure 5.7 Comparison of the expression of MAPK (ERK1 and 2 measured together) in human female and male frontal cortex between different age groups of young (male n=10, female n=8), middle-aged (male n=10, female n=9) and elderly (male n=12, female n=11) subjects and between ND (male n=11, female n=12) and AD (male n=10, female n=9) subjects. **(a)** representative blotting data for MAPK and the house keeping protein βactin, **(b&c)** Densitometric analysis of MAPK immunoblots during brain ageing and between ND vs AD, respectively. **(d&e)** ratio of pMAPK/tMAPK during brain ageing and between ND vs AD. With two-way ANOVA revealing no significant effect of sex and age or sex and disease state on the investigated protein. Data are presented as mean ± S.E.M.

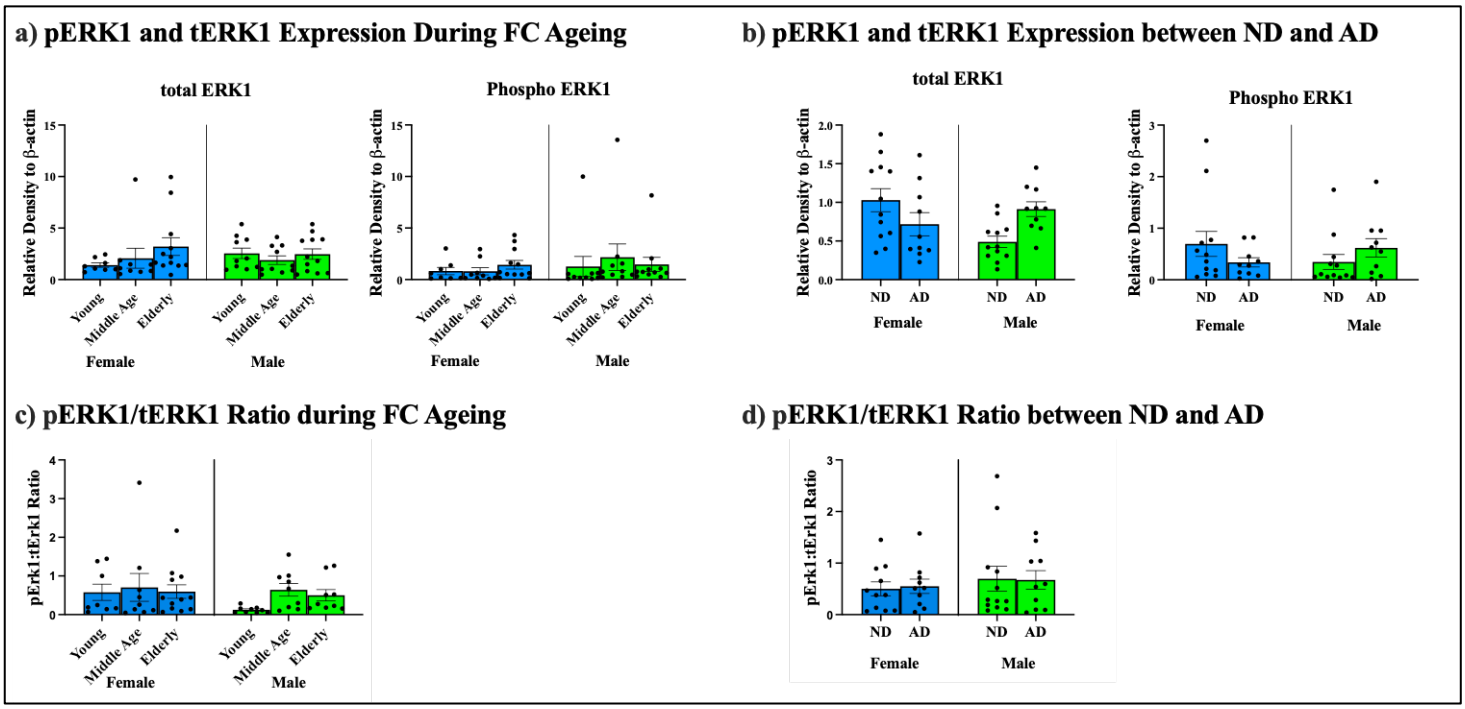


Figure 5.8 Comparison of the expression of ERK1 in human female and male frontal cortex using the blots for the data in Figure 5.6. **(a&b)** ERK1 densitometric analysis of immunoblots during brain ageing and between ND vs AD, respectively. Ratios of **(c&d)** pERK1/tERK1 ratio during brain ageing and between ND vs AD. With two-way ANOVA revealing no significant effect of sex and age or sex and disease state on the investigated protein. Data are presented as mean ± S.E.M. *P<0.05.

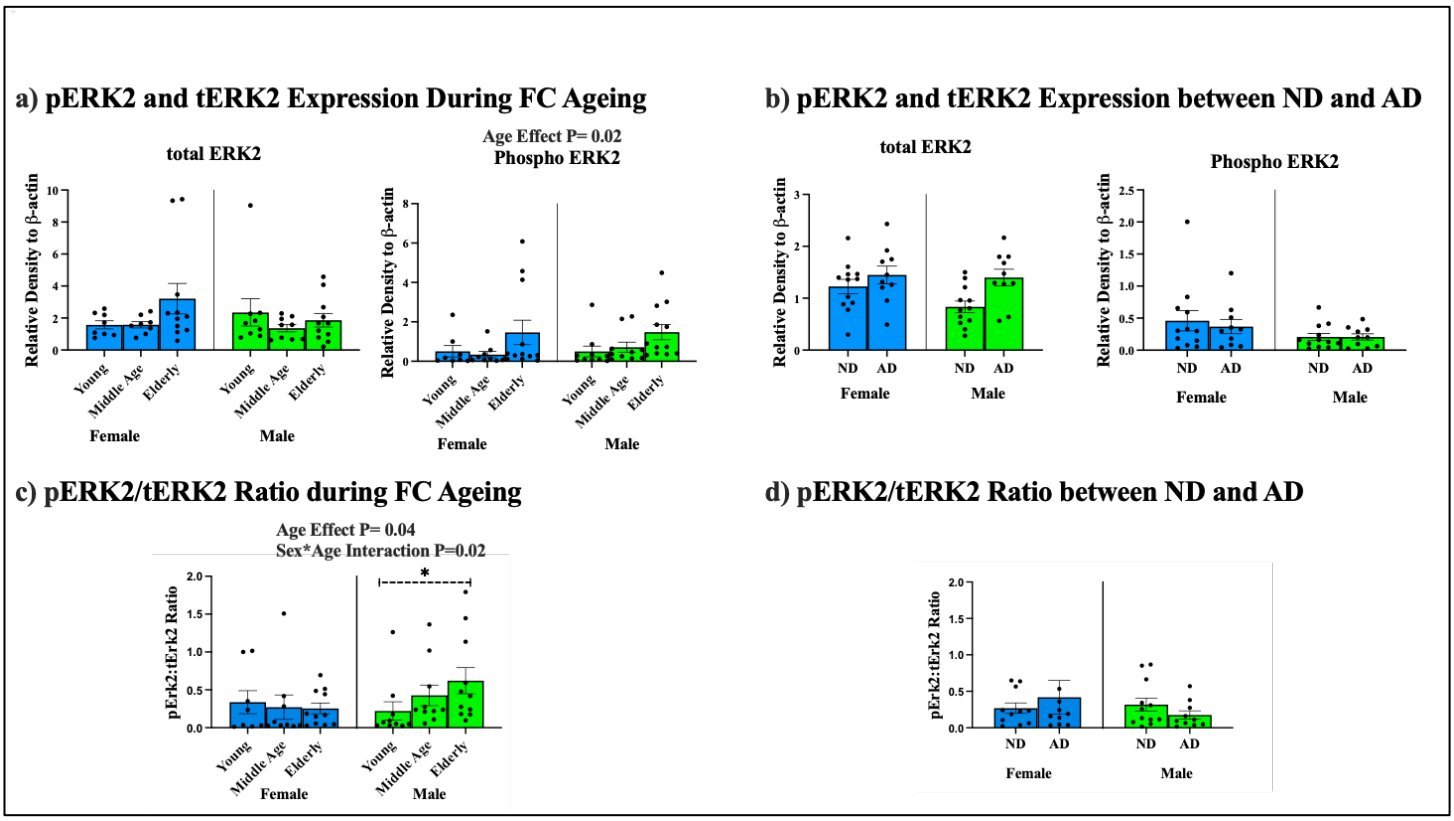


Figure 5.9 Comparison of the expression of ERK2 in human female and male frontal cortex using the blots for the data in Figure 5.6. **(a)** ERK2 densitometric analysis of immunoblots during brain ageing, two-way ANOVA reveal a significant age-related increase in pERK2 expression (Age $F(2,55)=4.08$, $p=0.02$) . **(b)** ERK2 densitometric analysis of immunoblots between ND vs AD, two-way ANOVA revealing no significant effect of sex and disease state on the investigated protein. **(c&d)** Ratio of pERK2/tERK2 ratio during brain ageing and between ND vs AD, respectively, with two-way ANOVA showing an age-related increases of this ratio only in male samples (Sex*Age $F(2,54)=4.5$, $P=0.02$) and (Age $F(2,54)=3.46$, $P=0.04$). Data are presented as mean ± S.E.M. *$P<0.05$.

5.5 Discussion

This Chapter described an age-related increase in ERβ activity and an upregulation of downstream signalling pathways, ERK2 and Akt, only in male ageing frontal cortex and not in females. While in AD, ERα was upregulated in both sexes. These findings are novel to the best of my knowledge and might suggest different roler for ERα and ERβ in the brain. Also, the age-dependent result of ERβ in male brains could suggest a possible neuroprotective effect of ERβ which could reduce or slow AD development. Table 5.2 presents a summary of the findings of this Chapter.

Table 5. 2 Chapter 5 age-related findings summary

	ERα				ERβ					MAPK								Akt				
	tERα	pERα	pERα/tERα ratio	pERα vs. tERα	tERβ	pERβ	pERβ/tERβ ratio	pERβ vs. tERβ	Prog.	tErk1	pErk1	pErk1/tErk1 ratio	pErk1 vs tERK1	tErk2	pErk2	pErk2/tErk2 ratio	pErk2 vs tErk2	tAkt	pAkt	pAkt/tAkt ratio	pAkt vs tAkt	GPER1
During Frontal Cortex Ageing																						
Female	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Male	-	-	-	-	-	↑	↑	-	-	-	-	-	-	-	↑	↑	-	-	-	↑	-	-
ND vs AD																						
Female	-	-	↑	↑	↓	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Male	↑	↑	↑	↑	↓	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

5.5.1 ERα expression in the frontal cortex of both sexes during ageing and in AD

5.5.1.1 Age as a risk factor

When the expression of total and phospho ERα and the ratio of pERα/tERα was investigated in ageing human frontal cortex, neither males nor females had any changes in their expression or ratio with age.

As far as I know, the age-related expression of ERα in human brain has not been investigated to date. However, its expression level during ageing has been studied in rodent brain but with contradictory results. In male and female rat cerebral cortex, ERα levels did not change during ageing (Cai et al., 2014, Sharma PK and Thakur MK, 2006). On the other hand,

another study reported an age-related decline of ERα in male rat hippocampus (Wu D, 2010). A decline was also reported in ERα in female mouse cortex (Cai et al., 2014). However, these latter two studies looked at the protein expression in animals which were either gonadectomized or ovariectomized followed by E2 treatment. So, these *in vivo* models do not represent what really happens during human brain ageing. It is well documented that as humans get older, serum E2 levels decline. Serum E2 declined significantly to a greater extent in females than in males, with serum E2 showing an 85–90% decline in postmenopausal women (Khosla S. et al 1997).

Female E2 levels in the brain are reported to be qualitatively similar to circulating E2 and, in postmenopausal women, both circulating and brain E2 levels show a significant decline compared to pre-menopausal women (Henderson and Popat, 2011, Rosario et al., 2011, Trevoux et al., 1986). So, it was expected that, with a decline in the brain E2 level in female samples, one of its target proteins (ERα) should show an age-related decline. The previous assumption has been proven by several *in vivo* studies (Zhang et al., 2011a, Zhang et al., 2009, Qu et al., 2013) where long-term E2 deprivation was the lack of changes in phospho and total ERα levels or their ratio in ageing female frontal cortex in this Chapter does not support this project hypothesis, where I expected that E2-associated protein activity will alter in the female brain with advancing age, thus increasing female susceptibility to develop AD earlier in life compared to males.

The lack of changes in ERα levels in males could be the result of males not experiencing a severe decline in steroidal hormones in middle age. Male testosterone falls by approximately 2-3% annually once the age of 30 years is reached (Feldman et al., 2002, Muller et al., 2003). Since testosterone can be converted to E2, males do not exhibit the steep drop in E2 levels evidenced in females following the menopause or post-premenopausal surgical bilateral ovariectomy. Furthermore, aromatisation of androgen to oestradiol can occur and thus stimulate E2 pathways (Feldman et al., 2002, Muller et al., 2003).

However, the main function of ERα in cognitive function is not yet clear. Preclinically, administration of a selective ERα antagonist or ERα knockout led to a diminution of the cortical protective effects of E2 in mice (Dubal et al., 2006, Shin et al., 2011). Interestingly, when Sampei et al. (Sampei et al., 2000) evaluated ERα gene-deficient female mice against wild type female mice, similar damage from stroke insults was revealed. These observations implied that

the neuroprotective impact of E2 may not occur via ERα. Nonetheless, it is well documented in *in vivo* studies that ERα receptors have a role in learning enhancement and memory preservation (Corbo et al., 2006, Ji et al., 2000, Olsen et al., 2006).

An additional explanation for not seeing differences in ERα with advancing age in female cortex is the wide range of ages within each of the ageing groups used here which were determined by sample availability in the Brain Banks. For instance, Ishunina's group (Ishunina et al., 2007) investigated the hippocampal levels of ERα during the menopause transition window (perimenopause 49-50 years old and postmenopausal > 58 years old) using immunohistochemistry. They reported that levels of nuclear ERα were enhanced during the female menopause transition phase (Ishunina et al., 2007). Also, they highlighted that ERα splice variant expression was increased in elderly women (Ishunina et al., 2007).

The expression of the long ERα variant, also referred to as $ERα_{66}$, has been the subject of most of the work within the brain, including this project. A new smaller ERα splice variant ($ERα_{36}$) was reported in breast cancer in 2005 (Wang et al., 2005). This isoform has been demonstrated to co-localise and engage with caveolin-1 expressed in caveolae membranes, which may influence a broad spectrum of signalling compounds (Zhang et al., 2012). Regarding its expression in the brain, some researchers indicated cortical and hippocampal $ERα_{36}$ expression in mice (Liu et al., 2013b) and humans (Zou et al., 2015). The expression of the isoform was especially elevated in the hippocampus, cortex and cerebellum, implying a role in the CNS, and potentially in AD. Additionally, this splice variant could act as a negative regulator of ERα-induced transcription function (Ishunina and Swaab, 2008, Ishunina and Swaab, 2009). In this Chapter, ERα investigation did not identify another ER variant in Western blotting, possibly because the antibodies used against phospho- and total- ERα were monoclonal and thus only recognised a specific epitope not found in $ERα_{36}$. It would be interesting to look at the levels of this small ERα splice variant during brain ageing as it inhibits the function of the long ERα variant. Nonetheless, the finding of no changes in ERα expression, and thus possibly activity, during brain ageing in both sexes could suggest that ERα maintains a stable neuroprotective function in ageing brains, even when there is an increase in Aβ burden and tau hyperphosphorylation with advancing age (reported in Chapters 3 & 4).

5.5.1.2 In Alzheimer's Disease

In AD, clinical studies have pointed to an alteration in extranuclear and nuclear ERα levels in female hippocampus, with either a decline (Hu et al., 2003) or no change in its levels being found (Savaskan et al., 2001). Here, in contrast to the findings for ERα expression during frontal cortex ageing, a significant increase in ERα in AD samples was seen in both sexes. Female AD samples had a significant increase in both total and phosphorylated ERα and the ratio of pERα/tERα, also male AD samples showed an increase in both total and phosphorylated ERα and the ratio of pERα/tERα. The important difference between the findings of the previous clinical studies and those presented in the Chapter, is that ERα was investigated here in the frontal cortex while the other studies investigated the expression of hippocampal ERα (Savaskan et al., 2001, Hu et al., 2003). Thus, the rise in ERα in AD seen here could suggest that it is more important for neuroprotection in the frontal cortex and that its neuroprotective function is increased in both men and women.

In AD preclinical studies, it has been suggested that ERα can protect neurons from $A\beta_{1-42}$ toxicity through stimulation of the Akt pathway (Mateos et al., 2012) via inhibition of the apoptosis signal-regulating kinase 1 pathway or by modulation of thioredoxin-1, a multi-purpose redox protein which has anti-apoptotic properties (Holmgren, 2000). Moreover, it has been demonstrated that glutamate excitotoxic injury can be prevented by E2 via ERα signalling cascades (Jelks et al., 2007, Xia et al., 2009). Excitatory glutamatergic neurotransmission through NMDA receptors is essential for synaptic plasticity and neuron longevity, but excessive NMDA receptor stimulation leads to excitotoxicity and facilitates cellular demise, representing a route of neurodegeneration in AD (Jelks et al., 2007, Xia et al., 2009). *In vivo*, Xia et al. evaluated the protective function of ERα on glutamate-induced neuronal damage in mice following administration of Propyl-Pyrazole-Triol (PPT), a selective ERα modulator; glutamate-induced cell death was aborted (Xia et al., 2009). This effect, with respect to NMDA receptor excitotoxicity, was limited to females and Bryant and Dorsa (Bryant and Dorsa, 2010) postulated that PPT protects cortical nerve cells from glutamate-induced injury in females only, since *in vitro* there is a higher level of immunoreactive ERα in female neurons compared to males. Thus, looking at NMDA receptors as ERα activity changes during female brain ageing and in AD would be a good target for future work.

5.5.2 *ERβ expression in the frontal cortex of both sexes during ageing and in AD*

5.5.2.1 Age as a risk factor

When ERβ expression was screened, an age-related increase in the ratio between pERβ/tERβ was seen only in male frontal cortex. However, even if females did not show an increase in ERβ during ageing in the frontal cortex, its expression did not decrease suggesting that ERβ activity is still maintained in the ageing female brain. As with ERα, expression of ERβ levels during brain ageing has only been studied in rodents and again contradictory results were found. Rat cerebral cortex in both sexes showed a decrease in ERβ levels with advancing age (Cai et al., 2014, Sharma PK and Thakur MK, 2006). The decline in ERβ in female rat cortex was correlated to a decrease in cognitive function with ageing (Frick KM, 2009). However, in contrast to the previous study with a decline in ERβ, (Yamaguchi-Shima and Yuri, 2007) reported that in female rats ERβ expression was unaltered during ageing in the cortex, however, in the hippocampus there was an age-related decrease.

The findings in male brains in this project indicated that the pERβ/tERβ ratio increased with ageing in male brain. It is possible that this upregulation of ERβ levels in males could arise through the conversion of testosterone to E2 as males do not suffer a severe decline of sex steroid hormones during ageing (Feldman et al., 2002, Muller et al., 2003). This upregulation in the ERβ ratio could suggest a protective role for ERβ against the effects of the accumulation of Aβ in the elderly male frontal cortex (Chapter 3).

In preclinical studies, ERβ activity was linked to several neuroprotective actions. ERβ can stimulate the release of IDE, which aids in the catabolism of Aβ, through the PI3K-Akt pathway. Thus, this pathway may govern the expression of IDE in normal brain ageing and in people exhibiting the early stages of AD (Zhao et al., 2011). Thus, the increase in ERβ activity in male samples, and the lack of change in female samples, might partially explain the reason for higher Aβ accumulation in elderly female brains compared to males (Chapter 3). Another function of ERβ is its positive impact on object recognition and placement memory tests through enhancing 3-methoxy-4-hydroxyphenylglycol (MHPG) levels, and dopamine and serotonin breakdown products, homovanillic acid and 5- hydroxyindole acetic acid, respectively, in the rats prefrontal cortex (Jacome et al., 2010).

An important neuroprotection of ERβ is its potential action in prophylaxis for AD. A number of preclinical studies have demonstrated that diarylpropionitrile (DPN), an ERβ agonist, can provoke a notable decrease in hippocampal ApoE mRNA and protein expression, which is a recognised indicator of late-onset AD (Han et al., 2013, Wang et al., 2006, Zhao et al., 2013). Moreover, heightened inflammation impacts both the ageing brain and AD, and so suppressing neuroinflammation may additionally protect the brain. Several cytokines and chemokines or interleukins, e.g. IL-1β, IL-6 and IL-12, are reported to increase in the serum during the menopause transition phase (Abu-Taha et al., 2009). ERβ has the ability to maintain BBB permeability and reduce its break down (Shin et al., 2013). Thus, ERβ could have a protection function against neuroinflammation through reducing transportation of inflammatory proteins to the brain through the BBB with advancing age. Furthermore, another neuroprotective mechanism of ERβ against Aβ toxicity is inhibition of apoptosis which was highlighted in an *in vitro* study (Zhang et al., 2010). Zhang et. al. reported that, in the absence of E2, ERβ can bind to Bcl2-associated death (BAD) promoter and restrict its involvement with downstream targets and thus inhibit apoptosis. These data reinforce the concept that ERβ protects the brain against the neurotoxicity resulting from Aβ accumulation, especially given that Bcl2 was upregulated in both sexes with ageing in the previous chapter (Chapter 4).

5.5.2.2 In Alzheimer's Disease

With regards to the AD data in this Chapter, tERβ expression significantly decreased in male samples and in female samples a reduction in the tERβ and pERβ was seen. Overall, however, there was no change in the pERβ/tERβ ratio in any AD subjects. A contrasting finding was reported in a clinical study using immunohistochemistry (IHC) where it was reported that cytoplasmic and nuclear ERβ increased in AD hippocampus for both sexes (Savaskan et al., 2001). The difference between the results here and the previous clinical results is likely to be due to the brain location examined, frontal cortex here which is enriched with ERβ, while Savaskan investigated the hippocampus (Savaskan et al., 2001). In Chapter 6, I will discuss my own IHC findings for ERβ.

The results presented here are consistent with other preclinical findings in the literature. An *in vivo* study demonstrated that lack of ERβ is associated with AD as ERβ$^{-/-}$ mice exhibited different AD processes, e.g. Aβ and ApoE accretion in the cortex (Zhang et al., 2004a). Furthermore, mitochondrial impairment can arise owing to low ERβ levels, subsequently

enhancing the Aβ build-up underlying AD. Wang et al. observed that overexpression of ERβ protected adrenal PC12 cells against toxicity from Aβ (Wang et al., 2012b). The AD samples investigated in this project are from late stage patients (Braak stages V & VI) who had an abundance of insoluble and soluble Aβ and NFTs in their brains (see Chapters 3 and 4). Thus, it is unclear why ERβ levels were not affected more significantly by sex in this study. One possible explanation is that, as for ERα, only the long isoform of ERβ was investigated here and the other splice variants were not studied. This theory is supported by the finding from a clinical study that higher levels of ERβ splice variants were more likely to elevate the risk of AD in females (Pirskanen et al., 2005).

At present, five experimentally authenticated human ERβ isoforms, labelled ERβ1 to ERβ5, have been recognised. The ERβ antibody used in this chapter detect the long form (ERβ1) according to the band size; 59 kDa (González-Mora and Garcia-Lopez, 2021), and was selected for this project due to availability issues. ERβ2 is the best-described human variant and is generally thought to act as a major negative regulator for ERα (Ogawa et al., 1998). Thus, the discussion will focus on this variant. ERβ2 has the capacity to attach to DNA and to trigger oestrogen receptor element (ERE) promoter function independently of E2 stimulation (Leung et al., 2006, Mott and Pak, 2012). It has been reported that the quantity of alternative splicing events within the human brain rises with advancing age; it can therefore be inferred that ERβ2 splice variant expression is also elevated with age (Tollervey et al., 2011). In keeping with this theory, *in vivo* work has documented markedly enhanced hypothalamic ERβ2 mRNA expression in female rat aged 18 months compared to 3 month-old rat (Shults et al., 2015). However, the situation is more complicated in human females as ageing is related to the menopause transition phase when serum E2 levels drop. The combination of age and lack of E2 differentially changes brain ERβ2 expression. An *in vivo* study showed that E2 deficiency was associated with a rise in mouse brain and serum leucocyte ERβ2 expression (Wang et al., 2012d). Furthermore, *in vivo*, amplification of the expression of ERβ2 was associated with a fall in E2 therapy efficacy in depressive disorders using the forced swim test model in rats (Wang et al., 2012d). In combination, these results suggest that E2 suppresses brain ERβ2 splice variant expression, but the mechanisms underlying this process remain obscure.

At present, there has been no research to evaluate the relationship of ERβ splice variants with human brain ageing and AD. Here in this study, as ERβ1 was not altered in the ageing female brain, it would be interesting to look at the ERβ2 variant. As shown in Chapter 3,

females express AD biomarkers earlier than male samples, in particular an age-related increase in APP and the significantly higher level of Aβ in aged female brains compared to age-matched males. Thus, investigations into the alternative splice variants will expand our understanding of the role of ERβ in brain ageing and related pathological conditions, since these variants may have a range of functional characteristics that differ from the original isoform. It is important to note that the preclinical studies above that looked at the cerebral ERβ2 variant used quantificative RT-PCR which measures ERβ2 mRNA and not the protein itself (Wang et al., 2012d, Shults et al., 2015). As far as I can tell, there is no commercially available selective antibody against the cerebral ERβ2 protein which doesn't cross-react with other ERβ variants. Wang and colleagues study used a selective antibody against the brain ERβ2 protein which was a gift from Dr. Robert Handa (Wang et al., 2012d).

5.5.3 GPER expression in the frontal cortex of both sexes during ageing and in AD

5.5.3.1 Age as a risk factor

In this Chapter, no alteration in GPER1 expression was seen in both sexes in the ageing frontal cortex. The consequence of ageing on GPER1 activities is not well-delineated in humans, however, *in vivo* studies have reported variations in its expression according to its brain region location. GPER1 expression in the hypothalamus of ovariectomized (OVX) female rhesus monkeys was unaffected by age (Naugle and Gore, 2014). However, in the hypothalamus the number of cells exhibiting GPER1 expression and the degree of GPER1 expression for each cell were both elevated in older OVX animals (Naugle and Gore, 2014). In contrast, compared to young adult mice, elderly OVX female mice exhibited diminished hippocampal GPER1 mRNA (Wu et al., 2018). The latter finding in the hippocampus was related to the lack of E2; reduced E2 levels were correlated with less GPER1 mRNA expression (Wu et al., 2018). This could suggest that, as the E2 concentration falls, a drop in GPER1 expression during ageing in females might be seen. However, that did not happen in my data, which could suggest that humans are different to lower order mammals and/or that the brain region examined is crucial to detect differences.

No sex disparities during brain ageing were reported clinically. In preclinical studies, males showed equivalent results to females in *in vivo* observations with the expression of GPER1 in the hippocampus being diminished in elderly male mice compared to younger adults

(Xu et al., 2018). However, in males and females *in vivo*, the GPER1 decrease did not seem to impact GPER1-mediated functions since G1, a GPER1 agonist, was able to improve memory in the older animals (Wu et al., 2018) . Nevertheless, in OVX females, long-term E2 deprivation caused G1 to loss its GPER1-mediated neuroprotective function (Wu et al., 2018). GPER1 interactions with ERα and ERβ have also been described (Prossnitz and Barton, 2011, Filardo and Thomas, 2012). Such crosstalk between GPER1 and ERα/ERβ encompasses a number of types of interplay, i.e. cooperative, antagonistic and dependent (Hadjimarkou and Vasudevan, 2018). Thus, in this project, the age-related increase in ERβ in human male samples might stimulate/or antagonise some of the functions of GPER1.

5.5.3.2 In Alzheimer's Disease

When the expression level of GPER1 in AD samples was investigated, as with the ageing findings, no alteration in its expression was seen in both sexes. Brain GPER1 expression in humans with AD has not yet been explored. However, in the 5xFAD mouse model, chronic stimulation of GPER1 by G1 mitigated memory dysfunction in the novel object identification test in females but was ineffective in males (Kubota et al., 2016). In females, this appears to be analogous to E2-mediated prophylactic influences, as reviewed in Chapter 1 (Section 1.10).

Despite the preclinical sex disparities for GPER1 stimulation described above, G1 therapy was also shown to protect rat neuron cells from toxicity provoked by $A\beta_{1-42}$ via alterations in oxidative measures, diminishing nitrous oxide and hydrogen peroxide concentrations (Kurt et al., 2019). Interestingly, when Rosario et al. (Rosario et al., 2011) investigated the clinical level of E2 in healthy and AD patients ageing 60 to > 80 years old, AD patients showed a decline in E2 levels in female brains > 80 years old with no differences in those aged 60-79 years, while males showed a decline in androgen levels at similar ages to females (Rosario et al., 2011). Taken together, even if GPER1 levels in female AD samples did not change, their activity would be expected to be lower since the female AD samples used in this project were > 80 years old. However, most of the male AD samples used in my project were < 80 years old, so GPER1 activity might not change.

5.5.4 PR expression in frontal cortex of both sexes during ageing and in AD

5.5.4.1 Age as a risk factor

As far as I can discern, there have been no investigations of PR-B expression in either the human ageing brain or in AD. In this Chapter, PR did not show any age-related alterations in its expression in both sexes. Additional, no *in vivo* PR variations between the sexes have been identified in the bed nucleus of stria terminalis (BST) (Auger and De Vries, 2002), resembling the findings here even if in a different brain region.

In clinical studies, Bixo et al. have performed post-mortem assays in brain tissue from young and postmenopausal human females for progesterone and its active isoforms, 5α–DHPROG and 3α-5α-THPROG. Progesterone was most concentrated in the hypothalamus, amygdala and cerebellum; 5α–DHPROG and 3α-5α-THPROG were highly present in the substantia nigra and hypothalamus compared to the cortex (Bixo et al., 1997). In addition, progesterone and its active isoforms were reported to be higher in young fertile subjects compared to postmenopausal samples in all brain regions (Bixo et al., 1997), implying that brain levels of the three steroids may rely on peripheral hormone synthesis and fall during the ageing process. These results suggest that the activity of PR should decrease with age in female samples but that was not seen here. This could be because the brain region investigated here, the frontal cortex, was different to those reported previously (Bixo et al., 1997), in addition the variant of PR investigated here in this chapter was PR-B and not PR-A.

5.5.4.2 In Alzheimer's Disease

For the disease state, levels of PR did not change in AD male and female samples. However, an *in vivo* study using the 3xTransgenic-AD (3xTg-AD) mice model, showed higher levels of 5α–DHPROG in the brain limbic areas (Caruso et al., 2013). The neuroprotective influence of progesterone has been shown in various experimental representations of neurodegenerative conditions, e.g. 3xTg-AD OVX mice administered progesterone either alone or with E2 for 12 weeks had notably diminished tau hyperphosphorylation (Carroll et al., 2007). Progesterone also assisted with the maintenance of cognitive skills in the 3xTg-AD model (Frye et al., 2007). Frye and colleagues have reported a positive influence of progesterone in an object placement test in rats when compared to controls that had undergone OVX (Frye et al., 2007). Therefore, even if PR-B did not change in AD in both sexes in this study, it would be interesting to investigate of PR-A.

5.5.5 MAPK/ERK and Akt expression in the frontal cortex of both sexes during ageing and in AD

5.5.5.1 Age as a risk factor

As mentioned previously, ER, GPER1 and PR all stimulate the activation of MAPK and Akt. Thus, it is not possible to identify definitively which of those receptors are responsible for the age-related increases of MAPK and Akt seen here in the male brain samples. Nonetheless, the samples used here are from the frontal cortex where ERβ is highly expressed. Also, the male samples showed an age-related increase in ERβ, resembling what is seen here with MAPK and Akt. On the other hand, the female brain samples lacked upregulation of ERβ activity and no increases in Akt and ERK were seen. Thus, it is possible that the significantly higher activity of Akt and MAPK observed here in male ageing brains relates to ERβ activity which could be involved in neuroprotection.

The non-genomic activity of ERβ occurs in relation to its phosphorylation status and stimulation of numerous cerebral signalling pathways, e.g MAPK/ERK and Akt. ERβ stimulation *in vivo* appears to be linked with heightened immunoreactivity and expression of MAPK/ERK and Akt phosphorylation in the brains of female rats (Benmansour et al., 2016, Ábrahám et al., 2004, Le and Belcher, 2010, Spencer-Segal et al., 2012). A further study implicated ERβ involvement in ERK1/2 phosphorylation in gonadotrophin-releasing hormone (GnRH) neuronal cells (Cheong et al., 2012). MAPK/ERK signalling governs a range of cellular functions, including replication, differentiation, longevity and demise, which are likely to contribute to keeping the brain healthy during advanced ageing (Kim and Choi, 2010). Also, Akt activity is an indicator of synaptic plasticity within the hippocampus (Nakai et al., 2014). Spencer-Segal et al. showed that ERβ is a mediator for the rise in phosphorylated Akt expression following a 6-hour administration of E2. Cerebral kinase pathways are implicated in cell differentiation, longevity, apoptosis, and proliferation; these processes are likely to play a role in healthy brain ageing (Spencer-Segal et al., 2012).

As mentioned above in section 5.5.2, ERβ was reported to potentiate ERK phosphorylation, believed to be one mechanism by which E2 regulates its cerebral neuroprotection activities (Zhao and Brinton, 2007). ERK1 and ERK2 share 84% of their amino acid sequences, thus it is hypothesized by researchers that ERK1 and ERK2 are homologs and compensate for each other. Indeed, it has been shown that brain levels of ERK1

phosphorylation in ERK2-KO mice were higher than in controls (Samuels et al., 2008). However, the increase in ERK2 activity in the ageing male brain seen here with no change in ERK1 might indicate a different pathway in their signalling processes to maintain normal brain function. To date, no study has been conducted to identify differences in the signalling pathways of ERK1 and ERK2 in the brain, but research into cancer has reported disparities between ERK1 and ERK2 function (Gagliardi et al., 2020).

5.5.5.2 In Alzheimer's Disease

In this chapter, Akt activity measured through pAkt was not altered in the frontal cortex of both sexes with AD. This result is in agreement with a previous clinical study where pAkt did not differ in the frontal cortex of individuals with AD compared to age-matched healthy subjects (Rickle et al., 2004). A preclinical *in vivo* study using mice cortex, reported that neuroprotection from E2 occurred through an Akt-mediated cascade (Honda et al., 2000). The neuroprotective effect of ERβ overexpression against apoptosis induced by Aβ was eradicated after Akt inhibition (Wang et al., 2012b). These observations support an earlier study reporting that stimulation of Akt by oestradiol in hippocampal nerve cells mitigated Aβ-mediated toxicity (Zhang et al., 2001). In this project, the lack of change in its activity levels suggests that Akt neuroprotection might be insufficient to counteract the large accumulation of Aβ seen in the AD samples in Chapter 3.

With regards to total MAPK, along with ERK1 and ERK2, expression in AD, no alterations in their levels were seen in this Chapter for both sexes. However, in other studies, overactivation of ERK was associated with NFT formation and Aβ protein deposition in the early stages of AD, resulting in impairment of hippocampal function and memory deficits in both mouse models and AD patients (Pei et al., 2002, Feld et al., 2014). The AD samples used here were in the later stages (Braak V & VI), which might be the reason why no changes in MAPK/ERK were seen in this project. Also, the samples used here were from the frontal cortex, while the increases in ERK described above were seen in the hippocampus.

5.6 Conclusion

In conclusion, the increase in the pERβ/tERβ ratio in ageing male but not female samples could result in a neuroprotective effect against AD in men. This ERβ increase in male samples was associated with an increase in the activity of the kinase signalling pathways, ERK2 and

Akt. Thus, the findings presented here suggest that ERβ is the significant oestrogen receptor responsible for mediating the neuroprotective effect of E2 in the human frontal cortex. Importantly, the lack of changes in ERβ expression and its associated signalling pathways during ageing in the female brain are likely to contribute to the higher incidence of AD in females compared to males.

The fact that the Western blotting (WB) data revealed no change in ER expression in the female frontal cortex in this chapter led to the hypothesis that the complexity of the frontal cortex structure, comprising varying cell types within the grey and white matter, could mask location-specific alterations in these receptors during the ageing and in AD. Thus, in the next Chapter, an in-depth investigation using immunohistochemistry of the cell types where ER are localised and their cellular distribution is described to provide a clear picture about ER expression in the female frontal cortex during brain ageing and in AD.

CHAPTER 6 _ Expression and Cellular Distribution of Oestrogen Receptors in Grey and White Matter in Females During Brain Ageing and in Alzheimer Disease.

6.1 Introduction

The cerebral frontal cortex plays a critical role in executive functions, e.g. behavioural inhibition, planning and problem solving, and working memory (Kane and Engle, 2002). Structurally, the frontal cortex is composed of outer and inner layers referred to as grey (GM) and white (WM) matter, respectively. The cell components of both areas differ; the GM is mostly comprised of neurons whereas the WM predominantly contains myelinated neuronal axons and glial cells (Jiang et al., 2015a). E2 has been reported to regulate a variety of cognitive processes including working memory (Rapp et al., 2003b, Keenan et al., 2001).

Glia are the immune cells of the brain and CNS. There are two glial cell types; microglia and macroglia. For macroglia, the three major subtypes are astrocyte, oligodendrocyte, and ependymal cells. The ramified form of glia cells exists in a surveillance state, ready to respond to potential nerve cell challenges. Through their constant monitoring and interactions with various CNS components, glia make essential contributions to the maintenance of CNS homeostasis and development processes, including apoptosis, neurogenesis, neural network modelling and synaptic pruning (Paolicelli et al., 2011, Hoshiko et al., 2012, Pont-Lezica et al., 2014, Prinz and Priller, 2014). Being phagocytes, glia are tasked with removing protein aggregates, cell waste and degrading the Aβ peptide (Ries and Sastre, 2016). Stereological counts of glial cells have not revealed any population changes in the ageing human brain (Fabricius et al., 2013, Pelvig et al., 2008). However, morphological alterations lead to a decline in functionality, i.e. a dystrophic or senescent phase; glial cell activity gradually diminishes with advancing age, decreasing their capacity to protect and defend ageing neural tissue (Streit et al., 2004). Thus, their failure to safeguard the CNS may facilitate the development of neurodegenerative diseases.

The efficacy of glia to mop up Aβ from the extracellular space declines with age (Hickman et al., 2008). In AD the presence of Aβ aggregates are hypothesised to harm glial cell function, generating a vicious cycle in which additional Aβ accumulates, further reducing glial cell function and resulting in more damage (Hopperton et al., 2018). E2 treatment of microglial cells derived from elderly human cortices was observed to accelerate Aβ removal through heightened microglial uptake of aggregated Aβ (Li et al., 2000). It may therefore be hypothesised that, in females, the age-related attrition in the protective behaviour of glial cells could be correlated with an individual's decline in reproductive hormone levels with advancing years.

ERα and ERβ are mostly found within the cell nucleus and cytoplasm; less than 2% are sited within the cell membrane (Carroll and Brown, 2006, Klinge, 2000) and, according to an IHC study performed in various cell types collected from animals and humans, it was concluded that ERs are mostly located in the nucleus, thus they are called nuclear receptor proteins (Pelletier, 2000). The subcellular localizations of these two receptors aid in governing gene transcription via traditional genomic cascades by their nuclear localization (Prossnitz and Barton, 2009, Prossnitz and Barton, 2014, Schultz-Norton et al., 2011) or through influencing non-genome-related cellular signalling pathways by their cytoplasmic and cell membrane location, e.g. stimulating MAPK and ERK activity (Wade et al., 2001) (Figure 5.1).

The non-genomic route encompasses a swift cellular reaction that takes place over seconds or minutes (Prossnitz et al., 2008), which is in contrast to de novo transcription or protein manufacture which takes 1-2 days (Falkenstein et al., 2000). Such rapid E2-linked responses are stimulated by a broad spectrum of signalling pathways and may be initiated by ER in close proximity to or in association with the cell membrane (Mitterling et al., 2010), ER translocation from the cytoplasm to the cell membrane (Sheldahl et al., 2008), engagement of intracellular ERα and ERβ with integral membrane proteins (Boulware et al., 2005, Bourque et al., 2013) or via GPER1 stimulation (Filardo et al., 2007, Filardo et al., 2000, Zhang et al., 2004b).

The cellular localisation of ER during brain ageing has been investigated in vivo (Chakraborty and Gore, 2004). A number of studies have documented cerebral ER alterations in rodents with ageing, changes that were noted to be region-specific. For instance, ERβ and ERα mRNA levels decreased in the female rat hippocampus with age (Yamaguchi-Shima and Yuri, 2007, Wilson et al., 2002), whilst in the female rodent cortex only ERα mRNA exhibited a decline with no alteration in ERβ titres (Cai et al., 2014, Yamaguchi-Shima and Yuri, 2007). In clinical studies, only the cellular distribution of ERα during cerebral ageing has been examined. Utilising an observational method of analysis, one group reported a rise in cortical nuclear ERα expression in postmenopausal females (Ishunina et al., 2007). However, as far as can be discerned, no research has evaluated the cellular distribution of ERβ during ageing of the frontal cortex.

Although there have been numerous studies which have examined cellular ER expression in AD utilising immunohistochemical (IHC) techniques, controversy remains as to whether ER

expression rises or falls during this pathological process as varying results were found (Hestiantoro and Swaab, 2004, Hong-Goka and Chang, 2004, Lu et al., 2004, Savaskan et al., 2001, Ishunina and Swaab, 2001, Ishunina et al., 2007, Ishunina et al., 2003). Mostly, these studies evaluated the presence of ER within other brain areas, e.g. the hippocampus, and not the cerebral cortex, the focus of this project.

6.2 Hypothesis and Aims

Hypothesis: The complexity of the frontal cortex structure, comprising varying cell types within the GM and WM, could mask specific alterations in ER detected with Western blotting during the cerebral ageing process and in AD. Thus, it is expected that ER expression will vary within the GM and WM during female frontal cortex ageing and in AD and differences will be identified in the cellular distribution of ERs.

To investigate this hypothesis, this chapter aims to:

1- Examine ER expression in neurons and glia through measuring immunoreactivity in the GM and WM during the female transition phase from pre-menopausal to post-menopausal.
2- Investigate indirectly the levels of ER genomic and non-genomic function in neurons during female frontal cortex ageing and AD. This aim will be carried out through measuring ER localization in the nucleus and cytoplasm and determining the nucleocytoplasmic ratio.

6.3 Experimental Methods

All the methods used in this chapter have previously been detailed in Chapter 2.

6.3.1 *Human Brain Tissue Samples*

Tissue sections obtained from the frontal cortex of five females for each age group listed in Tables 2.1 and 2.2 were used in this Chapter. The tissue sections were 4 μm thick and dissected from different frontal cortex locations from paraffin-embedded brain samples by the Brain Banks prior to receipt.

The selected samples were divided into three age groups, young (20-35 years), middle-aged (40-46 years) and elderly (71-80 years), together with an additional fourth group with AD

(71-88 years). Mean ages ± standard deviation (SD) for the selected subject cohorts were 27.6 ± 6 years, 43 ± 2.83 years, 74.8 ± 4 years and 76.6 ± 7.5 years for young, middle-aged, elderly and AD subjects, respectively. The mean PMI was 59.13 ± 18.84 hours.

6.3.2 *Experiments and Target Proteins*

The primary rabbit antibodies used for immunohistochemistry (Chapter 2; Table 2.5) were: (1) monoclonal antibodies (mAb) that recognise: (a) the ERα carboxy terminus epitope (Abcam, cat# ab108398) and (b) the ERα phosphorylation Ser167 residue (Abcam, cat# ab31478); and (2) polyclonal antibodies (pAb) against (a) the epitope located at the carboxy terminus ligand-binding domain of ERβ (Abcam, cat# ab5786) and (b) the ERβ phosphorylation Ser105 residue (Abcam, cat# ab62257). Details of the immunohistochemical processing and specificity of the antibodies are described in Chapter 2, Section 2.7. The IHC optimization for target proteins was done by me, and the staining work was carried out by the assistance of Michal Tynor under my supervision. The image acquisition and analysis in this chapter was all carried out by myself.

Negative controls for a single healthy young and elderly female were conducted. These negative controls were crucial to demonstrate that the reactions visualized when investigating the target proteins were due to the interaction of the epitope of the target molecule and the paratope of the antibody. The negative control was carried out with the same process used in the IHC method (Chapter 2, section 2.7.1), excluding the primary antibodies.

6.3.3 *Image Acquisition and Intensity Quantification*

In order to examine a particular organ, tissue or cell type, microscopic sections should be selected as representative of the whole sample (Hsia et al., 2010). In the brain, selecting specific sections for image capture fails to provide an equal opportunity for analysis and misses the effect of regional heterogeneity which could lead to biased conclusions. Thus, image capture should encompass all the different regions of investigated samples so as to avoid selection bias. For each GM and WM region, five different areas of tissue were selected randomly, and a total of 20 images at 20X and 63X magnification were captured for analysis.

The intensity of DAB staining was evaluated semi-quantitatively with ImageJ Fiji. Given that the aim of this Chapter was to assess protein expression in the GM and WM of the frontal

cortex, with subsequent measurement of cellular protein location within the nuclei, two methods of data acquisition were utilised (Figure 6.1 and Figure 6.2).

6.3.4 *Statistical Analysis*

When calculating nuclear localisation, a concern is whether the macromolecule in question is more concentrated in the nucleus or in the cytoplasm (Kelley and Paschal, 2019). In this case, a mean nuclear intensity value would be accompanied by a comparative mean cytoplasmic value. A frequently utilised parameter for the nucleocytoplasmic distribution of an investigated protein is therefore the nuclear:cytoplasmic mean intensity ratio. Values of < 1 or >1 indicated that the investigated protein was mainly cytoplasmic or nuclear in distribution, respectively. If the ratio = 1, it reflected an equal concentration in the two compartments.

It is important to highlight that the nucleocytoplasmic ratio measurements of ER were only examined in neuronal cells. This method could not be performed for WM glial cells as the receptors were only found to be present in the nuclei (Figures 6.7 and 6.12).

All data in this chapter were analysed using a factorial two-way ANOVA to compare the main effects of age and brain location [WM vs GM] or cellular location [Nucleus vs Cytoplasm] (IV), and the disease state and brain location (IV), as well as the interaction effects of those factors against each other [Location*Age or Location*Disease state Interaction]. If the lateral interaction [Location*Age or Location*Disease state Interaction] is significant it indicates that male and female presented with different patterns in protein expression throughout ageing or in the disease state, thus, to investigate that further, a one-way ANOVA would be conducted for each sex separately. The statistical results of two-way ANOVA will be included in the figures legends, while the post-hoc test results will be discussed more deeply in the results section.

Finally, the statistical validity and precision of the study were assessed by calculating the coefficient of error (CE). The sampling density should be sufficient to produce a low CE value, in general, below 15% (0.15) (Peterson, 2010).

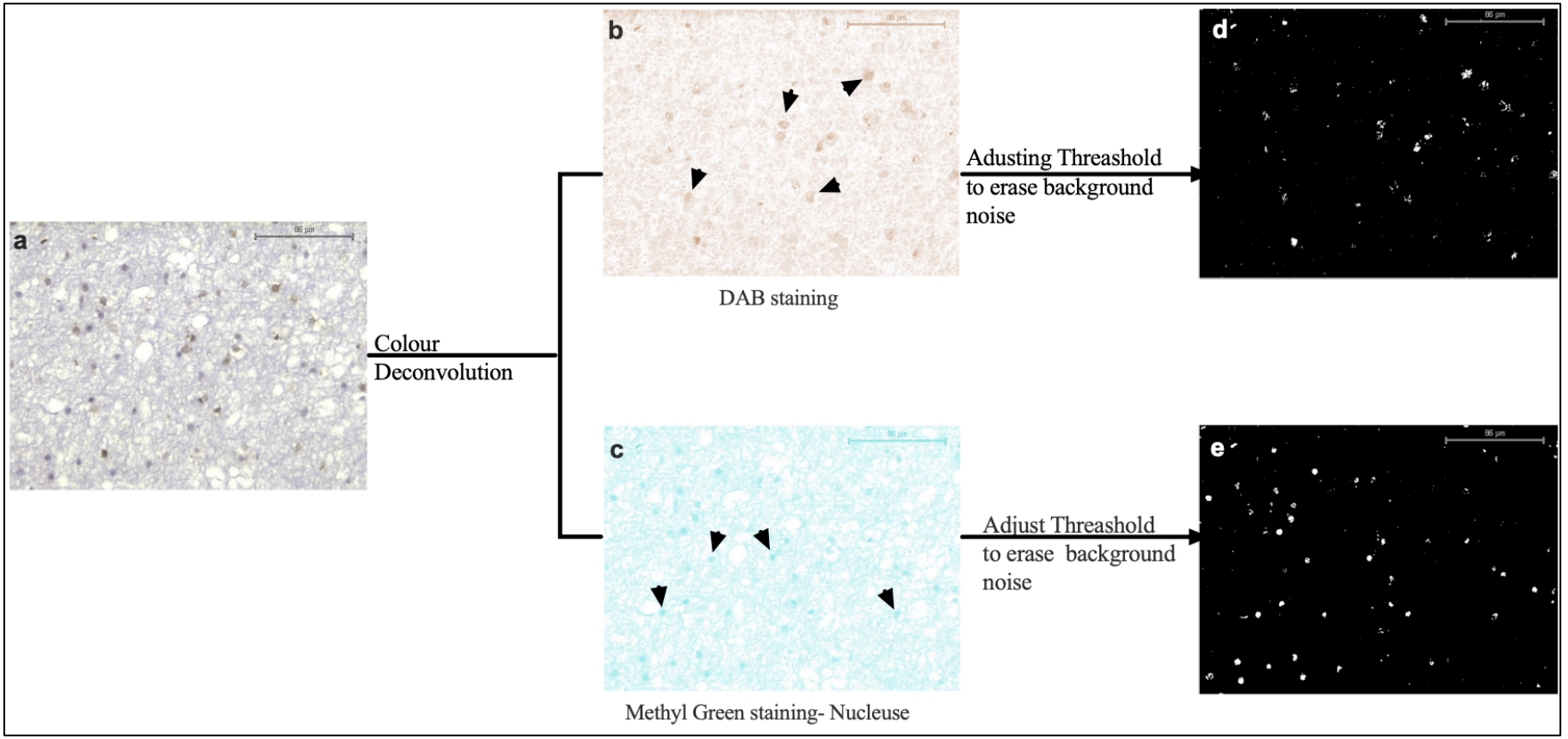


Figure 6.1 Schematic of the steps used for measuring DAB staining using ImageJ. The initial aim of this chapter was accomplished by employing a colour threshold technique. **a)** the original image was split into two channels, one representing DAB staining, black arrow identifying the DAB staining **(b)**, and another representing Methyl Green staining as an indicator of the nucleus, black arrow indicating the nucleus **(c)**. The DAB staining was measured through the colour threshold command, a different threshold adjustment was used for each image to minimize any background noise **(d)**. While the number of nuclei in the image **(c)** was counted either through using the cell counting tool or through using the Analyze particles command after adjusting the threshold in image **(e)**. Finally, the DAB intensity measurement was then normalized through dividing its value by the total number of nuclei in the image. For more detail about this method, refer to (Crowe and Yue, 2019). Scale bar = 85 μm

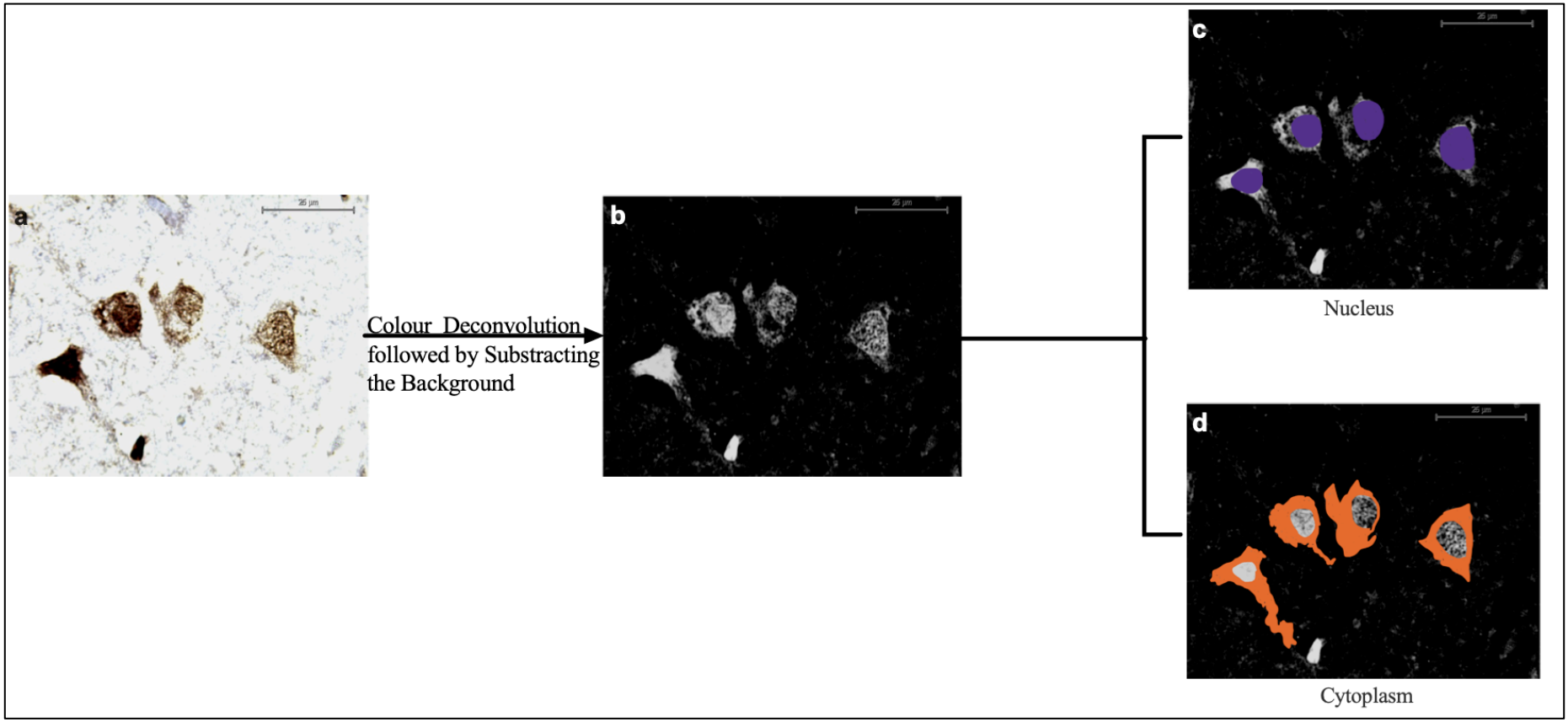


Figure 6.2 Schematic of the steps used for measuring the nucleocytoplasmic ratio using ImageJ. The cellular localization of ER was conducted utilising a region of interest (ROI) tool, which is a useful feature of ImageJ that enables the selection of specific areas for evaluation, or the deselection of areas of unwanted elements. (b) It is important to subtract the background intensity from the data, as the background can drastically change the cellular measurements (Kelley and Paschal, 2019). (c) The level of ER in the nucleus was measured through selecting the nucleus with the ROI tool and measuring the optical density (OD). (d) The cytoplasmic ER was measured also using ROI. Finally the nucleocytoplasmic ratio was calculated by dividing the measurements of the nuclei by the measurements of the cytoplasm (Kelley and Paschal, 2019). Scale Bar = 25µm.

6.4 Results

6.4.1 *Grey Matter and White Matter regions in Frontal Cortex Samples*

The fixed brain frontal cortex sections used in this project comprised both GM and WM regions (Figure 6.3A). Differences in the location and number of neuronal and supportive cells within GM and WM regions are well-documented (Jiang et al., 2015a). This was evidenced using a single healthy young female section (SD023/08) which was stained with neuronal, microglial and astrocyte/glial markers, i.e. FOXG1, CX3CR1 and GFAP, respectively.

FOXG1 is a transcription factor which is only found in neuronal cells (Adesina et al., 2015, Gunhanlar et al., 2018). Here, immunoreactivity for FOXG1 was seen in the nuclei of cells in the GM and WM. These nuclei are round in shape and are larger in size than glial cells, resembling the description of neuronal cell nuclei (Arsene et al., 2011). In addition, large numbers of neurones, identified by FOXG1, were found in the GM as expected (Figures 6.3 A and D), with much lower numbers in the WM (Figures 6.3 B and C).

CX3CR1, a marker of microglia cells (Hickman et al., 2019), produced the opposite result; the WM exhibited a higher number of microglial cells than the GM (Figures 6.4 A and B). In addition, as seen in the image for CX3CR1 labelling in the WM (Figure 6.4 C), inactive microglia cells presented with small nuclei and little cytoplasm, while active microglia had much larger nuclei and more extensive cytoplasm (Blaylock, 2013, Lier et al., 2021), which agrees with results from previous neurohistological studies (Arsene et al., 2011).

Finally, macroglia/astrocytes labelled with GFAP (Zhang et al., 2019) were strongly visible at the border between the GM and WM (Figures 6.5 A and B), and in the outermost layer of the GM (Figure 6.5 A) and had the classic star-shaped structure (Ferrer, 2017). Some astrocyte/glial cells were also seen within both the GM and WM (Figures 6.5 C and D).

Based on these data and the morphological findings, the GM and WM were considered as being representative of overall target protein expression in neuronal or microglial cells, respectively. Thus, during image acquisition and analysis, WM and GM territories were evaluated separately.

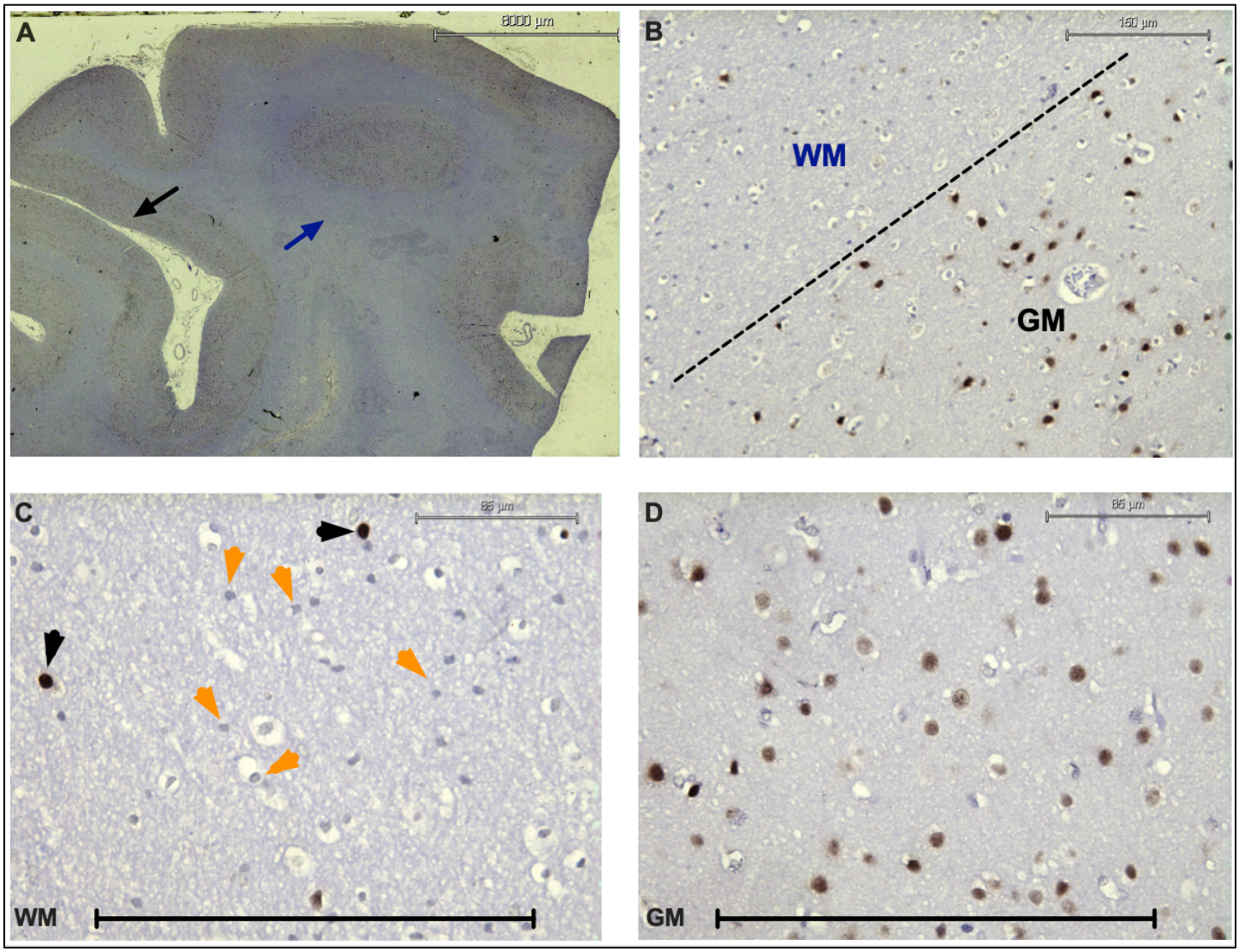


Figure 6.3 Neuronal cell location in a young, healthy, female frontal cortex. FOXG1, a transcription factor protein, was used as a neuronal marker, it stained the neuronal nuclei. Neuronal cells can be identified from the shape of their nucleus, the neuronal nuclei are larger in size and have a round shape compared to non-neuronal cells (**C**, black arrows indicating neuronal cells, orange arrows indicating non-neuronal cells). **(A)** Neuronal cells were found to be widespread in the grey matter, GM (black arrow) compared with the white matter, WM (blue arrow), scale bar = 8000 µm. (**B**) Image captured at the interface between the WM and GM regions, scale bar = 150 µm. (**C**) WM region, and (**D**) GM region; scale bar = 85 µm.

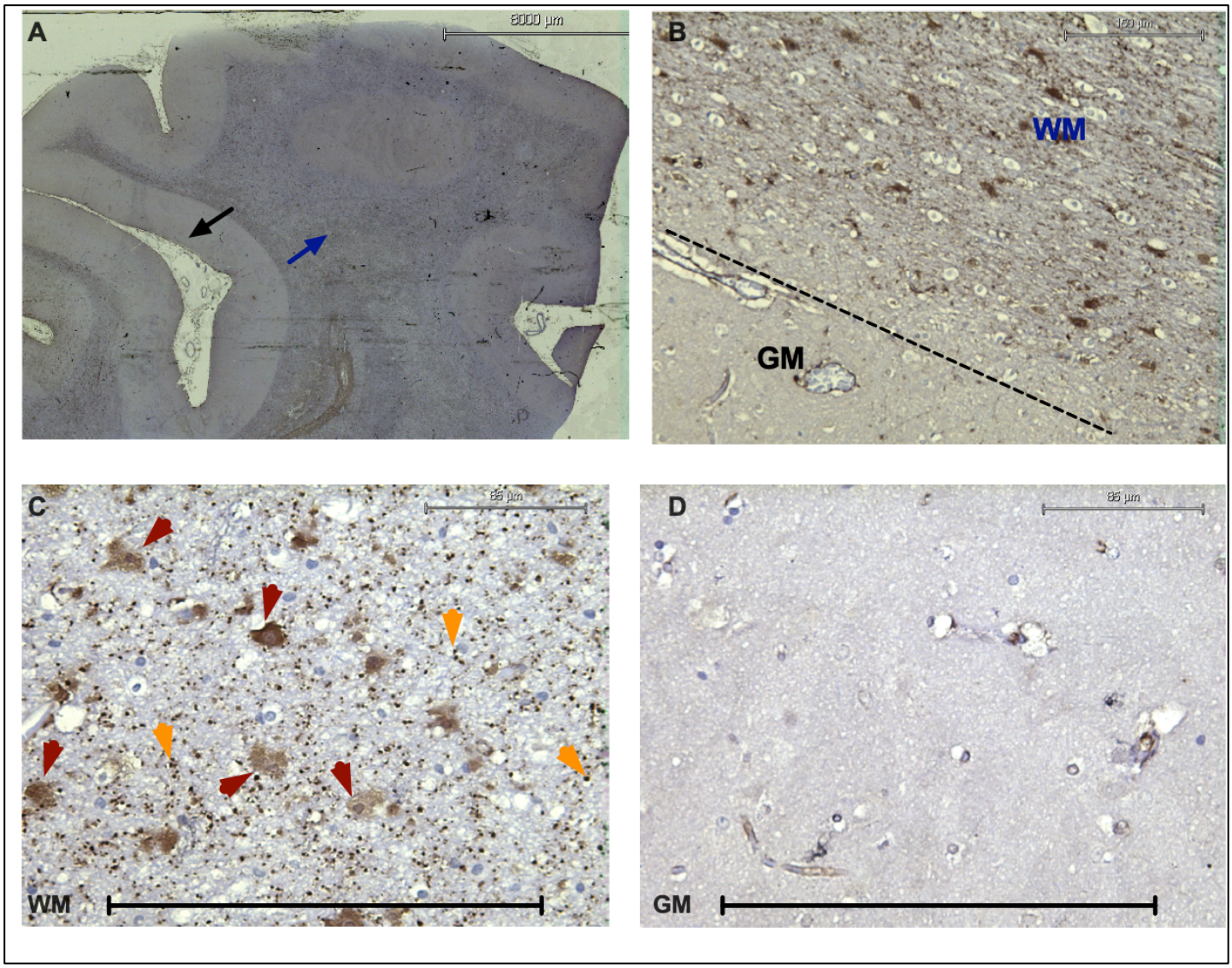


Figure 6.4 Microglial location in a young, healthy, female frontal cortex. CX3CR1 was used as a marker for microglial cells. **(A)** Microglial cells was much more common in the white matter, WM (blue arrow) compared with the grey matter, GM (black arrow), scale bar = 8000 µm. (**B**) Image captured at the interface between the WM and GM regions; scale bar = 150 µm. (**C**) WM region, and (**D**) GM region; scale bar = 85 µm. Inactive and active microglial cells were visible in the WM **(C),** the inactive microglial cells had small nuclei and little visible cytoplasm (orange arrows), while the nuclei and cytoplasm of the active cells were much larger (red arrows) (Blaylock, 2013).

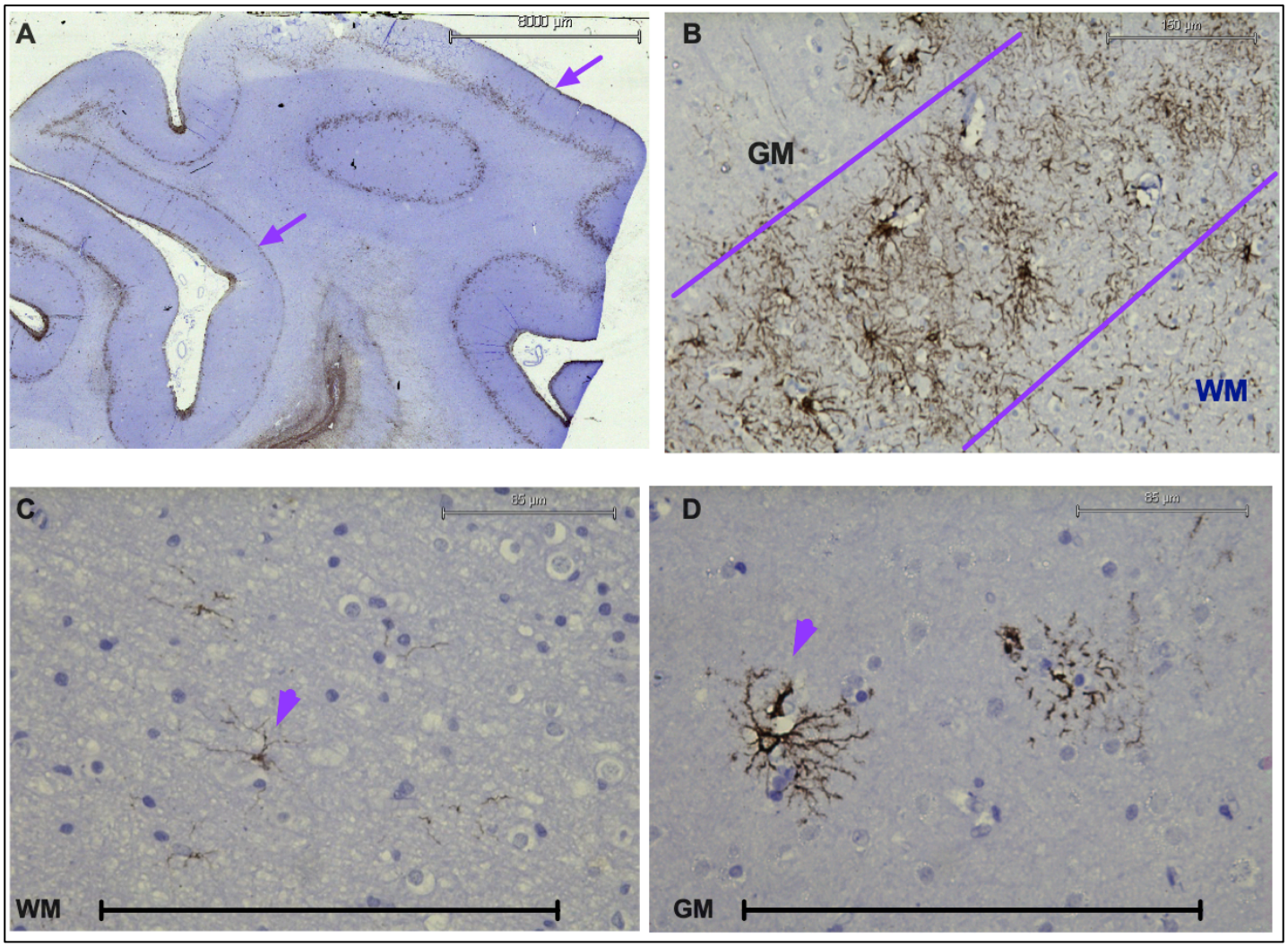


Figure 6.5 Astrocyte location in a young, healthy, female frontal cortex. GFAP was used as a marker for astrocytes and these cells were mainly found at the border between the white and grey matter (WM/GM) (**A**, purple arrows) scale bar = 8000 µm. (**B**) Image captured at the WM and GM border; scale bar = 150 µm. (**C**) WM region, and (**D**) GM region; scale bar = 85 µm. Astrocytes can be distinguished by their star-shaped structure (**C** & **D**, purple arrows).

6.4.2 *ERα Immunoreactivity in the GM and WM During Frontal Cortex Ageing and in AD*

ERα immunoreactivity was seen in neuronal cells (Figure 6.6) and in microglial cells (Figure 6.7). Furthermore, based on the FOXG1 staining of neuronal nuclei, for the ERα staining, the morphological shapes of neuronal cells could be identified in the GM with their large round nuclei, visible cell body and neuronal processes (Figure 6.6) and could be distinguished morphologically from the microglial cells in the WM (Figure 6.7). This supports the previous results of the GM mainly containing neuronal cells and the WM microglial cells.

Regarding semi-quantitative determination of ERα expression, the GM showed no differences in the mean immunoreactivity values for tERα levels during brain ageing, whilst pERα declined significantly ($p< 0.05$, Figures 6.6 and 6.8a). In comparison, the WM had a significant age-related decrease in tERα; however, no changes in pERα were observed in the WM ($p< 0.05$, Figures 6.7 and 6.8a).

In AD, the levels of total and phosphorylated ERα were significantly diminished in both the GM and WM compared to the ND samples ($p<0.05$; Figures 6.6, 6.7 and 6.8b).

The ratio of phosphorylated:total ERα was determined; no variations were seen during ageing or in AD in either in the GM or WM regions (Figures 6.8 c and d).

Finally, the immunoreactivity for both ERα forms was significantly higher in GM neurons than WM glial cells for all age groups and in AD ($p < 0.002$; Figures 6.8 a and b).

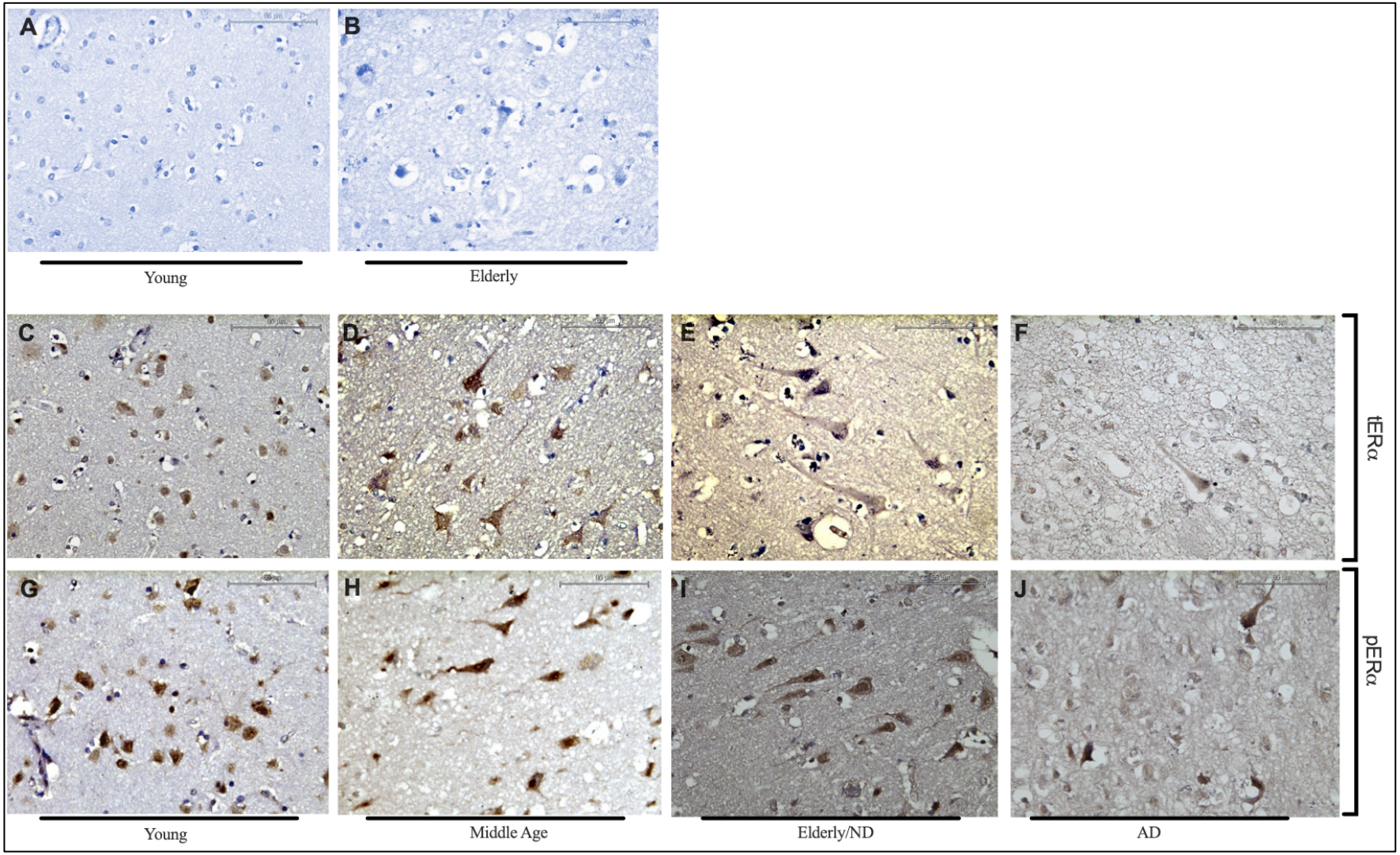


Figure 6.6 Representative Grey Matter (GM) region of female frontal cortex stained for total (C-F) and phosphorylated (G-J) ERα. Negative control of healthy young (**A**) and elderly (**B**) GM. Different age groups of young (**C** and **G**), middle-aged (**D** and **H**), elderly/ND (**E** and **F**) and AD (**F** and **J**) samples. n= 5, scale bar = 85 μm. AD = Alzheimer's disease, ND = no disease.

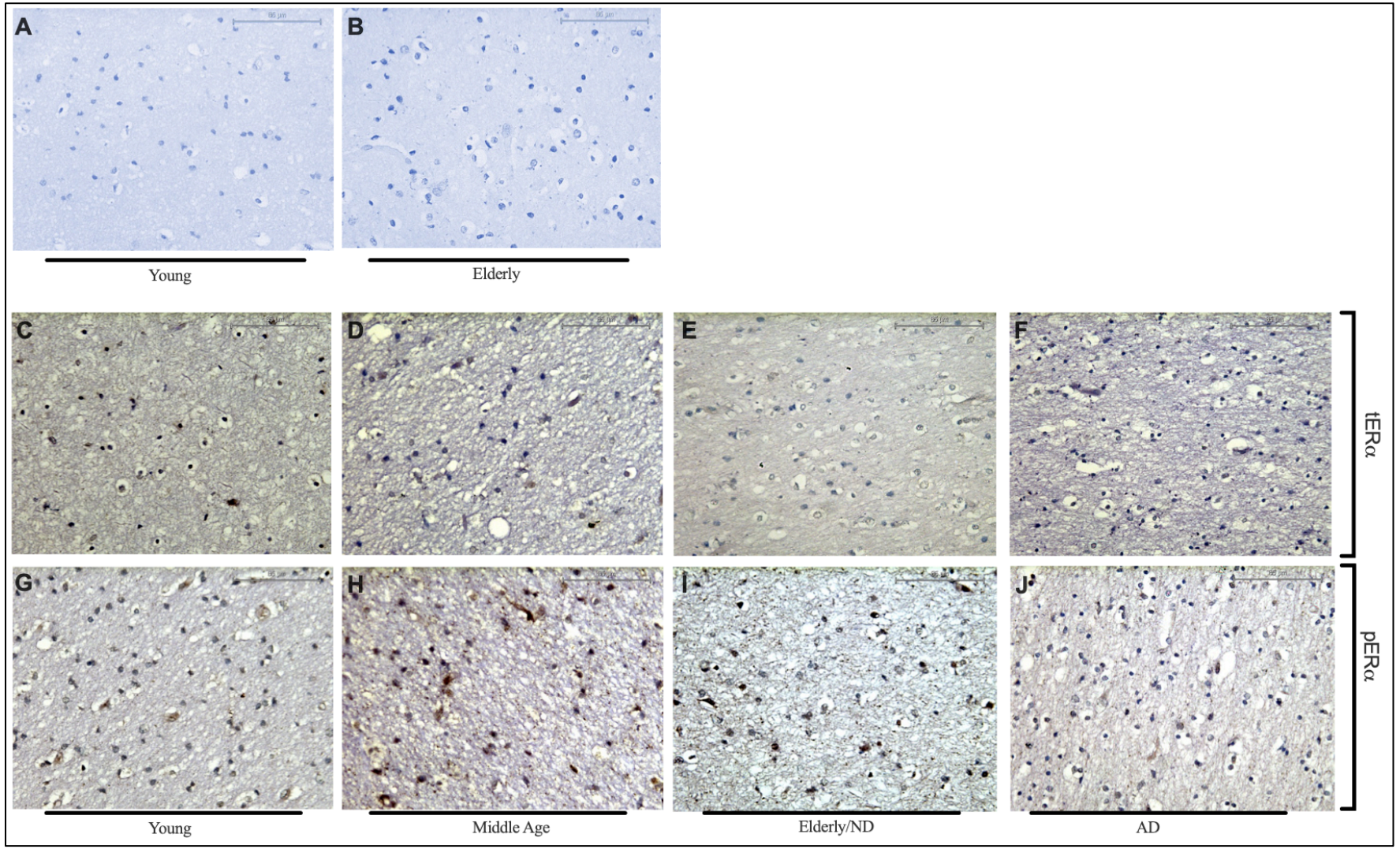


Figure 6.7 Representative White Matter (WM) region of female frontal cortex stained for total (C-F) and phosphorylated (G-J) ERα. Negative control of healthy young (**A**) and elderly (**B**) GM. Different age groups of young (**C** and **G**), middle-aged (**D** and **H**), elderly/ND (**E** and **F**) and AD (**F** and **J**) samples. n= 5, scale bar = 85 μm. AD = Alzheimer's disease, ND = no disease.

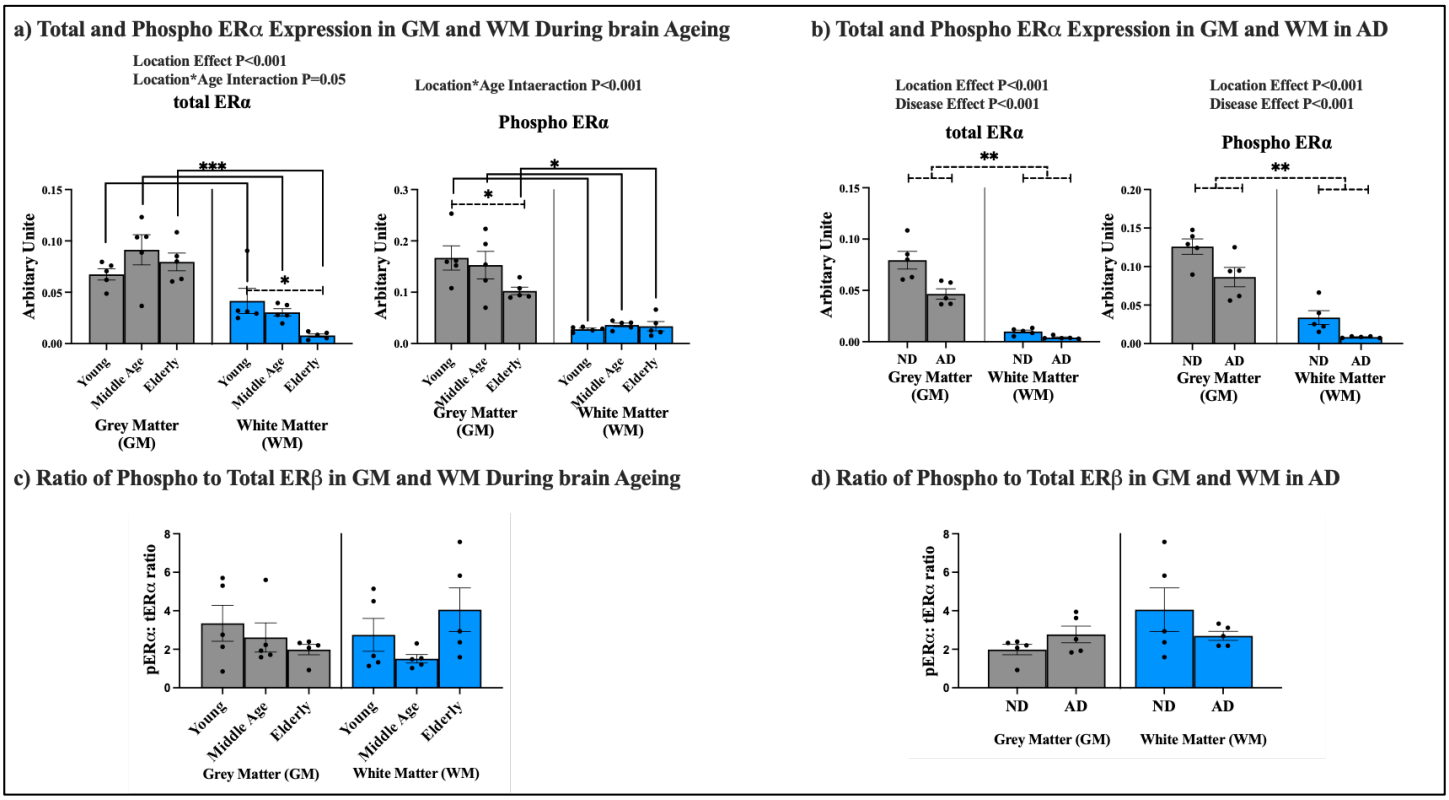


Figure 6.8 Expression of ERα in GM and WM of female frontal cortex during ageing and in AD. (a) represent quantification of total and phosphorylated ERα in GM and WM areas during frontal cortex ageing, two-way ANOVA revealed a higher level of ERα in GM than in WM area, in addition an age related decrease in tERα and pERα was calculated in WM and GM respectively (Location $F(2,24)=88.77$, $P<0.001$ and Location*Age $F(2,24)=3.51$, $P=0.05$ for tERα) and (Location*Age $F(1,24)=73.25$, $P<0.001$ for pERα). **(b)** Represent quantification of total and phosphorylated ERα in GM and WM areas in AD, two-way ANOVA revealed a higher level of ERα in GM than in WM area (Location $F(1,16)=122.6$, $P<0.001$ and $F(1,16)=85.51$, $P<0.001$ for tERα and pERα respectively), in addition, the expression of ERα decline significantly in AD in both areas of GM and WM (Disease State $F(1,16)=14.64$, $P=0.001$ and $F(1,16)=12.44$, $P=0.003$ for tERα and pERα respectively). (**c** and **d)** Show the ratio of phospho:total ERα in all groups, with two-way ANOVA revealing no significant effect of location and age or location and disease state on the investigated protein. n = 5 for each group; the analysis was conducted with 20X images. Data are presented as mean of DAB staining intensities standardised by nuclei number ± S.E.M. *p < 0.05, **p < 0.002, *** p < 0.001.

6.4.3 *Nucleocytoplasmic Immunoreactivity of ERα in Neurons During Brain Ageing and in AD*

A higher magnification of neurons in GM regions displayed the subcellular distribution of ERα staining (Figure 6.9). The immunoreactivity for ERα was observed in the nuclei and cytoplasm of the neurons in all age groups and in the AD cohort.

During brain ageing, the immunoreactivity of tERα significantly declined in both neuron nuclei and cytoplasm ($p < 0.05$; Figures 6.9 and 6.10a). However, no changes were seen in relation to the pERα staining (Figures 6.9 and 6.10a). The apparent change in the phospho:total ERα ratio in both neuronal nuclei and cytoplasm failed to reach significance. Nonetheless, a trend towards an increased phospho:total ERα ratio could be seen in the nuclei ($p = 0.12$; Figure 6.10c). For AD samples, interestingly, significant upregulation in the immunoreactivity of tERα and pERα compared to ND samples was found in both nuclei and cytoplasm ($p < 0.05$; Figure 6.10b), but no changes were seen in the nucleocytoplasmic distribution of the phospho:total ERα ratio in this cohort (Figure 6.10d).

When the levels of nuclear and cytoplasmic immunoreactivity were compared, tERα immunoreactivity was significantly higher in the nuclei from the young and middle-aged groups, but this increase was attenuated and not significant in the elderly and AD subjects ($p < 0.05$, Figures 6.10a and b). However, with respect to pERα, significant increases in immunoreactivity were seen in the nuclei compared to the cytoplasm in all age groups and in AD samples ($p < 0.001$; Figures 6.10a and b). The ratio of phospho/total ERα was apparently higher in the nuclei than in the cytoplasm, however, a significantly higher level of this ratio was only seen in middle age nuclei compared to cytoplasm (Figure 6.10c). Secondly, when looking at the ratio of nuclear to cytoplasmic distribution, it was noted that the expression levels of tERα and pERα were greater in the nucleus than in the cytoplasm for all groups as the nucleocytoplasmic ratio was > 1 (Figures 6.11a and b). However, no significant differences in the nucleocytoplasmic distribution during frontal cortex ageing or in AD were found.

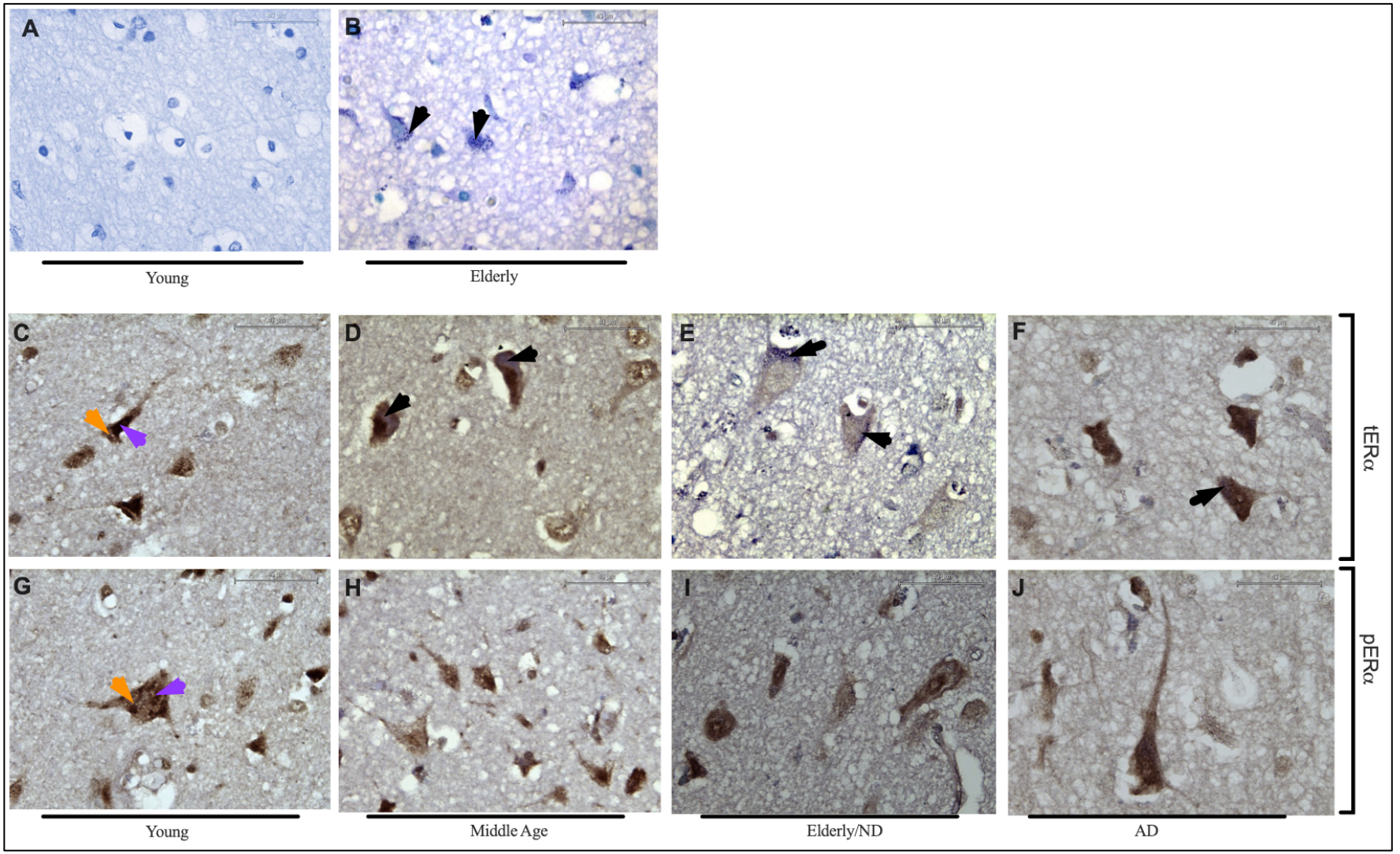


Figure 6.9 Representative Grey Matter (GM) region neuronal cells of female frontal cortex stained for total (C-F) and phosphorylated (G-J) ERα. Negative control of healthy young (**A**) and elderly (**B**) GM. Different age groups of young (**C** and **G**), middle-aged (**D** and **H**), elderly/ND (**E** and **F**) and AD (**F** and **J**) samples. n= 5, scale bar = 40 µm. AD = Alzheimer's disease, ND = no disease. Purple and orange arrows indicate the nucleus and cytoplasm, respectively. Some methyl green precipitation was identified in the cytoplasm of older neurons (black arrows)

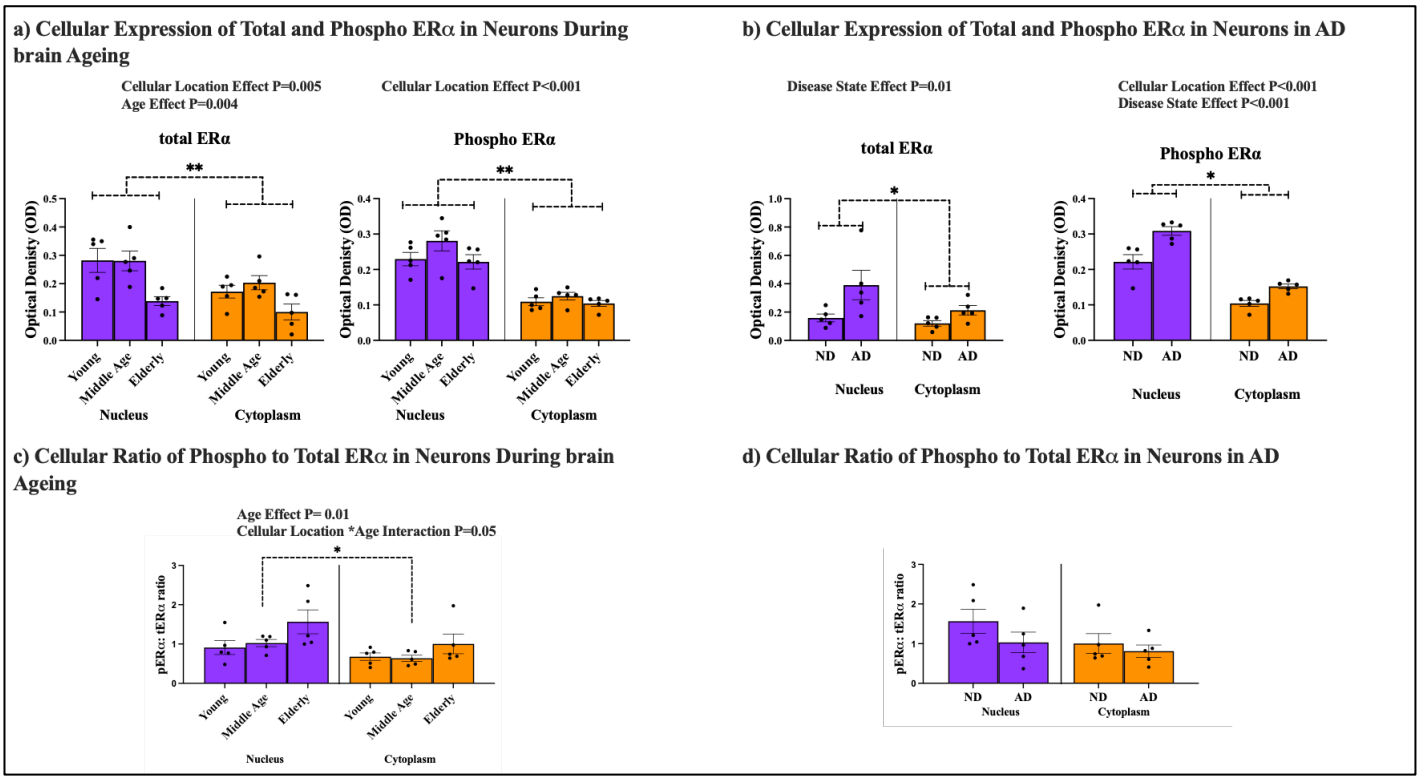


Figure 6.10 Quantification of total and phosphorylated ERα expression in the nucleus and cytoplasm of neuronal cells in the GM during female frontal cortex ageing and in AD. (a) Represent optical density measurements of both total and phosphorylated ERα in neuronal nuclei and cytoplasm during frontal cortex ageing, two-way ANOVA revealed a higher level of ERα in nucleus than in cytoplasm (Cellular Location $F(1,24)=9.68$, $P=0.005$ and $F(1,24)=82.91$, $P<0.001$ for tERα and pERα respectively), in addition an age-related decrease of tERα was calculated (Age $F(2,24)=7.03$, $P=0.004$ for tERα). **(b)** Represent optical density measurements of both total and phosphorylated ERα in neuronal nuclei and cytoplasm in AD, two-way ANOVA revealed a higher level of pERα in nucleus than in cytoplasm (Cellular Location $F(1,16)=111.1$, $P<0.001$ for pERα), in addition the expression of ERα increase significantly in AD in both areas of nucleus and cytoplasm (Disease State $F(1,16)=8.12$, $P=0.01$ and $F(1,16)=27.22$, $P<0.001$ for tERα and pERα respectively). (**c** and **d)** illustrate the ratio of phospho:total ERα in the nucleus and cytoplasm, two-way ANOVA revealed a higher level of pERα/tERα ratio in nucleus than in cytoplasm in middle age group (Age*Cellular Location $F(2,24)=2.81$, $P=0.05$) and(Age $F(2,24)=3.21.44$, $P=0.01$). n = 5 for each group; the analysis was conducted with 63X images. Data are presented as mean DAB staining intensities ± S.E.M. *p<0.05, **p<0.002. AD = Alzheimer's disease, ND = no disease.

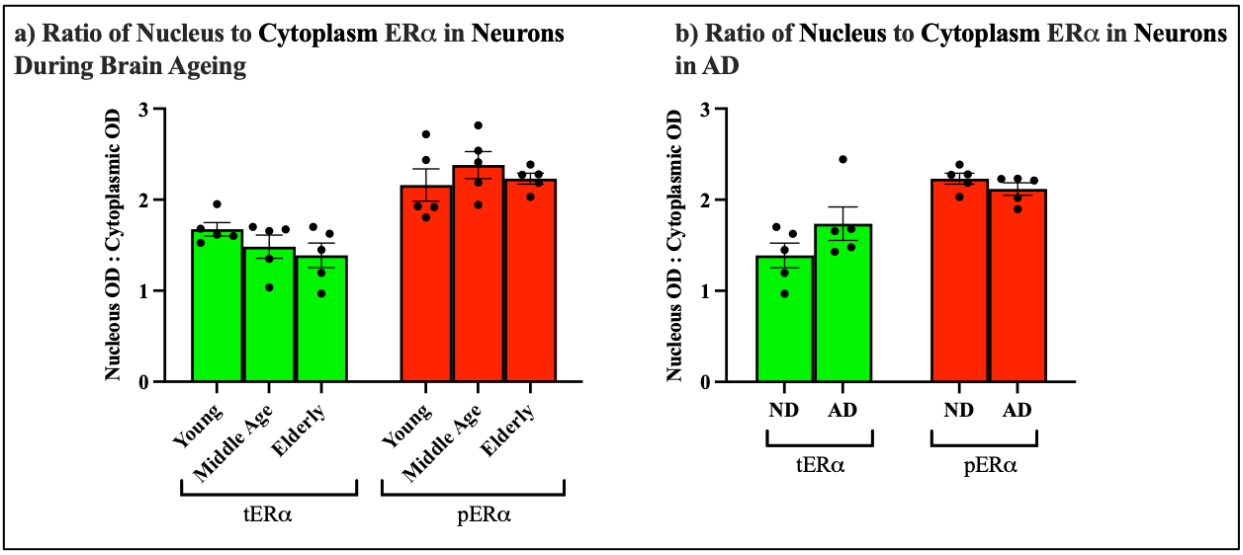


Figure 6.11 Nuclear to cytoplasmic ratio of total and phosphorylated ERα in neurons in the GM during female frontal cortex ageing (a) and in AD (b), two-way ANOVA revealing no significant effect of nucleus/cytoplasm ratio during age or in disease state on the investigated protein. n=5 for each group; the analysis was conducted with 63X images. Data are presented as mean DAB staining intensities of nucleocytoplasmic ratio of ERα ratio ± S.E.M.

6.4.4 *ERβ Immunoreactivity in the GM and WM During Frontal Cortex Ageing and in AD*

Similarly to ERα, ERβ immunoreactivity was positive in GM neuronal cells (Figure 6.12) and WM microglial cells (Figure 6.13).

ERβ semi-quantification in the GM revealed significantly higher immunoreactivity for both total and phosphorylated ERβ in young and elderly subjects compared to the middle-age group ($p < 0.05$; Figures 6.12 and 6.14a). On the other hand, there were no changes in tERβ or pERβ in the WM (Figures 6.13 and 6.14a). When the ratio of phospho:total ERβ was calculated, a significant decline was seen in the WM with age but not in the GM ($p<0.05$; Figure 6.14c).

With respect to the AD samples, both total and phosphorylated ERβ immunoreactivity fell significantly in the GM and WM compared to the ND samples ($p < 0.05$; Figure 6.14b). In addition, the phospho:total ERβ ratio decreased significantly in the GM compared to the ND samples ($p < 0.05$; Figure 6.14d) but not in the WM (Figure 6.14d).

Finally, in the elderly cohort only, both tERβ and pERβ demonstrated significantly higher degrees of immunoreactive staining in the GM neurons than in the WM glial cells ($p<0.002$; Figure 6.14a). In AD, only total ERβ immunoreactivity was significantly greater in the GM compared with the WM ($p<0.002$; Figure 6.14b).

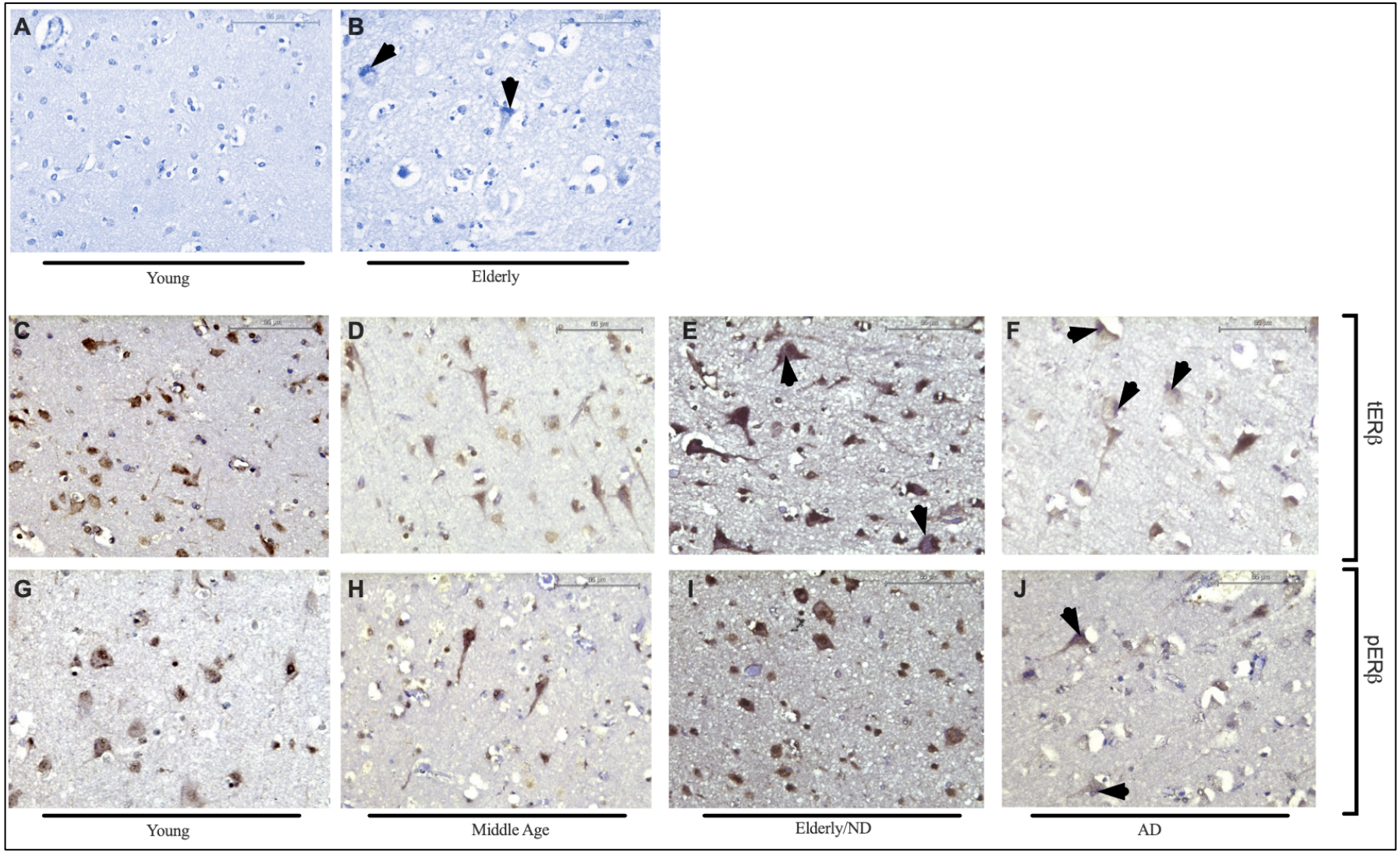


Figure 6.12 Representative Grey Matter (GM) region of female frontal cortex stained for total (C-F) and phosphorylated (G-J) ERβ. Negative control of healthy young (**A**) and elderly (**B**) GM. Different age groups of young (**C** and **G**), middle-aged (**D** and **H**), elderly/ND (**E** and **F**) and AD (**F** and **J**) samples. n= 5, scale bar = 85 μm. AD = Alzheimer's disease, ND = no disease.

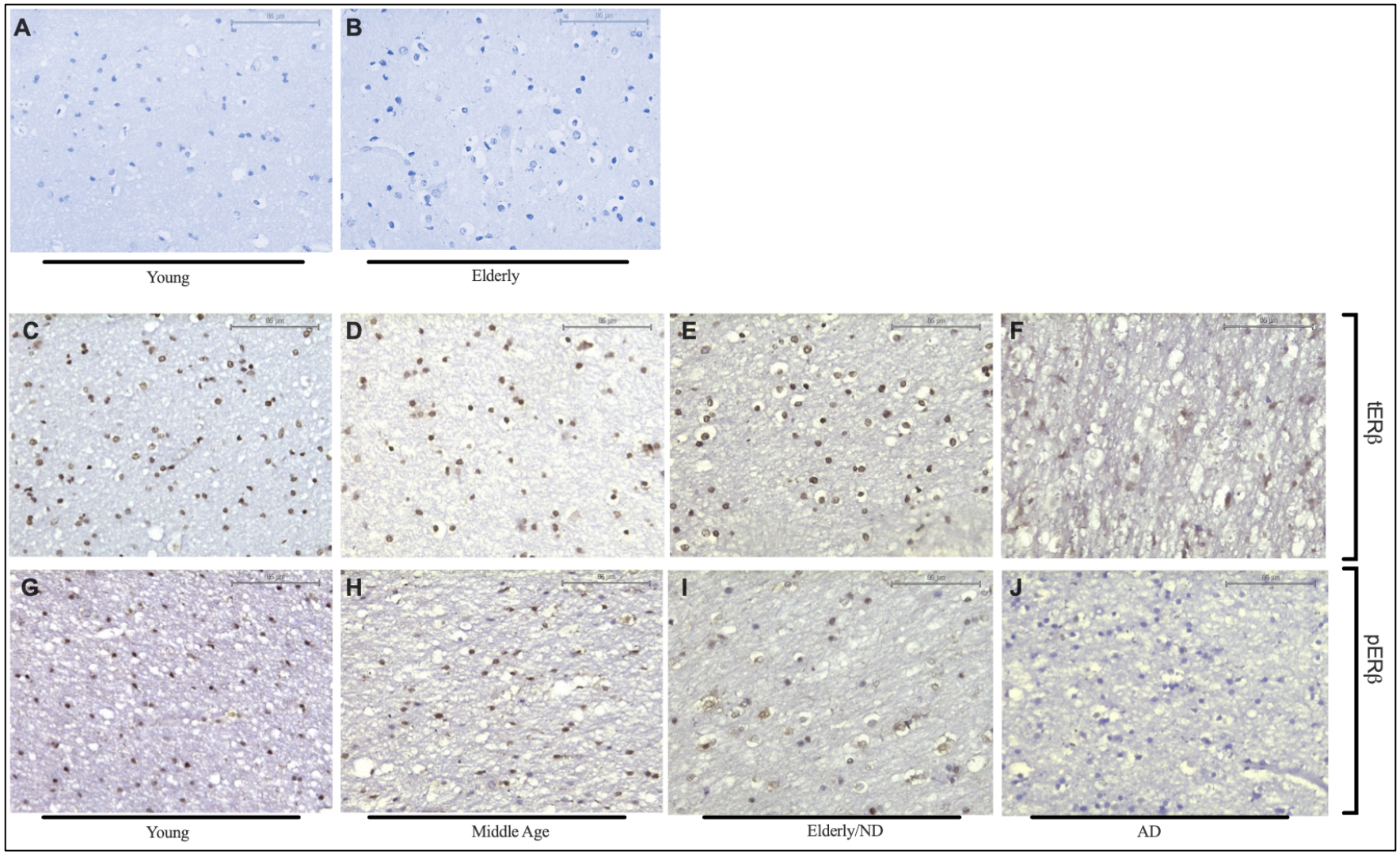


Figure 6.13 Representative White Matter (WM) region of female frontal cortex stained for total (C-F) and phosphorylated (G-J) ERβ. Negative control of healthy young (**A**) and elderly (**B**) GM. Different age groups of young (**C** and **G**), middle-aged (**D** and **H**), elderly/ND (**E** and **F**) and AD (**F** and **J**) samples. n= 5, scale bar = 85 μm. AD = Alzheimer's disease, ND = no disease.

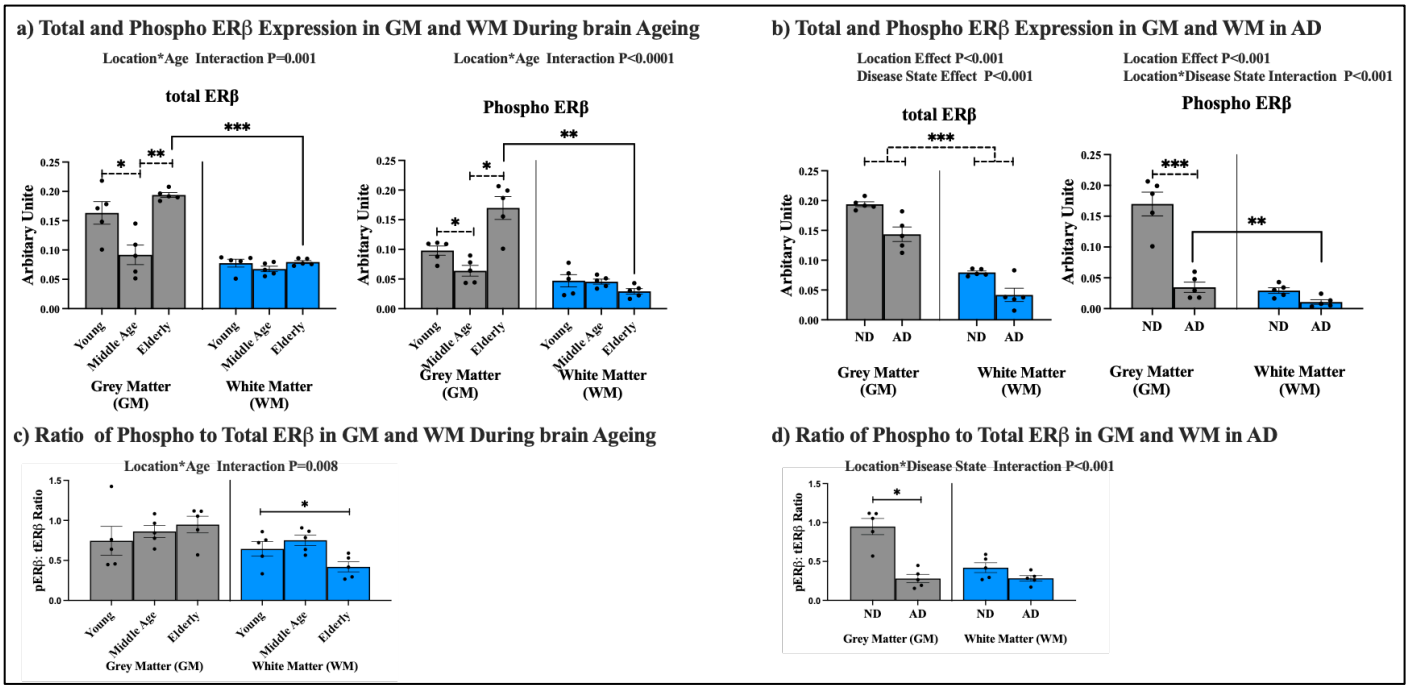


Figure 6.14 Expression of ERβ in GM and WM of female frontal cortex during ageing and in Alzheimer's disease (AD). (a) Represent quantification of total and phosphorylated ERβ in GM and WM areas during frontal cortex ageing, two-way ANOVA revealed that ERβ decline significantly from young to middle age group followed by increasing from middle age to elderly group in the WM area, in addition the levels of ERβ was significantly higher in GM than in WM area of the elderly age group (Location*Age $F(2,24)=8.64$, $P=0.001$ and $F(2,24)=18.19$, $P<0.0001$ for tERβ and pERβ respectively). (**b)** Represent quantification of total and phosphorylated ERβ in GM and WM areas during frontal cortex ageing in AD, two-way ANOVA revealed that ERβ levels was significantly higher in GM than in WM area in AD samples (Location $F(1,16)=157.5$, $P<0.001$ and $F(1,16)=56.79$, $P<0.001$ for tERβ and pERβ respectively), in addition, expression of ERβ did decline significantly in AD (Disease State $F(1,16)=26.06$, $P<0.001$ and Location*Disease state $F(1,16)=28.55$, $P<0.001$ for tERβ and pERβ respectively). (**c** and **d)** depict the ratio of phospho:total ERβ in all groups, two-way ANOVA revealed a significant age-related decline in the ratio of phospho:total ERβ in WM area (Location*Age $F(1,24)=8.35$, $P=0.008$), along with a significant decline of this ratio in AD GM (Location*Disease State $F(1,16)=34.36$, $P<0.001$). n=5 for each group; the analysis was conducted with 20X images. Data are presented as mean DAB staining intensities standardised by nuclei number ± S.E.M. *p<0.05, **p<0.002, *** p<0.001. AD = Alzheimer's disease, ND = no disease.

6.4.5 *Nucleocytoplasmic Immunoreactivity of ERβ in Neurons During Brain Ageing and in AD*

A higher magnification of neurons in GM regions displayed the subcellular distribution of ERβ staining (Figure 6.15). The immunoreactivity for ERβ was observed in neuronal nuclei and cytoplasm in all the age groups and in AD.

Neither the immunoreactivity for tERβ, or pERβ nor the phospho:total ERβ ratio were altered in either the nucleus or cytoplasm of neurons during frontal cortex ageing (Figures 6.15, and 6.16a and c). However, a trend towards an increase in the phospho:total ERβ ratio was evident in neuronal nuclei ($p = 0.06$) and cytoplasm ($p = 0.08$) during ageing.

In the AD samples, a significant decrease in the immunoreactivity of pERβ compared to the ND samples was seen for the nuclei alone but not in the cytoplasm ($p<0.05$; Figures 6.15 and 6.16b). No changes were seen in the ratio of phospho:total ERβ in either nuclear or cytoplasmic locations (Figure 6.16d). In one sample, strong staining indicating total ERβ accumulation within the GM was observed in some areas of the tissue, but without any discernible cell structure within that sample and there was no corresponding pERβ staining (Figure 6.18). However, the analysed images were captured in areas which did not contain any protein accumulation to minimize bias in the net results.

When the levels of nuclear and cytoplasmic immunoreactivity were compared, tERβ immunoreactivity was apparently higher in the nuclei for all groups, but this was only significant in the middle-aged group ($p < 0.05$, Figures 6.16a and b). However, with respect to pERβ, significant increases in immunoreactivity were seen in the nuclei compared to the cytoplasm in all age groups and in AD samples ($p<0.05$, $p<0.002$; Figures 6.16a and b). Finally, the nucleocytoplasmic distribution ratio was >1 indicating that the levels of total and phosphorylated ERβ were higher in the nucleus than in the cytoplasm for all groups (Figures 6.17a and b). The ERβ both forms nucleocytoplasmic ratio was significantly reduced during the menopause transition phase, i.e. between young and middle-aged groups and in AD compared to ND samples ($p < 0.05$; Figures 6.17a and b).

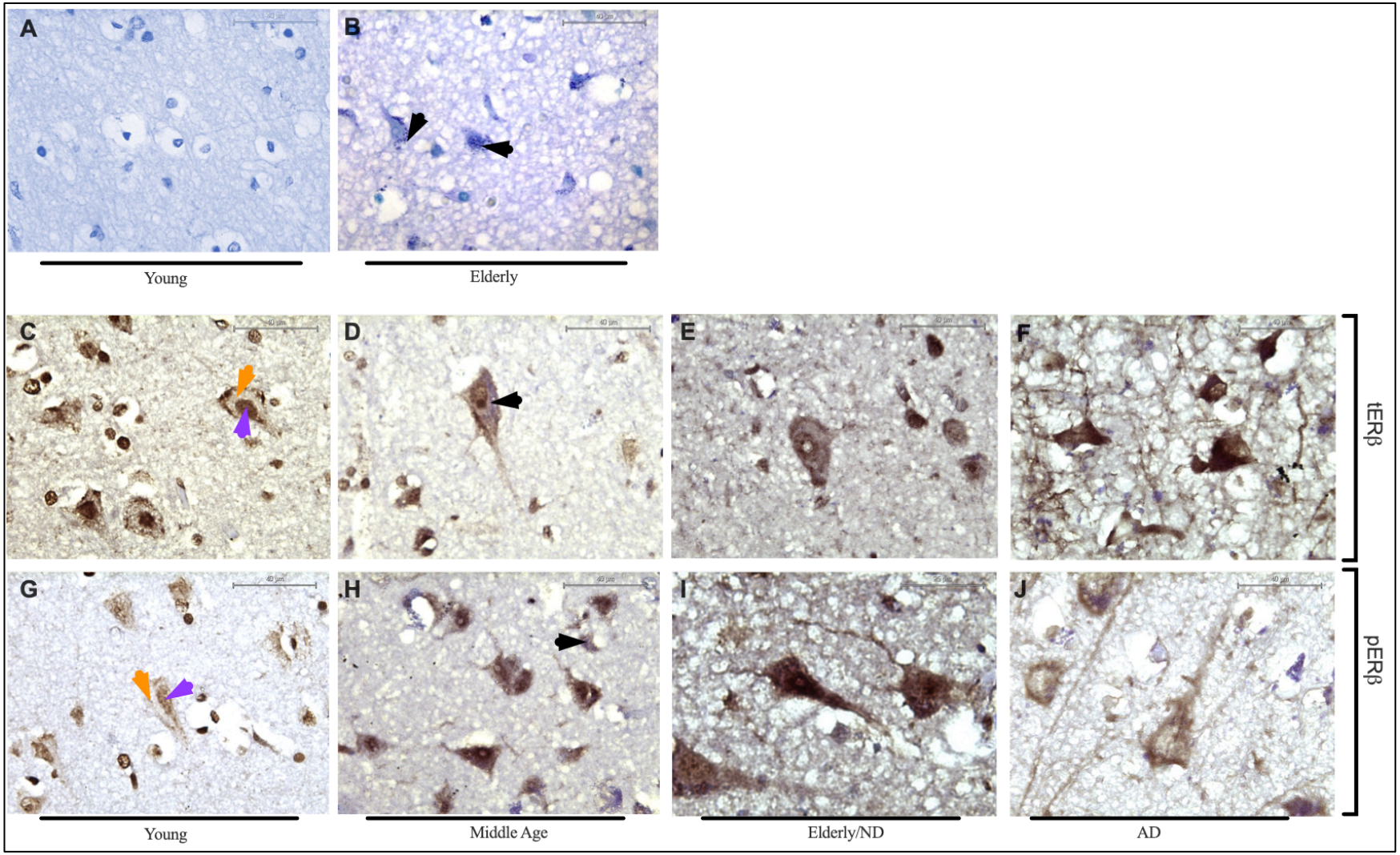


Figure 6.15 Representative Grey Matter (GM) region neuronal cells of female frontal cortex stained for total (C-F) and phosphorylated (G-J) ERβ. Negative control of healthy young (**A**) and elderly (**B**) GM. Different age groups of young (**C** and **G**), middle-aged (**D** and **H**), elderly/ND (**E** and **F**) and AD (**F** and **J**) samples. n= 5, scale bar = 40 μm. AD = Alzheimer's disease, ND = no disease. Purple and orange arrows indicate the nucleus and cytoplasm, respectively. Some methyl green precipitation was identified in the cytoplasm of older neurons (black arrows)

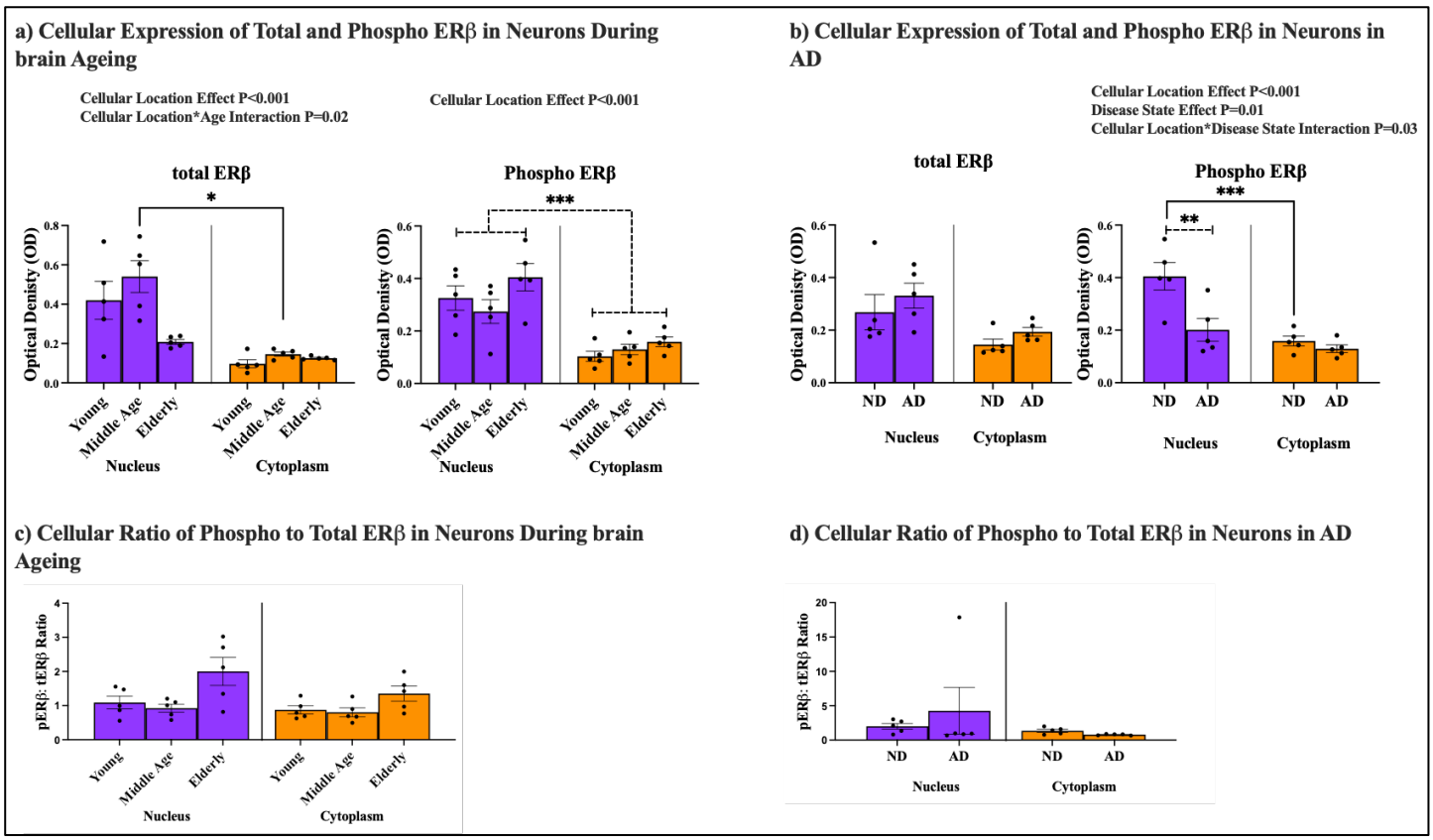


Figure 6.16 Quantification of total and phosphorylated ERβ expression in the nucleus and cytoplasm of neuronal cells in the GM during female frontal cortex ageing and in AD. (a) Represent optical density measurements of total and phosphorylated ERβ in the nucleus and cytoplasm of neurons during frontal cortex ageing, two-way ANOVA reveal significant higher levels of ERβ in nucleus than in the cytoplasm of neurons (Cellular Location*Age $F(2,24)=4.83$, $P=0.02$ for tERβ) and (Cellular Location $F(1,24)=38.58$, $P<0.001$ and $F(1,24)=46.64$, $P<0.001$ for tERβ and pERβ respectively). **(b)** Represent optical density measurements of total and phosphorylated ERβ in the nucleus and cytoplasm of neurons in AD, two-way ANOVA reveal significant higher levels of pERβ in nucleus than in cytoplasm of neurons in AD samples (Cellular Location $F(1,16)=27.62$, $P<0.001$ for pERβ), in addition levels of pERβ decline significantly in AD samples neuronal nucleus (Disease State $F(1,16)=8.04$, $P=0.01$ for pERβ and Cellular Location*Disease State $F(1,16)=5.94$, $P=0.03$). (**c** and **d)** show the ratio of phospho:total ERβ in the nucleus and cytoplasm, with two-way ANOVA reveal no significant effect of cellular location and age or cellular location and disease state on the investigated protein. n=5 for each group; the analysis was conducted with 63X images. Data are presented as mean DAB staining intensities ± S.E.M. *p<0.05, **p<0.002. AD = Alzheimer's disease, ND = no disease.

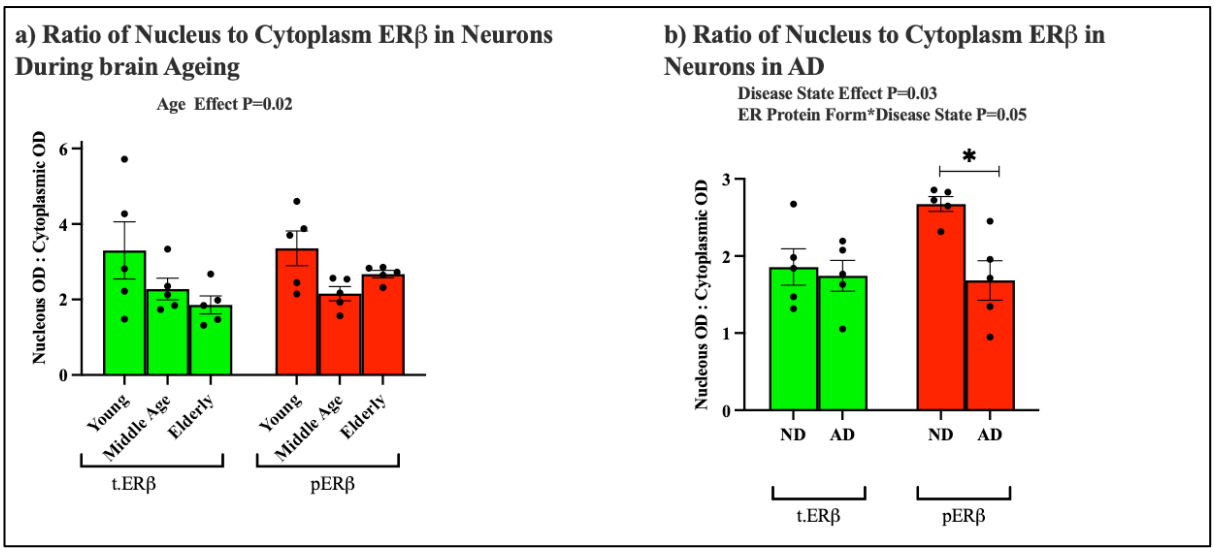


Figure 6.17 Nuclear to cytoplasmic ratio of total and phosphorylated ERβ in neurons in the GM during female frontal cortex ageing (a) and in AD (b). Two-way ANOVA revealed a significant decline of pERβ nucleus/cytoplasm ratio in middle age group (Age $F(2,24)=4.88$, $P=0.02$). In addition, pERβ nucleus/cytoplasm ratio also decline significantly in AD (ER protein form*Disease State $F(1,16)=4.51$, $P=0.05$) and (Disease State $F(1,16)=5.93$, $P=0.03$ for ER). n=5 for each group; the analysis was conducted with 63X images. Data are presented as mean DAB staining intensities for nucleocytoplasmic ratio of ERβ ± S.E.M. *$p<0.05$. AD = Alzheimer's disease, ND = no disease.

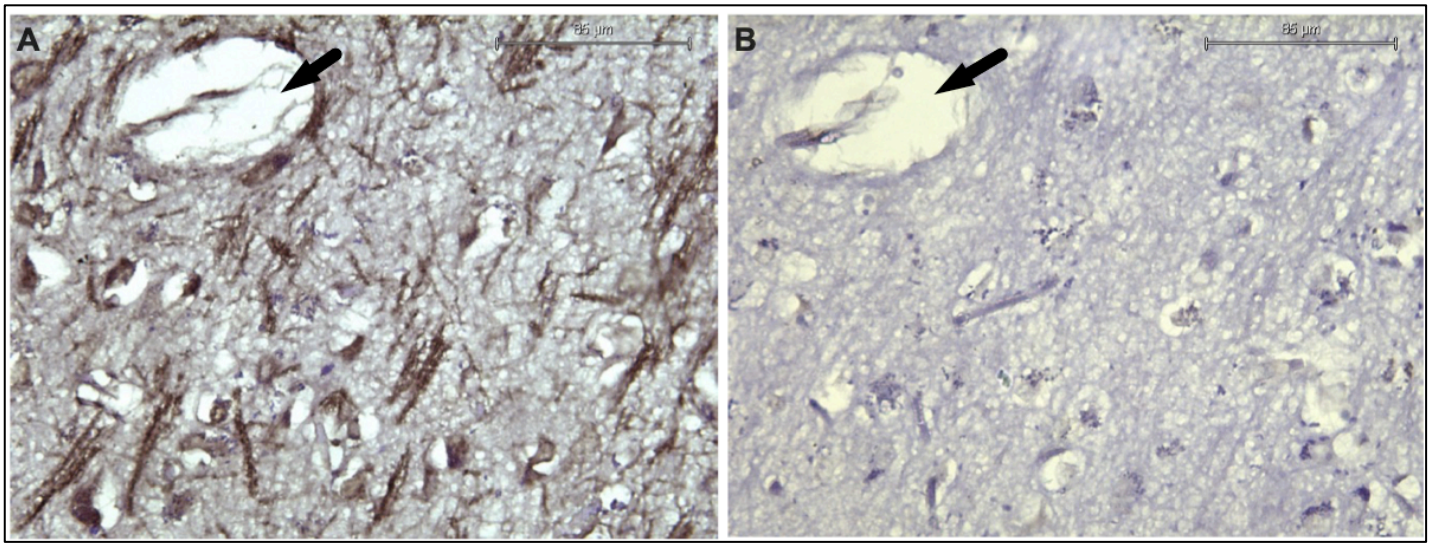


Figure 6.18 Accumulation of ERβ labelling in the GM of one of the AD samples. Image capture at 20X, scale bar = 85 µm. Black arrow points at a blood vessel which indicates the same brain location which was stained with total (**A**) and phosphorylated (**B**) ERβ.

6.4.6 *Statistical Validity and Precision of the Study*

In order to test the validity of the results obtained in this chapter, the coefficient of error (CE) was calculated for each of the groups by dividing the standard error of the mean over the mean of each sample [*CE = SEM / Mean*]. The measurements for each cohort are presented in Table 6.1. The red highlighting indicates CE values > 15% and occurs principally in the AD samples.

Table 6. 1 Coefficient of Error for all samples used in this Chapter.

	GM				WM			
Samples	**tERα**	**pERα**	**tERβ**	**pERβ**	**tERα**	**pERα**	**tERβ**	**pERβ**
Young_1	0.108	0.395	0.042	0.172	0.049	0.118	0.062	0.142
Young_2	0.146	0.124	0.137	0.110	0.109	0.060	0.071	0.091
Young_3	0.224	0.122	0.081	0.132	0.085	0.061	0.075	0.237
Young_4	0.159	0.094	0.091	0.176	0.158	3.193	0.076	0.114
Young_5	0.105	0.071	0.069	0.094	0.115	4.803	0.114	0.084
*Total Samples_CE	0.146	0.368	0.186	0.121	0.174	0.603	0.114	0.208
Middle-Aged_1	0.100	0.089	0.075	0.089	0.107	0.105	0.057	0.058
Middle-Aged_2	0.071	0.122	0.176	0.134	0.099	0.104	0.065	0.072
Middle-Aged_3	0.147	0.096	0.096	0.081	0.131	0.133	0.061	0.093
Middle-Aged_4	0.072	0.061	0.166	0.133	0.169	0.110	0.111	0.134
Middle-Aged_5	0.176	0.156	0.074	0.071	0.135	0.075	0.086	0.079
*Total Samples_CE	0.183	0.155	0.189	0.131	0.097	0.088	0.133	0.149
Elderly _1	0.178	0.084	0.080	0.081	0.095	0.109	0.097	0.126
Elderly _2	0.108	0.115	0.058	0.090	0.123	0.134	0.101	0.155
Elderly _3	0.083	0.080	0.056	0.051	0.091	0.105	0.053	0.097
Elderly _4	0.115	0.117	0.093	0.118	0.092	0.111	0.154	0.119
Elderly _5	0.152	0.098	0.069	0.061	0.103	0.145	0.081	0.103
*Total Samples_CE	0.132	0.077	0.099	0.145	0.059	0.065	0.171	0.085
AD _1	0.126	0.131	0.198	0.228	0.046	0.108	0.117	0.161
AD _2	0.126	0.111	0.127	0.211	0.561	0.119	0.202	0.243
AD _3	0.217	0.181	0.078	0.261	0.056	0.092	0.059	0.133
AD _4	0.178	0.078	0.079	0.205	0.053	0.141	0.175	0.219
AD _5	0.156	0.189	0.057	0.099	0.064	0.109	0.064	0.126
*Total Samples_CE	0.106	0.151	0.235	0.136	0.649	0.071	0.232	0.133

The **red highlighting** indicates CE values > 15%.

***** The CE value for each age group was calculated from the CE data of each sample, each group = Standard Error of the Mean (SEM)/Mean CE.

6.5 Discussion

This study is the first to use Image-J as a method for semi-quantifying the immunoreactivity of target proteins during frontal cortex ageing and in AD (Crowe and Yue, 2019, Kelley and Paschal, 2019). A previous clinical study used an observational IHC method reinforced by target protein mRNA quantification (Savaskan et al., 2001).

The current research has shown that, during the ageing process in healthy women, neurons in the GM exhibited a rise in ERβ immunoreactivity in elderly compared to young subjects. However, its nucleocytoplasmic expression level declined during the menopause transition phase. A fall in immunoreactivity for ERα phosphorylation at the Ser167 residue was observed in neurons during brain ageing, but with no changes in cellular distribution. Conversely, glial cells in the WM had a reduction in the phospho:total ERβ ratio and in total ERα immunoreactivity. The differences in ER immunoreactivity data, especially ERβ, in neurons (GM) and microglial cells (WM) might indicate a distinctive function of the respective ER within these cells during cerebral ageing. In sections from women diagnosed with AD, frontal cortex ERα and ERβ immunoreactivities were markedly diminished within both microglial cells (WM) and neurons (GM); this was associated with a fall in the nucleocytoplasmic distribution of both proteins.

6.5.1 *ER immunoreactivity in Neuron and Microglial Cells During Frontal Cortex Ageing and in AD*

6.5.1.1 Age as a Risk Factor

Both ERα and ERβ exhibited positive neuronal and glial immunoreactive staining in this Chapter. Previous immunohistochemical studies using observational methods have described the distribution of both receptors in mammalian neurons and glial cells in the brain (Azcoitia et al., 1999, Montague et al., 2008).

ERα has been shown to have neuroprotective functions as well as promoting synaptogenesis and synapse remodelling (Jelks et al., 2007). Studies have shown that a decline in synaptic density is associated with age (Masliah et al., 1993), which could be partially explained by the decrease in ERα seen in this study. Additionally, ERα has been reported to be the major ER responsible for neprilysin upregulation (Xiao et al., 2009), an enzyme which accelerates Aβ plaque degradation (Liang et al., 2010). Since pERα staining was lower in the

GM, where most cells are neurons, this decline in ERα phosphorylation may result in a fall in neuronal density during frontal cortex ageing and an increase in Aβ plaque formation.

With advancing age, serum E2 levels dramatically diminish in both human and rodent females; this E2 deprivation is associated with changes in mood, depression and evidence of cognitive impairment (Lamberts et al., 1997, Miranda et al., 1999, Chakraborty and Gore, 2004, Thakur and Sharma, 2006). In addition, the age-associated serum E2 drop in female rodents is associated with changes in intracerebral ER levels (Wise and Parsons, 1984, Wise et al., 1984, Wilson et al., 2002). Thus, although no study has reported any evidence of an early decline in cortical E2 levels during the menopause transition phase, titres have been observed to decline at a very advanced age, i.e. greater than 80 years old (Rosario et al., 2011). The decrease in neuronal ERα Ser167 phosphorylation, and thus likely receptor activity, observed in this Chapter suggest that cortical E2 activity might fall during the menopause transition phase.

The WM is the region in which neurons interact with microglial cells via their axons. The latter contribute to nerve cell survival through their role in neuronal ionic and metabolic homeostasis and synaptic transmission maintenance (Ma et al., 2016). In this study, WM microglial cell ERα levels showed the same pattern as for neuronal immunoreactivity with a decline in total ERα in elderly subjects. However, since no changes were observed with respect to its phosphorylated form, these results suggest that, in the cerebral ageing process, ERα may sustain its neuroprotection at the Ser167 site in the WM region of the frontal cortex. These ERα results are opposite to those reported by Ishunina et al. (Ishunina et al., 2007) who described an increase in hippocampal ERα immunoreactivity and mRNA during the menopause transition phase. The disparity between these two data sets potentially infers that ERα activity varies within diverse brain regions and that its expression is region-specific; this is in keeping with previous data from animal studies (Yamaguchi-Shima and Yuri, 2007, Wilson et al., 2002, Cai et al., 2014).

In contrast to ERα, ERβ immunoreactivity in neuronal cells from GM regions increased with age. Owing to the dominance of ERβ distribution in the cortex, hippocampus and basal forebrain nuclei, it has been suggested that ERβ is a critical protein which mediates the effects of E2 on cognitive functions (Boersma and Mosselman, 2000, Savaskan et al., 2001). Thus, the rise in both total and phosphorylated (active) ERβ might suggest significant stimulation of its neuroprotective actions against age-related physiological changes. In this project, this

stimulation could be the age-related build-up of Aβ in the brains of these patients reported in Chapter 3. Several clinical studies, which reported a neuroprotective action of E2 therapy in reducing the severity of AD (Gibbs and Aggarwal, 1998, Green and Simpkins, 2000), have postulated that this effect could be mediated through ERβ activation. These neuroprotective actions could occur through simulation of antioxidant activity, activation of MAPK and Akt signalling, alterations in anti-apoptotic Bcl-2 protein expression and adjustments in intracellular calcium homeostasis (Green and Simpkins, 2000), several of which were investigated in Chapter 5. In addition, E2 could stimulate the activity of cholinergic projections from basal forebrain neurons to the cortex and hippocampus, and may, therefore, assist in overcoming cholinergic deficits in AD (Gibbs and Aggarwal, 1998). Also, ERβ stimulated and promoted neuronal survival and synaptogenesis in aged cortical neurons at a more rapid rate than ERα (Sellers et al., 2015). Finally, the most important documented neuroprotective function of ERβ against AD biomarkers is its ability to assist in the breakdown of Aβ through stimulation of the synthesis/expression of the Aβ degrading enzymes such as IDE and neprilysin, although stimulation of the latter appears to a lesser extent than with ERα (Zhao et al., 2011, Xiao et al., 2009).

ERβ immunoreactivity within microglial cells in the WM fell during the ageing process. This was seen in the phospho:total ERβ ratio; these findings are in contrast to those observed in neuronal cells above. Preclinical studies have documented that Aβ internalisation was increased in cultured microglia cells following pre-treatment with E2 (Li et al., 2000). It has been demonstrated that E2 treatment of microglial cells derived from aged human cortex accelerated Aβ removal through enhanced microglial aggregated Aβ uptake; these results were mediated by both ERα and ERβ agonists (Li et al., 2000). In another study, 17β-oestradiol treatment of the murine microglial-like N9 cell line generated elevated Aβ uptake along with induction of phagocytosed Aβ degradation (Harris-White et al., 2001). Thus, age-dependent attrition of glial protective functionality against Aβ (Streit et al., 2004) could potentially be related to the reduction in ERβ expression observed in the current study.

From the results reported in this Chapter, whilst age-related amplification of ERβ expression was evident in neuronal regions, the decline in its activity within glial cells could act as an impediment to its capacity to eradicate frontal cortex Aβ accumulation. Future in vitro

and in vivo experiments should study glial cells alongside neuronal cells in order to clarify the importance of glial ERβ loss in the aetiology of AD.

The widespread subcellular distribution of these ER proteins in neurons as opposed to glial cells, suggests that they have a critical role in nerve cell mechanisms. Furthermore, it is well-documented in vivo that ER can form homodimers or heterodimers (Cowley et al., 1997, Pace et al., 1997, Pettersson et al., 1997). From the data in this Chapter, it is likely that both ERα and ERβ are colocalised in neurons and microglial cells in keeping with earlier *in vivo* observations (Shughrue et al., 1998), which would support their critical role in supporting neuronal actions. Since the ERs are nuclear receptors, it is highly likely that the genomic action of E2 depends on whether a cell contains ERα, ERβ or both. Thus, any change in the expression levels of one of these receptors, e.g. as seen here with a decrease and increase in ERα and ERβ, respectively, in the GM during ageing, might affect heterodimer formation and result in a partial alteration in their normal actions.

6.5.1.2 In Alzheimer's Disease

In the current study, immunoreactivity for both ERs was reduced in neurones and microglial cells. The decline in ERβ in the WM in the cortex has been reported previously (Honma et al., 2017). However, the latter study failed to observe any change in ER in the GM of individuals with AD, or of ERα in the WM. These differences in findings may have partly arisen due to methodological reasons. For instance, different IHC methods may yield varied results; (Honma et al., 2017) used an Autostainer method, whilst in this project a manual staining technique was employed. However, since the former study did not include age or PMI information with respect to their samples, comparison with the data presented in this Chapter was difficult.

GM neurons from the aged female AD subjects had a decrease in ERβ rather than the increase which was observed in the healthy aged subjects. This could give rise to the proposal that the post-menopausal decline in E2 is significantly higher in women who subsequently develop AD than in their healthy counterparts. However, Honma et al (Honma et al., 2017) measured E2 in aged brains and in AD subjects and no differences in E2 levels were reported. Thus, this fall in ER in AD could reflect neuronal degradation arising from early initiation of the disease environment, such as excessive cortical accumulation of NFTs and Aβ in elderly subjects. Of note, in preclinical studies in AD, E2 has been described as being capable of

reducing Aβ plaque generation in neuroblastoma cells by stimulating expression of degrading enzymes, e.g. IDE and neprilysin (Zhao et al., 2011, Xiao et al., 2009). Thus, the reduction in ER in AD may therefore hasten the accumulation of cerebral Aβ plaques observed in Chapter 3 in the same AD samples as those used in this chapter. When the data from the AD samples from the current study are considered alongside the previously described protective functions of ERs in nerve and glial cells, it could be inferred that the reduction in ERs in AD could attenuate cortical safeguarding activity giving rise to severe AD as a result of neuronal degradation arising from Aβ and NFT accumulation.

The discrepancy between the WB ER results reported in Chapter 5, where no changes in ERα and ERβ were seen during female frontal cortex ageing, and the alterations in their levels seen here within the GM and WM, are likely to partially result from methodological differences, such as tissue preparation and antibody specificity or concentration. For example, both the total and phospho ERα antibodies used in the IHC study were different from those used in WB as the WB total ERα antibody didn't give good immunostaining and the phospho ERα antibody were reported to not work well in IHC in paraffin-embedded samples according to the manufacturer (abcam.com). Different epitopes and phosphorylation moieties have therefore been investigated in this Chapter compared to those quantified by WB. However, whilst the ERβ antibody used in IHC was the same as that utilised in the WB experiments, it is important to note that this antibody detected immunopositive bands of molecular masses above and below 50kDa, i.e. full-length ERβ (Figure 6.19), and only the band at 50kDa was quantified in Chapter 5 as the precise identity of the other bands was not known. With respect to the larger bands > 50 kDa, various explanations may be postulated for their appearance in WB, including the formation of homodimers or heterodimers (Pasqualini et al., 1999), longer splice variants (Coleman et al., 2003), complexes of ERβ with any of its possible binding partners (Heldring et al., 2007) and phosphorylation or ubiquitination (Marin et al., 2006). In the IHC study, it was not possible to distinguish between the classical full-length ERβ immunoreactive band and additional alternative splice isoforms which could enhance the immunoreactivity signal at higher ERβ concentrations. Supporting this theory, *in vivo* studies have reported increases in the cortical ERβ splice variants with age (Shults et al., 2015, Tollervey et al., 2011). The most important reason for discrepancy in the ER results between WB and IHC data is likely to be regional variation. WB samples used homogenised whole tissue consisting of a variety of cell

types, e.g neuronal, astrocyte, microglial and endothelial cells. While in this chapter very specific locations in the cortex with specific cell types were examined.

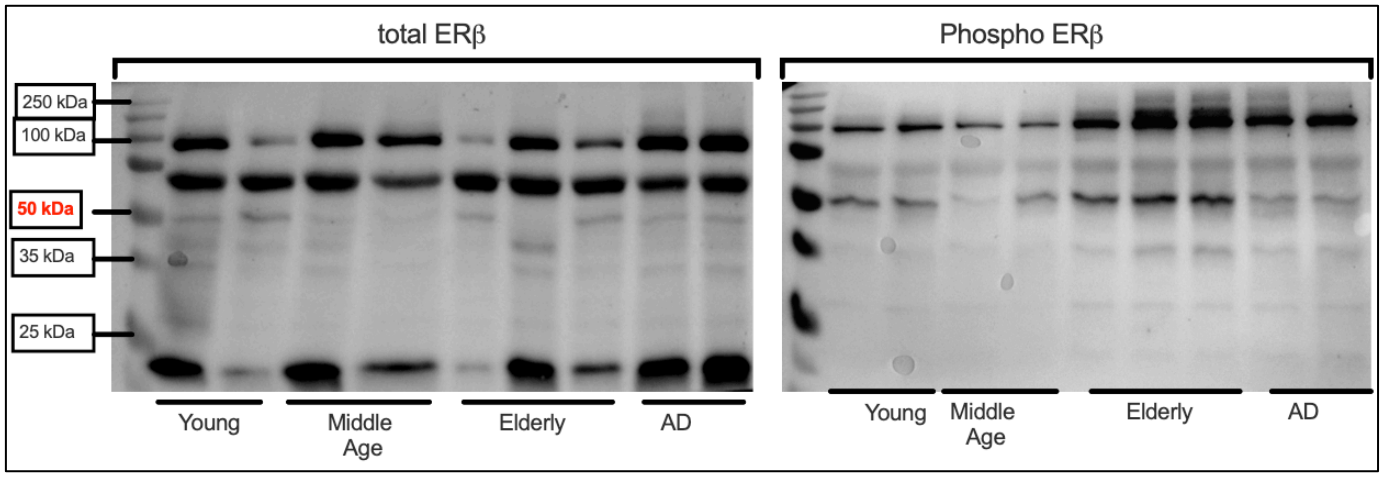


Figure 6.19 Immunoblotting membrane for female frontal cortex from different age groups and AD samples. The membrane was probed to detect tERβ (left panel), and then stripped and re-probed to detect pERβ (right panel). These antibodies revealed several bands which are larger or smaller than the full-length ERβ band, which is 50 kDa (indicated in red).

6.5.2 *ER Nucleocytoplasmic Distribution in Neuronal Cells During Frontal Cortex Ageing and in AD*

As far I can discern, the majority of AD studies have been conducted solely for the purpose of identifying nuclear ER expression, e.g. total ERα, and neglected cytoplasmic ER expression (Hestiantoro and Swaab, 2004, Hong-Goka and Chang, 2004, Lu et al., 2004, Savaskan et al., 2001, Ishunina and Swaab, 2001, Ishunina et al., 2007, Ishunina et al., 2003). In contrast, in the current Chapter, the nucleocytoplasmic distribution ratio was measured in order to identify any subcellular shift of those receptors evident in AD. Additionally, an ER phosphorylation site as well as the total protein was examined for each receptor to determine cerebral ER nuclear activity in AD; previously, this parameter has been ignored by the majority of studies (Hestiantoro and Swaab, 2004, Hong-Goka and Chang, 2004, Lu et al., 2004, Savaskan et al., 2001, Ishunina and Swaab, 2001, Ishunina et al., 2007, Ishunina et al., 2003).

In this project, the nucleocytoplasmic subcellular localisation of ERs was investigated only in neuronal cells. It was not possible to investigate the subcellular distribution of these proteins in microglial cells since ER staining was visible in their nucleus but absent in the cytoplasm, resembling results reported in a previous study (Honma et al., 2017)

Robust ER immunolabelling was present in the nucleus, cell body and the processes of neurons. These results concur with earlier reports of both nuclear and cytoplasmic ER immunolabelling that can extend into the dendrites of cerebral neurons (Kritzer, 2002, González et al., 2007, Montague et al., 2008).

Nuclear ER expression suggests that these receptors participate in the genomic or classical pathway as transcription factors that directly stimulate gene expression activity at ERE or indirectly stimulate gene activation through interaction with other mediators. Additionally, the extra-nuclear ER location in the cytoplasm suggests that they might also be mediating some non-genomic actions, for example, via the cAMP, MAPK or Akt pathways (see Chapter 5, Figure 5.1) (Pasqualini et al., 1999, Montague et al., 2008).

6.5.2.1 Age as a Risk Factor and Alzheimer's Disease

It is important to clarify that the whole picture with respect to genomic and non-genomic functions of ERs cannot be obtained simply through measurement of ERs within the nuclei and cytoplasm since the latter may initiate genomic mechanisms following activation by translocation to the nucleus (Prossnitz and Barton, 2009, Prossnitz and Barton, 2014, Schultz-Norton et al., 2011). However, the high nucleocytoplasmic ratio data in this chapter supports previous studies which have demonstrated that ER are predominantly localised in cerebral nuclei and that most of their functions occur via genomic pathways (Hestiantoro and Swaab, 2004, Hong-Goka and Chang, 2004, Lu et al., 2004, Savaskan et al., 2001, Ishunina and Swaab, 2001, Ishunina et al., 2007, Ishunina et al., 2003). The majority of the neurons from the sample groups in the current study displayed more ER within the nuclei than the cytoplasm, on average, by a factor of 1.5.

Whilst the total ERα nucleocytoplasmic distribution decreased in aged frontal cortex samples, no differences were seen in its phosphorylation at the Ser167 residue. This might indicate a sustained genomic neuroprotective action of ERα in neurons with advancing age. However, surprisingly in AD, there was upregulation of the levels of both total ERα and its

Ser167 phosphorylation form, a pattern which is similar to observations reported in human AD neurones within the hypothalamus (Hestiantoro and Swaab, 2004). However, in the human hippocampus, ERα nuclear expression did not change (Savaskan et al., 2001), inferring that cerebral ERα nuclear alterations in AD are region-specific. Wange et. al. reported entanglement of ERα within NFT in the hippocampus of human AD patients (Wang et al., 2016a), thus the upregulation of nuclear ERα in AD frontal cortex in this study could be a result of the attachments of proteins within NFT.

For ERβ, the trend towards an increase in immunoreactivity of its nuclear phosphorylated to total ratio during frontal cortex ageing might indicate stimulation of genomic neuroprotective activity through ERβ against the Aβ accumulation reported in Chapter 3. This function may be realised by epigenetic upregulation in the production of some Aβ degrading enzymes (Zhao et al., 2011, Xiao et al., 2009). Additionally, in AD, the decrease in ERβ activity in the nuclei, and the nucleocytoplasmic distribution in neurons through the downregulation of ERβ phosphorylation at the Ser105 residue may lead to reduced synthesis of Aβ degrading enzymes, thus promoting Aβ accumulation and the release of free radicals which cause neuron damage and disruption (Hellström-Lindahl et al., 2008).

As indicated previously, there is a lack of consensus regarding ER expression data in AD owing to the varied cerebral regions examined in the different studies (Hestiantoro and Swaab, 2004, Hong-Goka and Chang, 2004, Lu et al., 2004, Savaskan et al., 2001, Ishunina and Swaab, 2001, Ishunina et al., 2007, Ishunina et al., 2003). As far as I am aware, no study has previously investigated the subcellular localisation of ER in AD frontal cortex neurons.

In one brain tissue sample of GM from an AD patient, total ERβ was seen to be accumulated in distinct structures in some areas of the tissue (Figure 6.19). The identity of these structures was not clear although they could be NFT, as previously reported (Wang et al., 2016a). However, these receptors were not activated since no staining for phosphorylated ERβ was identified. This phenomenon was not associated with ERα. As reported previously in the results, these images were excluded during the semi-quantification of ERβ to minimize bias in the results.

Finally, one of the main theories relating to my project rests on the hypothesis that, during the menopause transition phase, ER-mediated neuroprotective function might decline.

Intriguingly, total and phosphorylated ERβ immunoreactivity in GM neuronal cell regions did diminish in the middle-aged group, representative of the perimenopause phase, compared to the young group (Figure 6.13a). The nucleocytoplasmic ratio of pERβ also decreased in this cohort indicating a potential shift of receptors from the nucleus to the cytoplasm during this life stage (Figure 6.16a). To some extent, this could reinforce the theory of an age window within female cerebral ageing prior to the menopause during which the neuroprotective activity of E2 becomes attenuated and potentially exposes the brain to premature initiation and development of preclinical AD. However, interestingly, ERβ immunoreactivity did increase in the post-menopausal elderly group compared to the perimenopausal or middle-aged cohort; this could help in attenuating the early negative symptoms of preclinical AD on cognitive functions.

6.6 Conclusion

The validity of the results in this chapter needs to be highlighted. From the CE results (Table 6.1), additional samples are needed to enhance the precision of these data particularly for the AD group, and to make sure that type II errors are not present. Cerebral structural heterogenicity has been well-documented previously (Price et al., 2017). Thus, if the CE were a reflection of the non-uniformity of cell distribution and clustering within certain tissues, e.g. the brain, it might not be possible to attain low CE values, regardless of the sampling density. However, even with brain heterogeneity, CE measurements of > 15% were only seen in some of the healthy sample groups and in AD (Table 6.1), suggesting that the method used here offered sufficient rigor in terms of random location selection for image capture and analysis to represent the entire tissue section. In addition, with regards to the higher CE values in AD, these may be due to variations in the degree of disease pathology between the different subjects used.

The complex structure of human frontal cortex could mask some alterations in investigated target proteins as seen here with ER. Thus, researchers need to include methods which distinguish between these cell types in brain studies.

The variation in the results between ERα and ERβ reported in GM and WM and their cellular distribution during female brain ageing could indicate different functions of these ER subtypes in the ageing brain. However, in female AD, both ERs subtypes declined significantly in GM and WM which suggests a decline in their neuroprotective functions.

In this Chapter, only female samples were used and so future studies could include inter-sex comparisons of the GM, WM and cellular locations of ER, along with investigating progesterone receptor. Such work would add valuable information regarding sex disparities in the development of AD, particularly with respect to the differences in cerebral anatomy between males and females. For example, the latter have a larger proportion of grey matter in a number of brain regions while in males, white matter is predominant (Cosgrove et al., 2007). Furthermore, investigation of androgen receptors would shed further light on sex-specific levels of hormonal receptors during cerebral ageing.

The Chapters so far have focused on protein expression in post-mortem human brain material so it was not possible to evaluate functional effects of ER activation on proteins involved in AD pathogenesis. Therefore, the final objective of this thesis was to conduct an in-vitro investigation of the effects of oestrogen on AD biomarkers using neurones derived from stem cells from human control and AD subjects, and this study is described in the next chapter.

CHAPTER 7 _ Pilot Study of the Effect of Oestrogen on Alzheimer's Biomarkers in Human Induced Pluripotent Stem (hIPS) Cells

The final objective in this thesis was exploring the effect of oestrogen in human neurones derived from stem cells. Unfortunately, the COVID-19 pandemic resulted in the closure of the laboratory between 03/2019 and 09/2019, thereby introducing a significant delay in the conducting of the stem cell experiments and there was then only time to carry out limited work. This Chapter therefore describes this in-vitro pilot study using human Induced Pluripotent Stem (hIPS) cells, wherein they will be transformed into neuronal cells and used to study the effect of oestrogen on a number of proteins, including ERs, APP and its metabolic enzymes, MAPK, and Akt. The study used two hIPS cell lines, one from a healthy older female (ND) and the other from an elderly female with Alzheimer's disease (AD).

7.1 Introduction

The effect of PMI on human tissue proteins depends crucially on the temperature at which the bodies were stored prior to tissue retrieval. Empirical evidence indicates that when a tissue is stored at 4C^{o}, after 14 hours variable vulnerability among proteins is present compared to fresh samples immediately kept at -80C^{o}. Some of the proteins that are vulnerable to degradation include MAPK and βActin (Ferrer et al., 2007) which were involved in earlier parts of this project. These findings indicate that different PMI for human samples might affect results, as the levels of protein degradation are likely to be variable between different samples. The PMIs of the brain samples described in Chapter 2 exceeded 14 hours. Hence, protein degradation before storage at -80C^{o} was predicted. In addition, when the correlation between PMI and the expression of investigated proteins in each age group was calculated (Appendix III), some proteins did associate with an inverse correlation with long PMI, i.e BACE1 in female samples. This could be either related to the selectivity of the used antibody or to the susceptible of BACE1 to degradation with longer PMI (AppendixIII).

Therefore, it is essential to perform *ex-vivo* experiments under controlled conditions. There are multiple examples of *in-vitro* studies in which cells were transfected with an over-expressed disease biomarker (APP or Tau) in order to investigate the pathophysiology of AD (Moyano et al., 2020, Qin et al., 2021, Yan et al., 2019, Tang et al., 2018) and much has been learnt from such studies. Nevertheless, it is important not to overlook the fact that such transfected cells do not represent the normal brain environment. Therefore, in this chapter, non-transfected neurones derived from stem cells from an age-matched healthy female subject and an AD female patient were used to mimic more closely the cellular environment of the brain.

7.2 Hypothesis and Objectives

The hypothesis for this Chapter is that E2 treatment will have a protective function against changes in AD biomarkers in non-transfected neuronal hIPS-derived cells. With this Chapter being a pilot study, the initial objectives here were to:

1- Establish non-transfected neuronal cultures derived from stem cells taken from a control and an AD patient
2- Investigate the effect of oestrogen on Alzheimer's disease biomarkers in hIPS-derived neuronal cells.

7.3 Experimental Methods

7.3.1 Neuronal Stem Cells

Human induced pluripotent stem (hIPS) cells were cultured in accordance with the process outlined in Chapter 2. These cells are somatic cells that have been reprogrammed so that they revert to pluripotent stem cells through the introduction of selected genes (van der Sanden et al., 2010). Methods used here were hIPS cell culturing, W.B, ICC and ELISA. Statistical analysis was not possible giving that the number of cell samples used here was one samples for each ND and AD, and these cells were expanded only once. At least 2 technical replicates were carried out for each experiment. Thus, the results were judged by observation, i.e. qualitative analysis.

7.4 Results and Discussion

As this Chapter is a pilot study, the results and discussion section were written as a one section.

7.4.1 Transforming hIPS cells into neuronal cells and investigating the effect of E2

7.4.1.1 Expansion and Differentiation of hIPS cells into Mature Neurons

The number of cells received from the manufacturer was not sufficient to conduct multiple biochemical experiments. In order to remedy this deficiency, the hIPS cells numbers were expanded by as much as three times, as per the recommendations of the manufacturer. Accordingly, it was possible to achieve a suitable target number of cells. Thus, the $1x10^6$ cells

received from the manufacturer were expanded to approximately $35x10^6$ cells. Figure 7.1 A, Figure 7.1 B, and Figure 7.1 C present images of the hIPS cells from the healthy control subject from day one to day five, when they reached 80% confluency, and prior to their expansion. Expanded hIPS cells were then differentiated to mature neuronal cells (Figure 7.1D). This process took between 21 and 28 days, based on the manufacturer's recommendation along with observational judgement on visible neuronal cell structure formation under the microscope. The morphology of the cells presented in Figure 7.1 F indicated that they had differentiated to neuronal cells.

The neuronal transcription factor FOXGI was employed as a biomarker in order to confirm hIPS differentiation to a neuronal phenotype. As observed in Figure 7.2, FOXG1 was highly expressed in the nuclei of the differentiated cells from both the control and AD subjects and co-localised with the nuclear stain, DAPI, in most of the cells. This confirms that the cells had indeed differentiated into neurones and confirmed the morphological assessment above seen in Figure 7.1 F. However, according to the manufacturer's instructions, the expansion of the hIPS cells could increase glial contamination during the differentiation process. For this reason, glial cells biomarkers were used here to examine the percentage of glial cells formed after hIPS expansion. CX3CR1 was used as a microglial biomarker. Figure 7.3 suggests that there were no microglia cells in the differentiated cultures from either subject as no staining was seen. However, when the astrocyte biomarker, GFAP, was examined, some cells were positively stained with this marker for both control and AD subjects (Figure 7.4) indicating that there were astrocytes in the culture. Thus, it cannot be assumed that any results obtained are purely from neurones.

Overall, the hIPS cells employed in this study looked very similar regardless of whether they were obtained from a control or an AD subject and were principally differentiated into mature neuronal cells. Moreover, the presence of some astrocytes in the cell culture could mimic the environment of the grey matter (GM) in the human frontal cortex, as reported in Chapter 6, section 6.4.1. Here, neuronal cells were clearly abundant in the GM, alongside a lower number of astrocytes. Thus, the first objective of this study, the production of viable human neuronal cell cultures more closely mimicking the environment of the brain in a Petri dish, was accomplished.

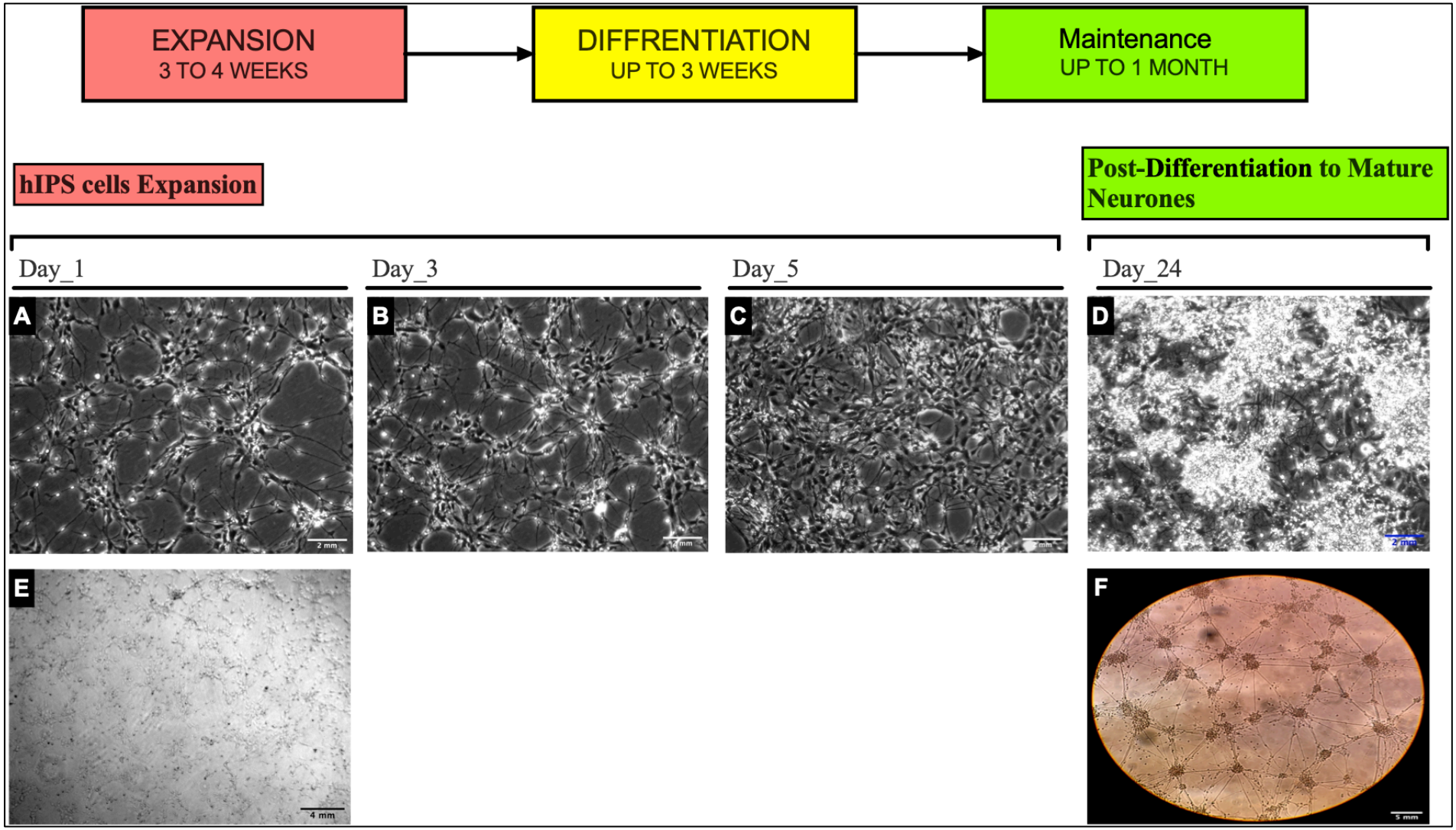


Figure 7.1 The process of expanding hIPS cells obtained from a healthy elderly female and differentiating them to mature neurons. A) hIPS cells at day 1where approximately 75,000 cells/cm2 were seeded at 2000 µm scale size, and **(E)** at 4000 µm scale size. **B)** Day 3 of seeded hIPSCs where they show an increase in the cell size at 2000 µm scale size. **C)** Seeded hIPS cells reach about 80% confluency and are ready for further expanding at 2000 µm scale size. **D)** Image of mature neurons captured at day 24 during stem cell differentiation at 2000 µm scale size. **F)** An expanded image, at 5000 µm scale size, on day 24 of differentiated hIPS cells which are seeded at a lower density on a coverslip showing mature neurons with visible axons.

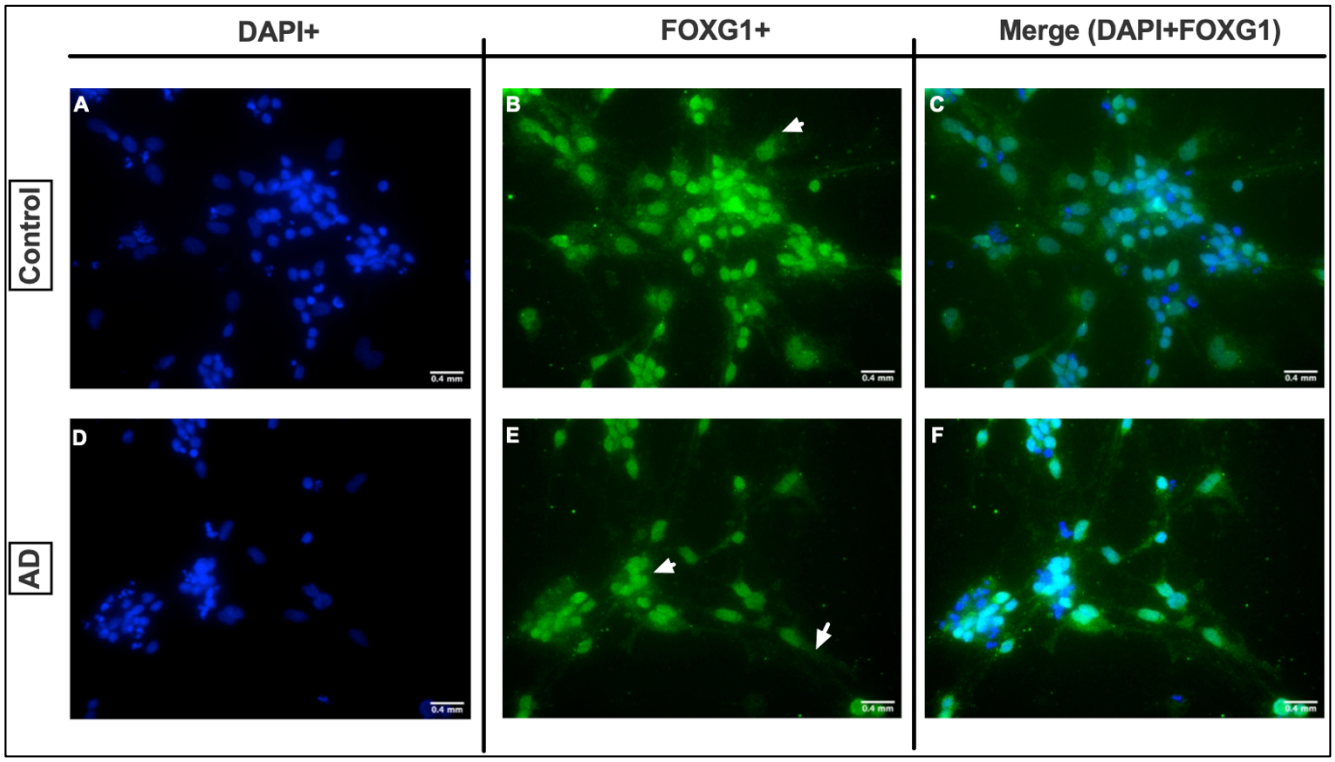


Figure 7.2 Evidence for the differentiation of hIPSCs to Neuronal Cells for the control (A,B,C) and AD (D,E,F) subjects. FOXG1 (**B,E**), a neuronal biomarker, was highly expressed in the cell nuclei also labelled with DAPI (**A,D**). (**C,F**) show the merged images for FOXG1 and DAPI with a high degree of overlap (pale blue cells) indicating that most of the cells were neurones. Also, FOXG1 staining could sometimes be seen in the neuronal cell body and axon (white arrows) (**E**). The cells were stained 5 days after differentiation to neurones. Scale bar 40 μm.

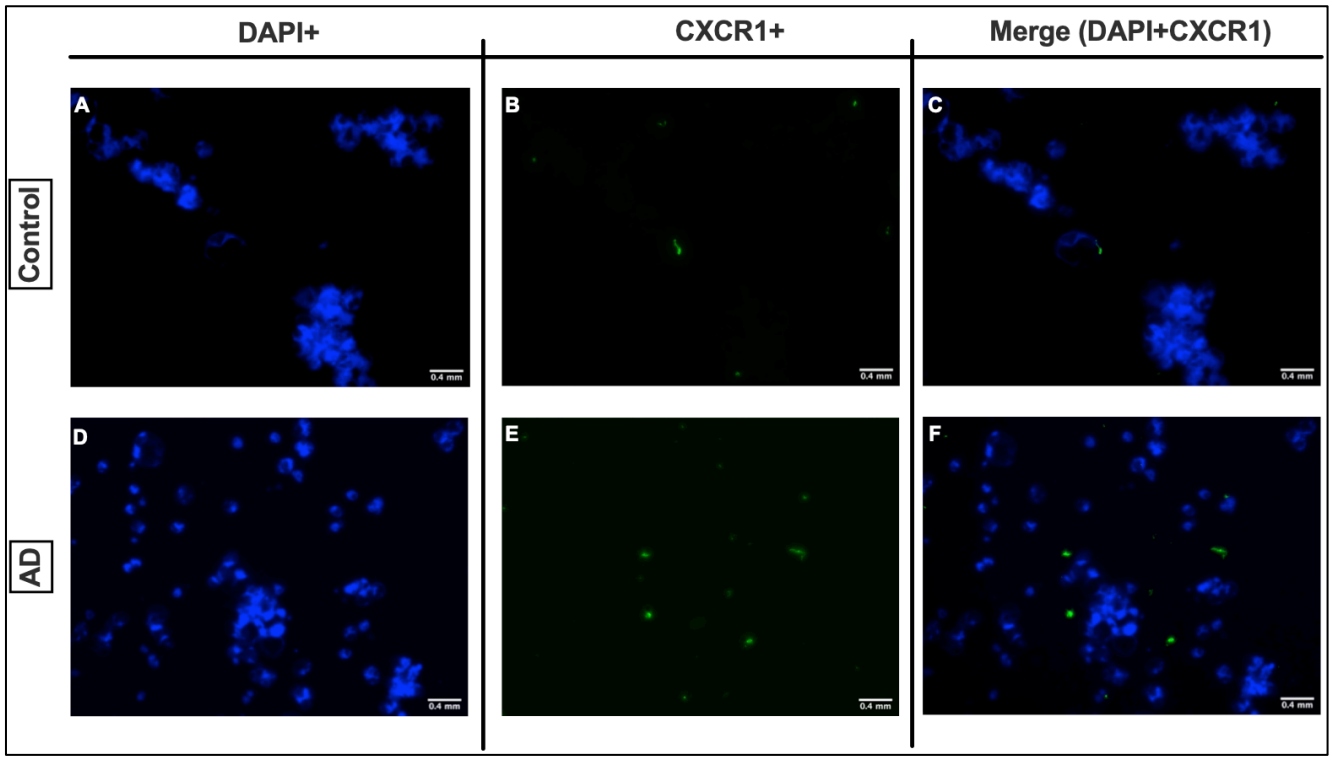


Figure 7.3 Absence of differentiation of hIPSCs to microglial cells for the control (A,B,C) and AD (D,E,F) subjects. No staining of microglia with the marker, CX3CR1, was seen in the differentiated cell culture (**B,E**), indicating that no microglia were present. The nuclei were labelled with DAPI (**A,D**). (**C,F**) show the merged images for CX3CR1 and DAPI with no overlap in staining. The cells were stained 5 days after differentiation to neurones. Scale bar 40 μm.

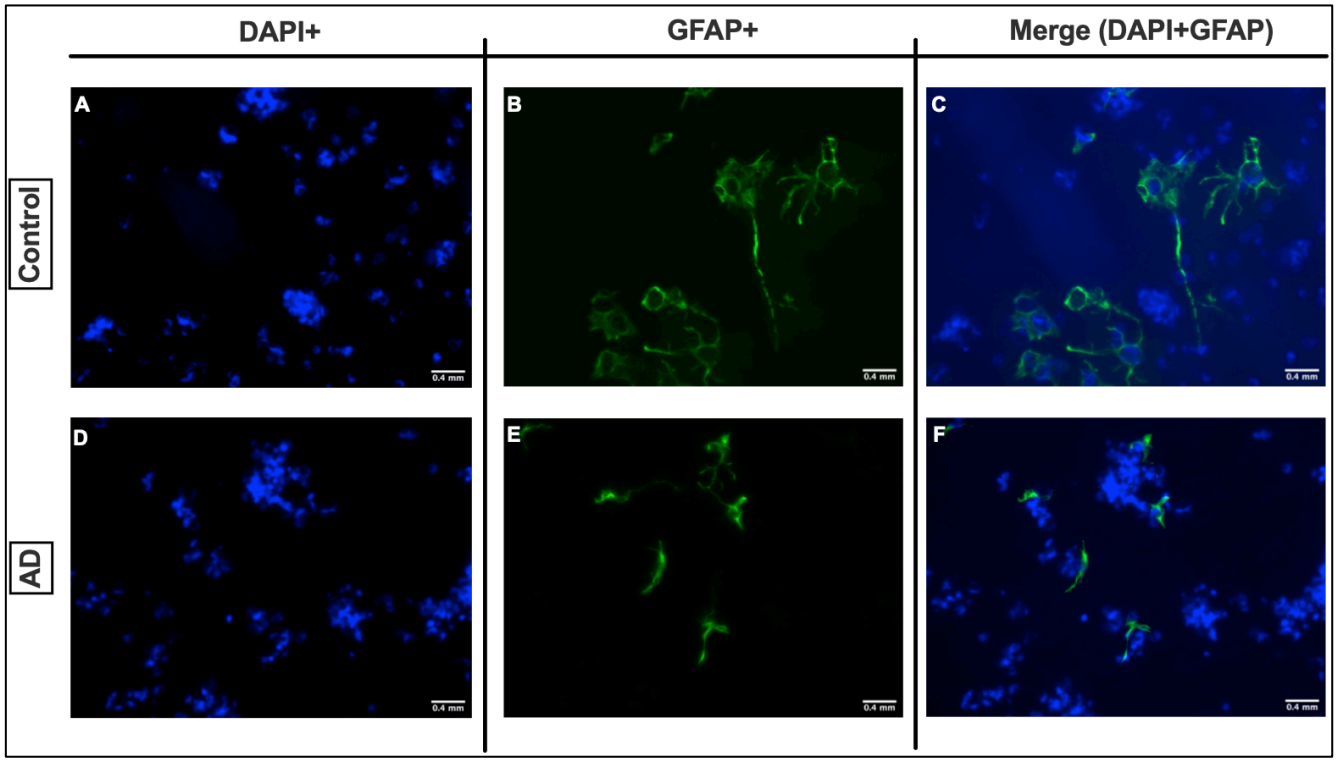


Figure 7.4 Evidence for the differentiation of hIPSCs to astrocytes for the control (A,B,C) and AD (D,E,F) subjects. Staining with the marker, GFAP, was seen in the cell bodies of some of the differentiated cells (**B,E**) indicating that astrocytes were present in the culture. The nuclei were labelled with DAPI (**A,D**). (**C,F**) show the merged images for GFAP and DAPI with some overlap in staining. The cells were stained 5 days after differentiation to neurones. Scale bar 40 μm.

7.4.1.2 Expression of ERs in the Differentiated Neuronal Cells

Both total and phosphorylated ERα (Figure 7.5) and ERβ (Figure 7.6) were highly expressed and to a similar extent in the hIPS neuronal cell cultures from both the control and AD subjects. These receptors are nuclear receptors so, as expected, they were predominantly present in the nucleus, rather than in the cell body and the axons. This accords with previous literature (Kritzer, 2002, González et al., 2007, Montague et al., 2008) and the observations regarding the human frontal cortex reported in Chapter 6.

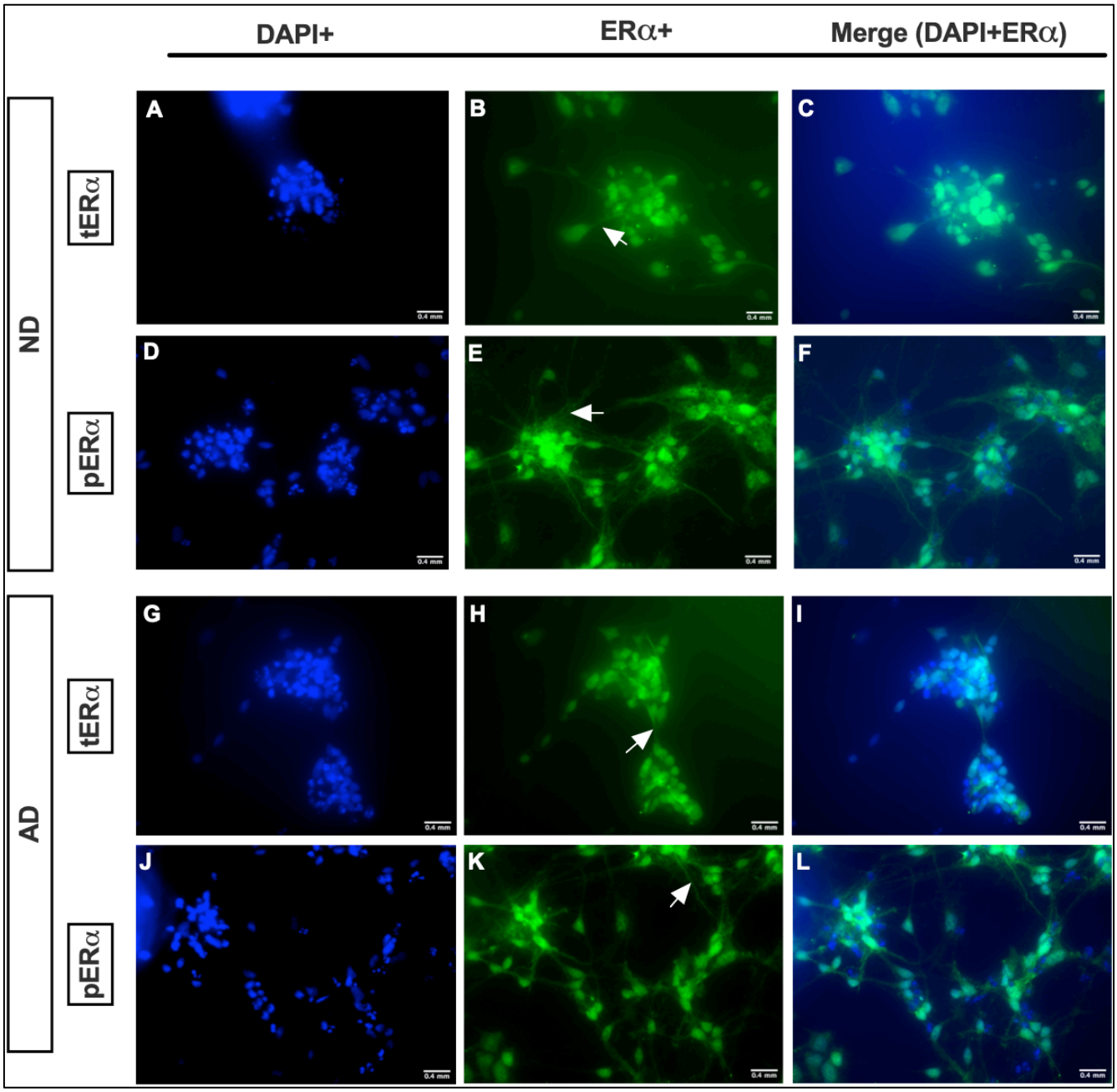


Figure 7.5 Expression of Total (t) and Phosphorylated (p) ERα in neurones differentiated from hIPS cells for the control (A-F) and AD (G-L) subjects. Both forms of the protein were localised predominantly in the nucleus rather than in the cell body and the axon of the neurones **(B,E,H,K),** the white arrows point at the neuronal cell body and axons. The nuclei were labelled with DAPI (**A,D,G,J**). (**C,F,I,L**) show the merged images for ERα and DAPI with extensive overlap in staining. The cells were stained 5 days after differentiation to neurones. Scale bar 40 μm.

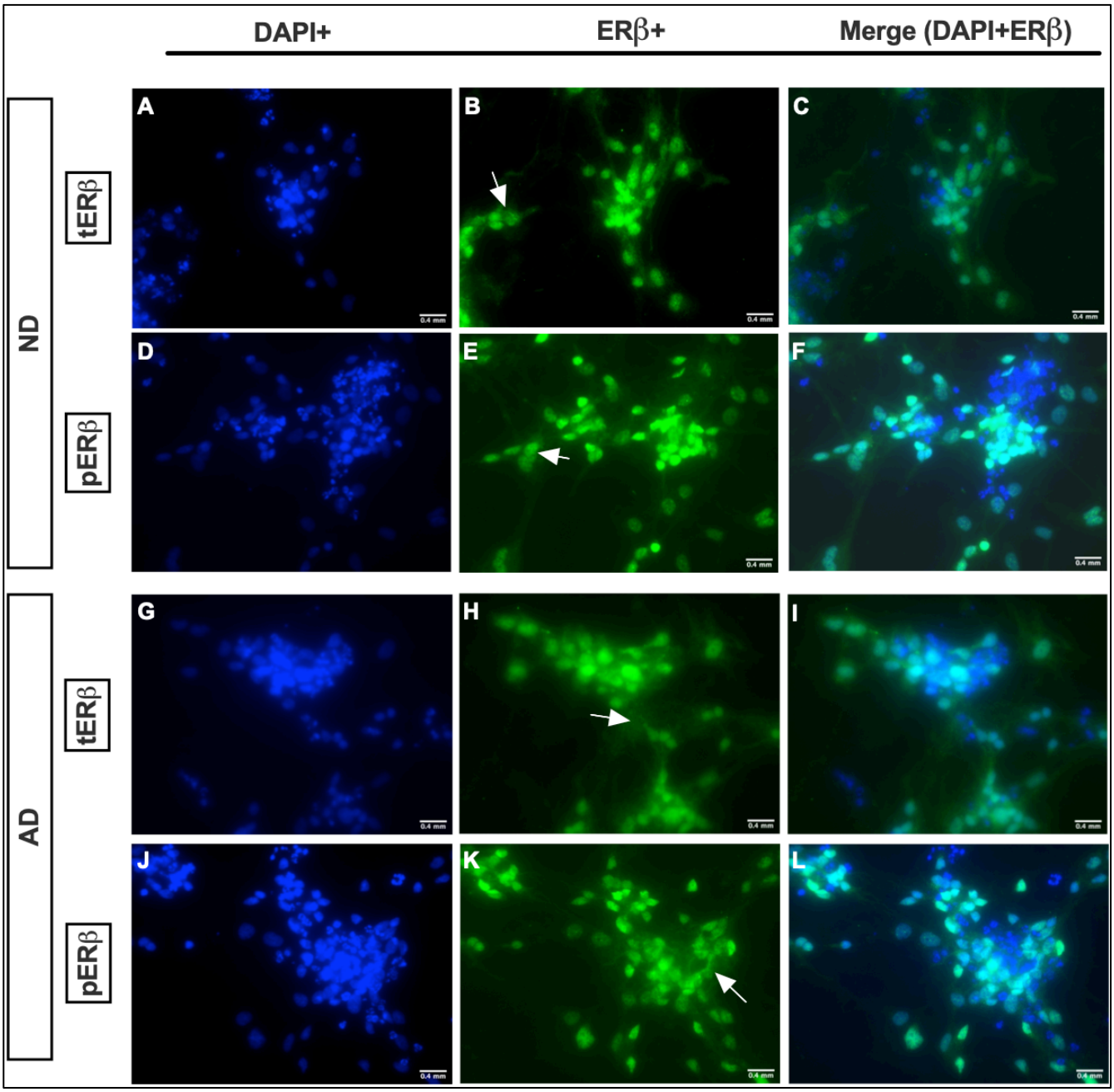


Figure 7.6 Expression of Total (t) and Phosphorylated (p) ERβ in neurones differentiated from hIPS cells for the control (A-F) and AD (G-L) subjects. Both forms of the protein were localised predominantly in the nucleus rather than in the cell body and the axon of the neurones **(B,E,H,K), t**he white arrows point at the neuronal cell body and axons. The nuclei were labelled with DAPI (**A,D,G,J**). (**C,F,I,L**) show the merged images for ERβ and DAPI with extensive overlap in staining. The cells were stained 5 days after differentiation to neurones. Scale bar 40 μm.

7.4.2 *E2 Dose Response Curve for Proliferation after Depriving the Cells of Growth Factors*

This experiment was carried out to determine the concentration of E2 to be used in the study to determine whether E2 treatment affected proteins involved in AD and responses to oestrogen. A concentration was required which activated ERs but was not toxic to the cells and, since E2 is known to cause proliferation of neurones (Okada et al., 2010), a cell proliferation assay was used to determine the E2 concentration.

Prior to E2 drug treatment, it was important to deprive the cells of growth factors to ensure all data collected were due to E2 stimulation. Since the media used in this Chapter were pre-prepared by the manufacturer (Axol) and could not be altered, the maintenance medium used to maintain the differentiated neuronal cells was switched to a medium free from growth factors, the proliferation medium (NeuroCult-XF, StemCell). Since the number of hIPS differentiated cells was limited due needing them for several biochemical investigations, a single 24-well plate was selected to test the effect of growth factor deprivation on neuronal stem cells before and after E2 treatment. The E2 dose used was 10nM according to published recommendations (Okada et al., 2010, Wang et al., 2008). From Figure 7.7 a, it can be seen that the proliferation of the ND cells declined when deprived of growth factors for 24 hours. While cell proliferation did increase slightly following E2 treatment, this was not majorly different to proliferation in the absence of growth factors. This could be due to the effect of E2 inducing proliferation of neuronal stem cells is significantly lower than the proliferation induction effects of growth factors as has been highlighted in rat neuronal stem cells (Brännvall et al., 2002). The earlier study did show that, while E2 slightly induced neuronal stem cell proliferation, Epidermal growth factor (EGF) obviously had a stronger stimulation of neuronal stem cell proliferation (about 4.5-fold higher than E2) (Brännvall et al., 2002). However, Fibroblast growth factor (FGF) was shown to have the same level as E2-inducing proliferation in neuronal stem cells (Okada et al., 2010).

The cells were treated with 17β-Oestradiol (E2) (Sigma-Aldrich, E2758) over the concentration range from 100pM to 100μM for 24 hours according to previous studies (Okada et al., 2010, Wang et al., 2008). Using an MTT assay which measures cell proliferation, the % of cell proliferation was calculated, and the half maximum effective concentration (EC50) was calculated using GraphPad Prism (Figure 7.7 b). A bell-shaped dose-response curve was seen

and an EC50 of log(-7.955) was found. This experiment was only performed once as this was a pilot study and time and resources were limited. Ideally, it would have been repeated 3 times to confirm the concentration used. Nonetheless, the EC50 value found in this chapter agrees with previous research into the effect of E2 on human neural stem cell proliferation at different concentrations (Wang et al., 2008). Thus, a concentration of 10nM of 17β-Oestradiol was used for all future studies.

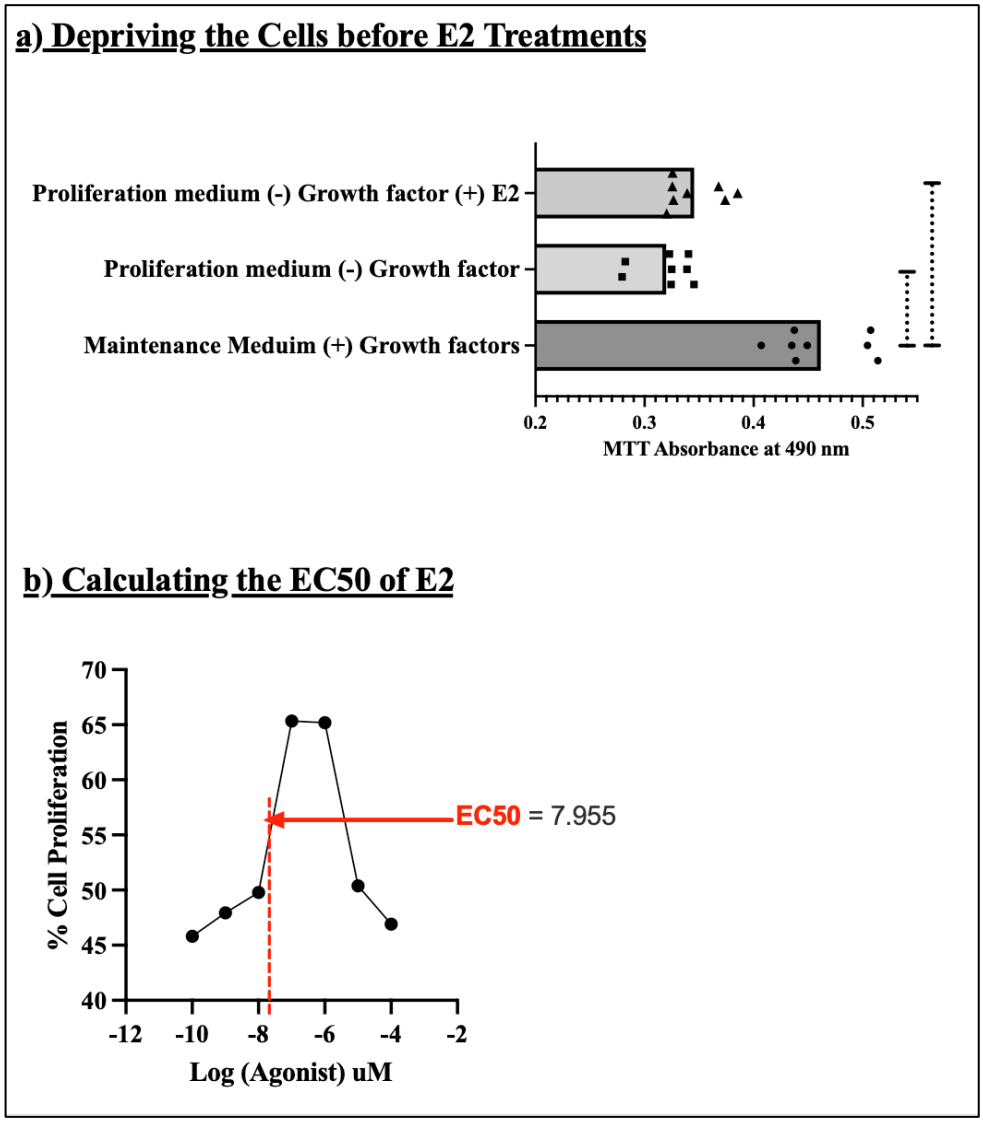


Figure 7.7 Oestradiol Dose-Response Curve. a) The level of cell proliferation before and after growth factor deprivation in neuronal hIPS cells from the ND donor. An observational reduction was seen when the medium was changed to one without growth factors or containing E2. **b)** The effect of different E2 concentrations from 100pM to 100µM on the proliferation of neuronal hIPS cells from female ND. The proliferation was expressed as a percentage of = (E2 Treated cell MTT proliferation / Control cell MTT proliferation) *100. E2 produced a bell-shaped dose-response curve with an EC50 value of Log (-7.955). n=1, was done once in octuplicate for each condition in the same 24-well plate of the same expansion. The analysis was qualitative, i.e observational.

7.4.3 W.B Results

7.4.3.1 ERα and ERβ Expression After E2 Treatment

The expression of tERα and pERα (phosphorylation at Ser118) and their ratio did not show any differences between ND and AD in the hIPS neuronal cells before E2 treatments (see Figure 7.8 b). This finding is at variance with the results reported in Chapter 5, wherein phosphorylated ERα levels at Ser 118 were shown to be elevated in AD brains. In Chapter 6, the investigation of different phosphorylation sites for ERα at Ser 167 revealed that levels were reduced in female AD human brains.

When the hIPS neuronal cells were treated with 10nM E2, the levels of total and phosphorylated ERα and their ratio did not change. Nor were there any apparent differences between ND and AD neurones. This finding does not accord with the expected results based on an *in-vivo* study which showed that an extended ovariectomy period was associated with a marked decrease of ERα in the murine brain (Zhang et al., 2011a, Zhang et al., 2009, Qu et al., 2013). Thus, it was expected that when the human neuronal IPS cells were deprived of growth factors, the levels of ERα would diminish and be followed by upregulation of phosphorylation activity during E2 treatments. The latter expectation was based on an *in-vivo* study, in which ERα in cerebral vessels declined after ovariectomy but increased significantly after chronic E2 exposure (Stirone et al., 2003).

While cell proliferation levels declined after growth factor deprivation (see Figure 7.7 a), ERα levels were unaffected by both conditions (growth factor deprivation and E2 treatments). This could indicate that the deprivation of growth factors was not completely efficient in the context of ERα activity. It is possible that a longer period would be required to affect ERα activity and this could be investigated in future studies. Furthermore, ER signalling can be stimulated by different pathways. Two of the main pathways were discussed in Chapter 6, namely: the genomic and non-genomic pathways (Prossnitz and Barton, 2009, Prossnitz and Barton, 2014, Schultz-Norton et al., 2011). In addition to those main pathways, ER can also be stimulated through an indirect pathway known as the ligand-independent pathway. In this pathway, ERα may be activated by growth factors or cAMP downstream signalling in the absence of E2 (VanHook, 2010). Thus, using an ERα antagonist such as methyl-piperidino-pyrazole (MPP) could be a more appropriate agent in future studies of ERα function and its downstream signalling in neuronal hIPS cells.

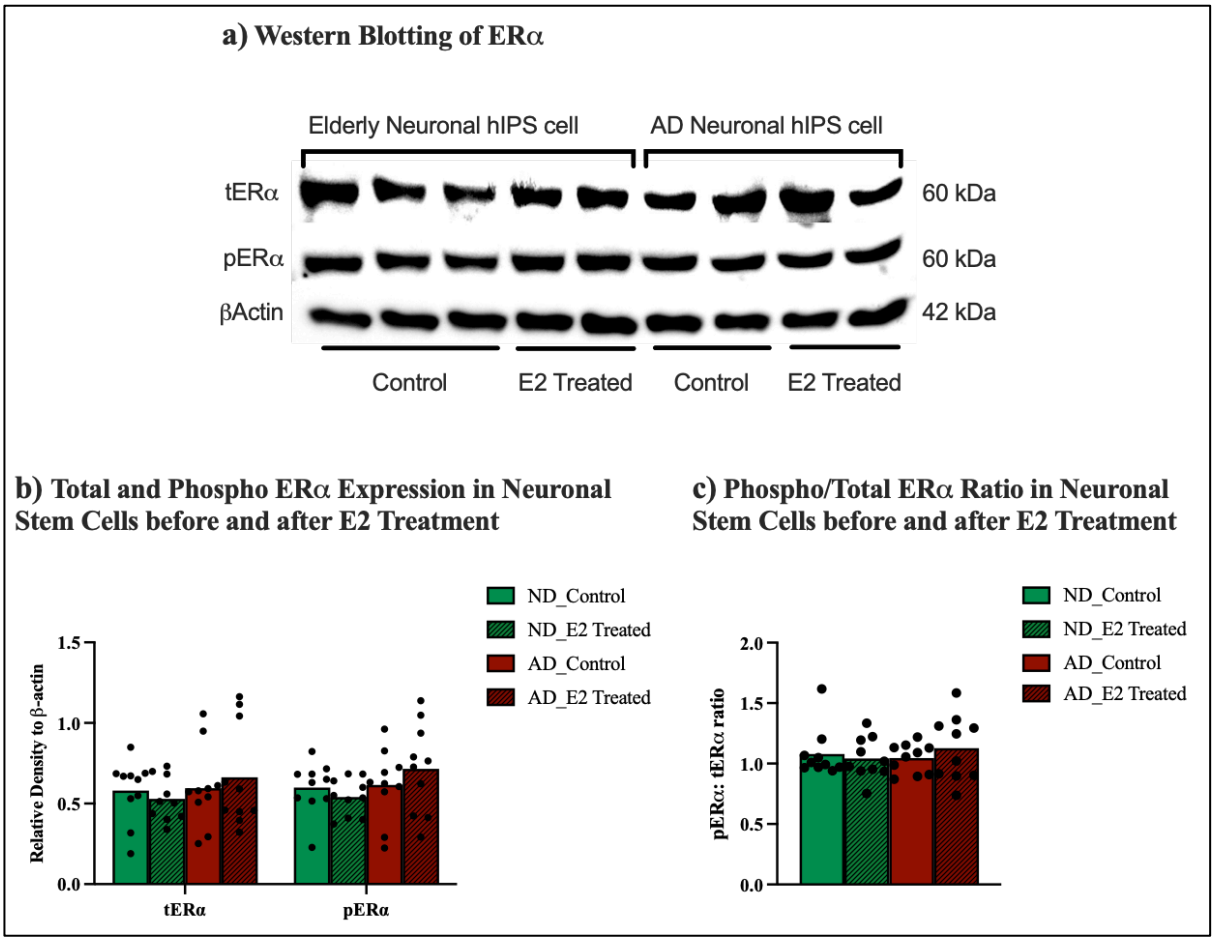


Figure 7.8 Densitometric analysis of ERα expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours. a) Representative immunoblot for ERα and the house keeping protein, βActin. **b & c)** No apparent differences in total or phsopho ERα or the pERα:tERα ratio were seen between ND and AD after E2 treatment. n=1 for ND and AD.

In contrast, levels of phosphorylated ERβ at Ser105 appeared to be higher in ND compared to AD female neuronal hIPS cells before E2 treatment (Figure 7.9 b). In Chapter 5, while differences in ERβ levels between ND and AD human female brains were found not to be altered statistically, a pattern of higher levels of this protein was observed in the ND brain subjects (see Figure 5.3 c and Figure 5.3 e). However, the wide variation in ERβ results between the ND samples in Chapter 5 (see Figure 5.3 c and Figure 5.3 e) could be the reason for the lack of statistical significance. Thus, the controlled cultured conditions for the female neuronal hIPS cells appear to have reduced the wide variation in ERβ expression (see Figure 7.9 b) compared to the human brain data in Chapter 5. Furthermore, in Chapter 6, the levels of ERβ did decline in AD female GM and, since the neuronal hIPS cells discussed in this Chapter more closely mimic the cell type and environmental conditions of GM as reported previously, the results presented in this Chapter can be considered to be comparable to the data discussed in Chapter 6 in respect of the decline in phosphorylated ERβ in AD. The data for ERβ obtained here will need to be confirmed by being repeated in fresh expansions of stem cells and in other ND and AD subjects

However, when the neuronal hIPS cells were treated with E2, the apparent differences between ND and AD were diminished due to an apparent decline in the phosphorylation of ERβ at Ser105 in ND cells (Figure 7.9 b) resulting in no apparent differences between ND and AD. This apparent decline in ND is at variance with an *in-vivo* study, where the E2 treatment of ovariectomized aged female rats was associated with an increase in hippocampus ERβ phosphorylation (Pinceti et al., 2015). Moreover, in the latter study, an *in-vitro* experiment using the mouse hippocampal-derived cell line HT-22, indicated that stimulating the phosphorylation of ERβ at Ser105 increased transcription activity at the ERE site. In addition, inhibition of ERβ Ser105 reduced its transcriptional activity (Pinceti et al., 2015). Thus, the reduction in ERβ phosphorylation at Ser105 in neuronal hIPS cells during E2 treatment could indicate an alteration in signalling pathways, the genomic pathway, and/or the non-genomic pathway. ERβ can be phosphorylated at other sites, such as Ser87 (Pinceti et al., 2015), Ser 106 and Ser 124 (Tremblay et al., 1999). Therefore, the exploration of those sites represents a possible avenue for future research into the functional pathways associated with ERβ in human neuronal stem cells.

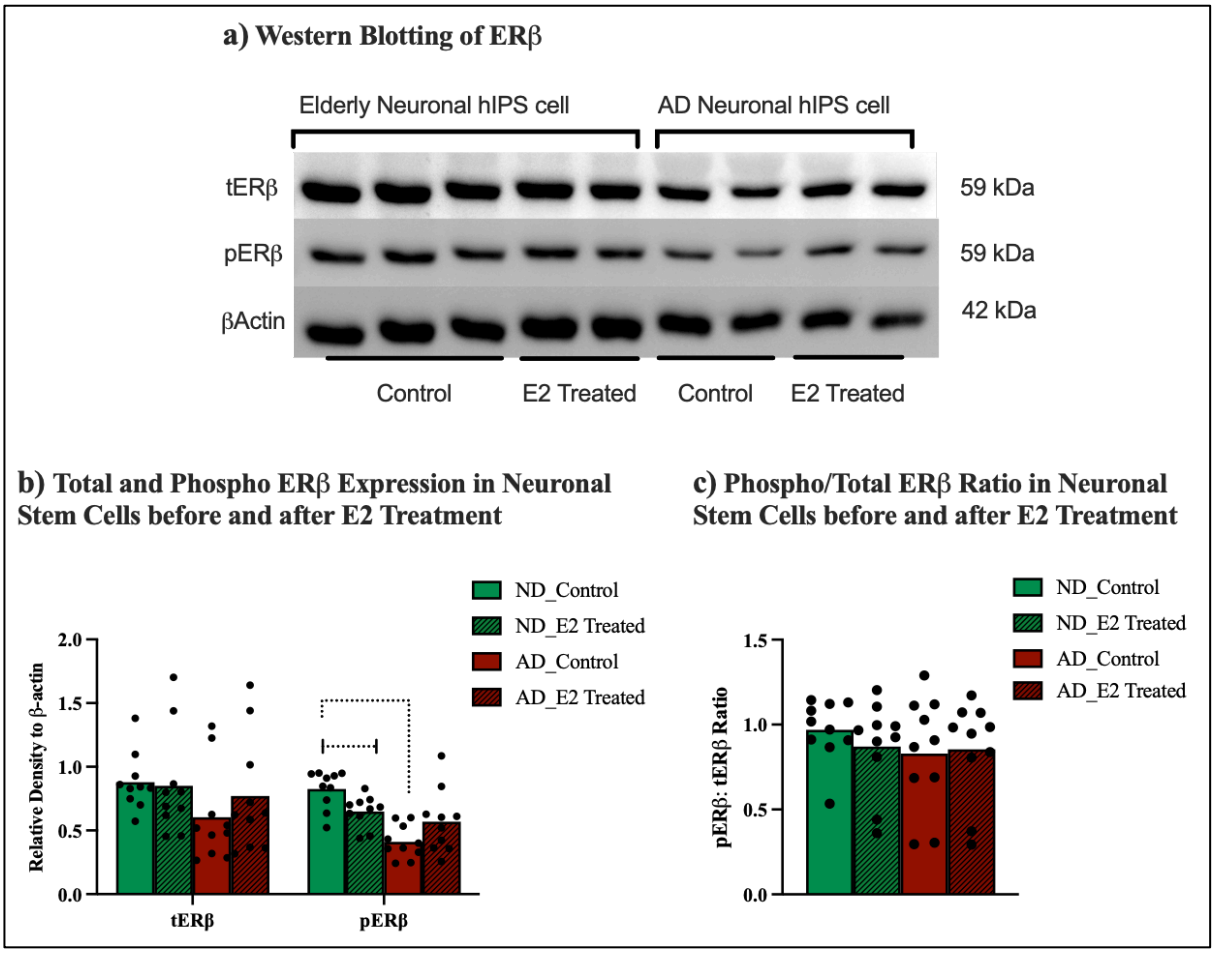


Figure 7.9 Densitometric analysis of ERβ expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours. a) Representative immunoblot for ERβ and the house keeping protein, βActin. **b & c)** An apparent decline in phosphorylated ERβ at Ser105 residue was seen between ND and AD in the control cells but this difference appeared to be lost after E2 treatment as a result of an apparent decrease in phosphorylated ERβ in the control condition after treatment with E2. The pERα:tERα ratio was not different between all the groups. n=1 for ND and AD.

7.4.3.2 ADAM10 Expression After E2 Treatment

ADAM10 cleaves APP at the α-cleavage site, thus directing the APP metabolic process towards the non-amyloidogenic pathway (Colciaghi et al., 2002, Manzine et al., 2015). The antibody used for ADAM10 identified two forms of the protein, namely the inactive form (proADAM10) and the active form (matADAM10). The neuronal hIPS cells apparently expressed lower levels of pro-ADAM10 and mat-ADAM10 in AD compared to ND cells before E2 treatment (Figure 7.10 b). In clinical studies, a significant reduction in the level of ADAM10 in the platelets of AD patients compared to control subjects was reported (Colciaghi et al., 2002, Manzine et al., 2015). In this thesis, it was not possible to identify any differences in ADAM10 expression in AD brains (see Chapter 3). One possible reason for the differing results is the use of an in-vitro study which was designed to study neuronal cells under controlled conditions and thus a decline in ADAM10 similar to the AD patients reported above (Colciaghi et al., 2002, Manzine et al., 2015) could be seen here in the *in-vitro* human neuronal cultures.

When neuronal hIPS cells were treated with E2, there was no apparent change in the level of ADAM10 in either the ND or AD cells. This accords with previous research conducted using human foetal brain neuronal cells where no changes in ADAM10 was observed after E2 treatment (Nord et al., 2010, Vetrivel and Thinakaran, 2006). However, the latter study did report a decline in one of the ADAM family proteins, ADAM17, after cell exposure to E2 (Nord et al., 2010, Vetrivel and Thinakaran, 2006). Nonetheless, the neuronal hIPS cells here did continue to have apparently lower levels of pro-ADAM10 and mat-ADAM10 in AD cells after E2 treatment compared to ND (see Figure 7.10).

It was planned to examine the expression of BACE1, the enzyme responsible for the initiation of the APP amyloidogenic metabolic pathway and the production of Aβ. However, due to a technical issue with the selectivity of the primary antibody and lack of time, it was not possible to look at it.

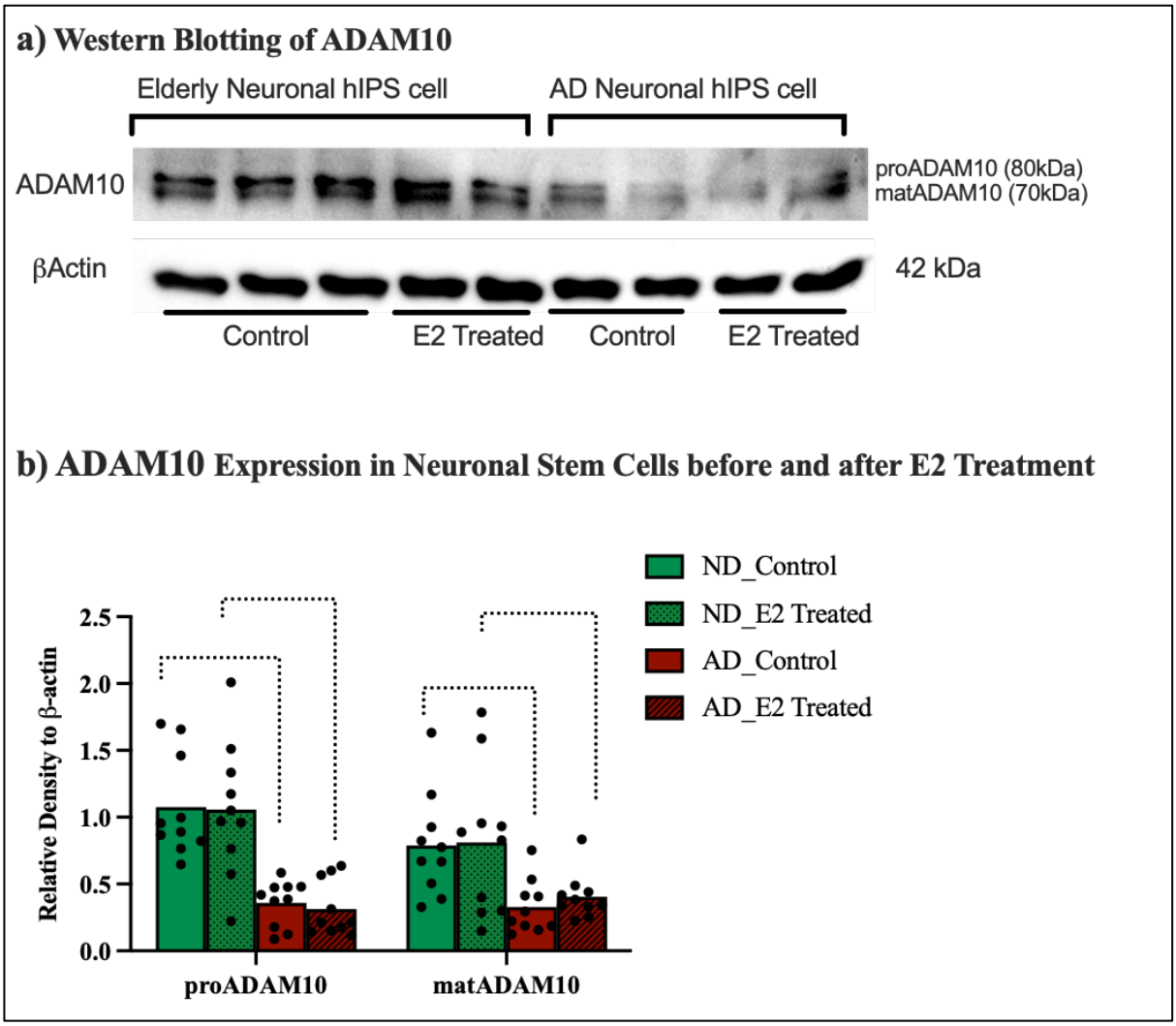


Figure 7.10 Densitometric analysis of ADAM10 expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours. a) Representative immunoblot for pro- and mat-ADAM10 and the house keeping protein, βActin. **b)** An apparent decline in both inactive (pro-) and active (mat-) ADAM10 was identified in AD cells compared to ND in both control and E2 treatment conditions. n=1 for ND and AD..

7.4.3.4 PS1 and PS2 Expression After E2 Treatment

PS1 and PS2 are components of the γ-secretase enzyme and contribute to the production of Aβ. An increase in their levels is regarded as a risk factor in the development of LOAD (Tong et al., 2011). The neuronal hIPS cells showed no apparent differences in PS1 or PS2 levels between ND and AD cells before E2 treatment (Figure 7.11 b). This finding concurs with the results reported in Chapter 3, where no differences in their expression levels were found in female AD brains. However, a clinical study did report higher levels of PS1 in the CSF of AD patients (Sogorb-Esteve et al., 2016).

Under E2 treatment conditions, an apparent decline in PS1 expression levels was seen in both ND and AD neuronal hIPS cells, along with an apparent diminished level of PS1 in AD compared to ND cells (Figure 7.11 b). This finding is in agreement with the results of a previous study that reported a downregulation in PS1 after E2 treatment in human foetal brain neuronal cells (Nord et al., 2010, Vetrivel and Thinakaran, 2006). However, PS2 expression levels were apparently not impacted in ND and AD neuronal hIPS cells after E2 treatment (Figure 7.11 b).

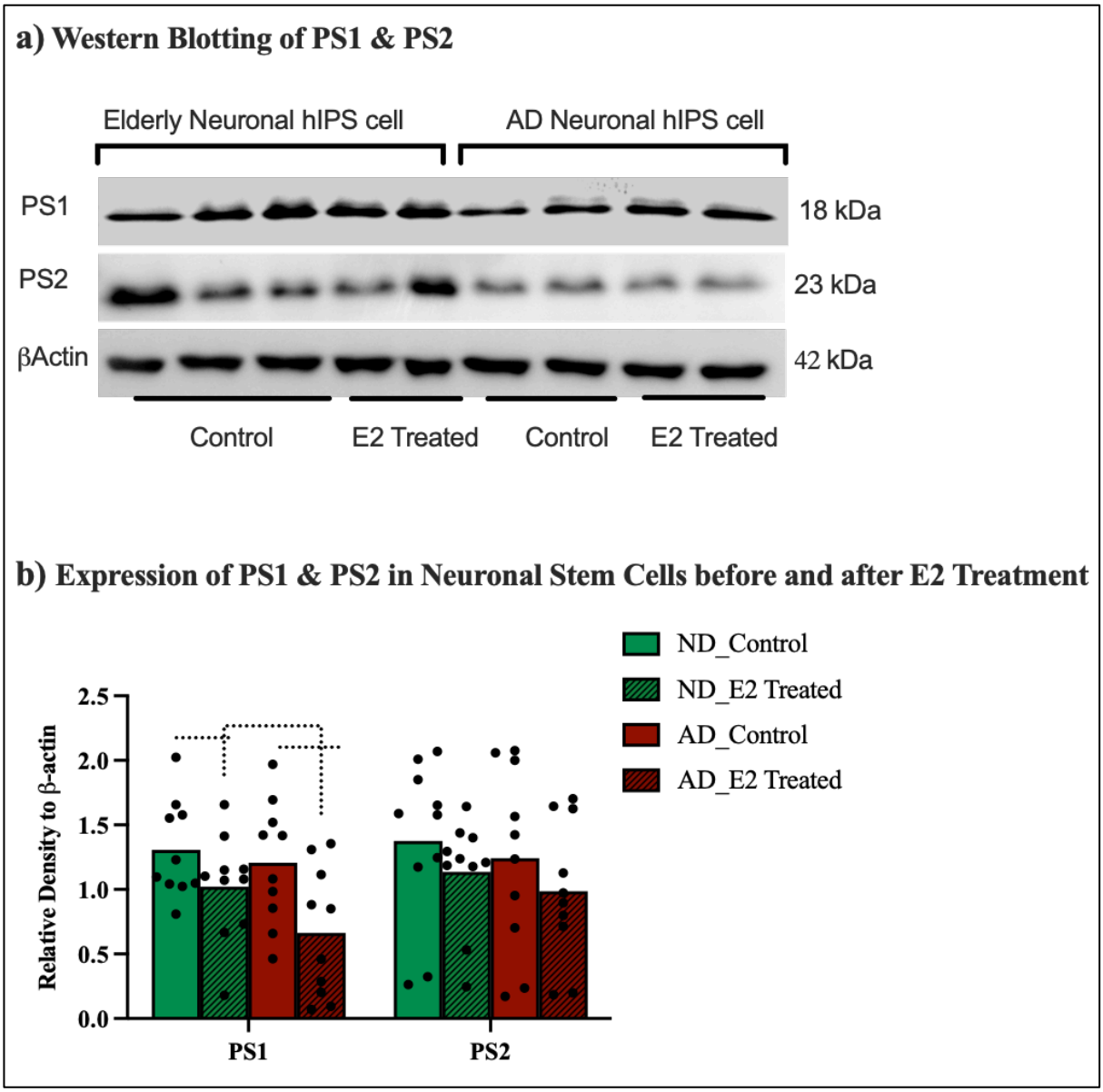


Figure 7.11 Densitometric analysis of PS1 and PS2 expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours. a) Representative immunoblot for PS1 and PS2 and the house keeping protein, βActin. **b)** No apparent differences were seen in both PS1 and PS2 levels between ND and AD before E2 treatment. While after E2 treatment, PS1 levels showed an apparent decline in both ND and AD cells and also a visibly lower level of PS1 was apparent in AD compared to ND cells after the drug treatment. n=1 for ND and AD.

7.4.3.5 Bcl2 Expression After E2 Treatment

Bcl2, an anti-apoptotic protein, was measured in the neuronal hIPS cells before and after E2 treatment and no alterations in its levels were seen between ND and AD cells (Figure 7.12). This accords with the results described in Chapter 4. An *in-vivo* study reported a direct relationship between E2 and Bcl2 in the brain as the treatment of ovariectomized rats with E2 was associated with an upregulation of Bcl2 mRNA and protein levels in the brain (Alkayed et al., 2001).

Previously, it was suggested that Bcl2 is regulated by ERβ, thereby indicating that ERβ can regulate the cerebral anti-apoptotic activity of Bcl2 (Zhao et al., 2004, Fan et al., 2008), Therefore, a marked decline in ERβ phosphorylation at Ser105 in the ND neuronal hIPS cells post-E2 treatment (Figure 7.9 b) could be expected to be associated with reduced Bcl2 levels. However, the level of Bcl2 in the hIPS cells did not alter after E2 treatment, suggesting that other factors also regulate Bcl2 expression. The current study did not examine the phosphorylated form of Bcl2 and thus, future research could examine post-E2 treatment phospho Bc12 levels.

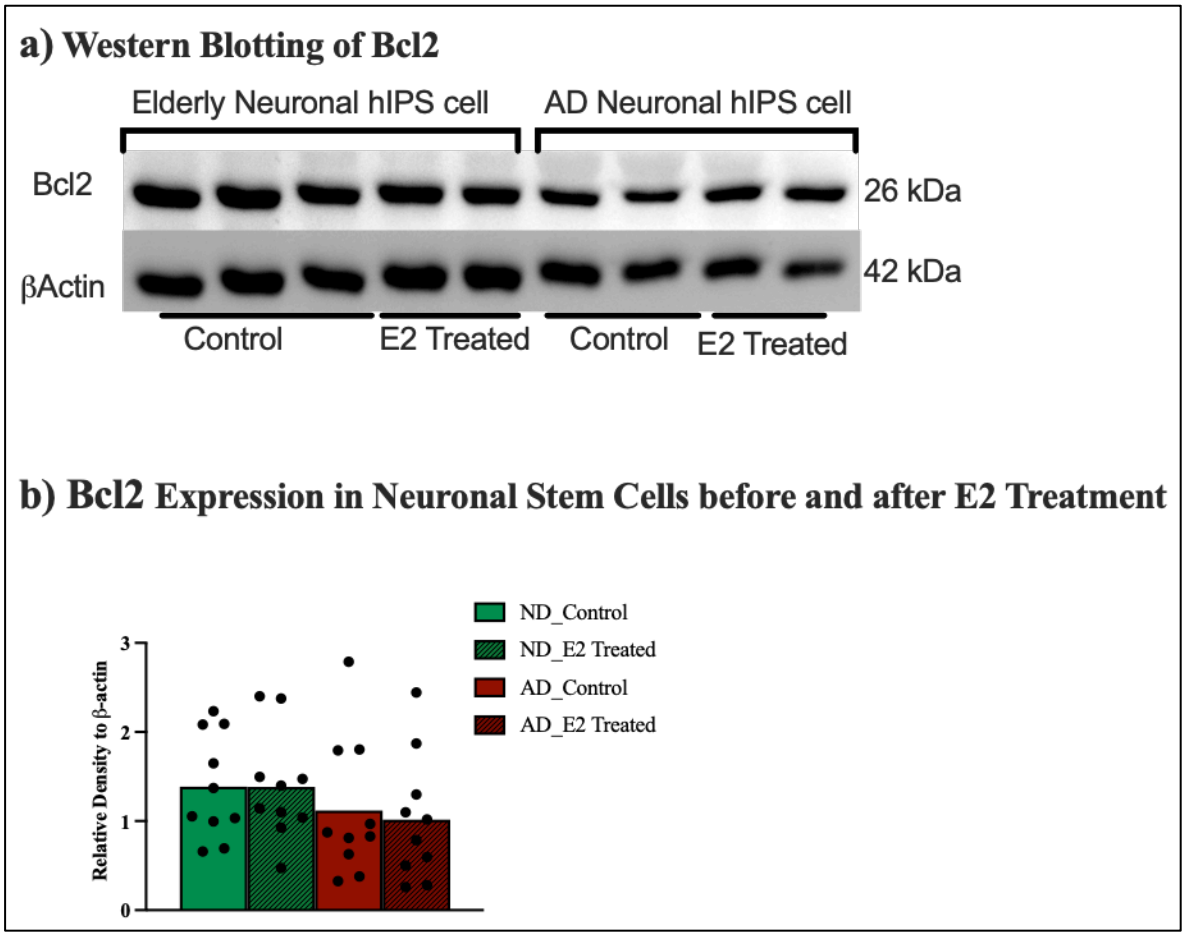


Figure 7.12 Densitometric analysis of Bcl2 expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours. a) Representative immunoblot for Bcl2 and the house keeping protein, βActin. **b)** No apparent differences were seen in Bcl2 levels between ND and AD in both control and E2 treatment conditions. n=1 for ND and AD.

7.4.3.6 MAPK (ERK1 and ERK2) and Akt Expression After E2 Treatment

MAPK and Akt are activated by ER through the non-genomic pathway (Taguchi et al., 2004). In this thesis, MAPK and Akt were investigated as indicators of the ER non-genomic pathway in the brain (see Chapter 5). Thus, these proteins were investigated here in the neuronal hIPS cells.

The ratio of phosphorylated/total MAPK had a pattern of apparent increases after E2 treatments in both ND and AD cells (Figure 7.13 c). This is similar to a previous study in which E2 was reported to stimulate the ratio of phospho/total MAPK in adult rats neuronal stem cells (Okada et al., 2010).

The MAPK antibody used here identified the MAPK subfamily proteins, ERK1 and ERK2. Before E2 treatment, neuronal hIPS cells had apparently higher levels of both the total and phosphorylated forms of ERK1 and ERK2 in the ND cells than in the AD cells (see Figure 7.14 a). This finding was not seen in the female brain samples (see Chapter 5). After adding E2, the apparent differences in ERK1 and ERK2 between ND and AD neuronal cells were still visible, except for phosphorylated ERK2 which appeared to increase in AD compared to ND cells (Figure 7.14 b). When the ratios of phosphorylated to total ERK1 and ERK2 were measured, only ERK2 had an apparent increase in the phospho/total ERK2 ratio after E2 treatment (Figure 7.14 c).

Interestingly, there was an apparent difference between the levels of the phosphorylated forms of ERK1 and ERK2. An apparently higher level of phosphorylated ERK2 compared to phosphorylated ERK1 was evident in AD cells before and after E2 treatment (Figure 7.14 b). It is thought that ERK1 and ERK2 are homologs that compensate for each other in the brain (Samuels et al., 2008). However, their signalling pathways in the brain have not been extensively investigated. Cancer research has also identified a disparity between ERK1 and ERK2 activity, with ERK2 the predominant regulator of cancer cell proliferation and metastasis (Gagliardi et al., 2020). Thus, phospho ERK2 levels exceeding phospho ERK1 levels in the neuronal hIPS stem cell could suggest that there are functional differences between them in neurons.

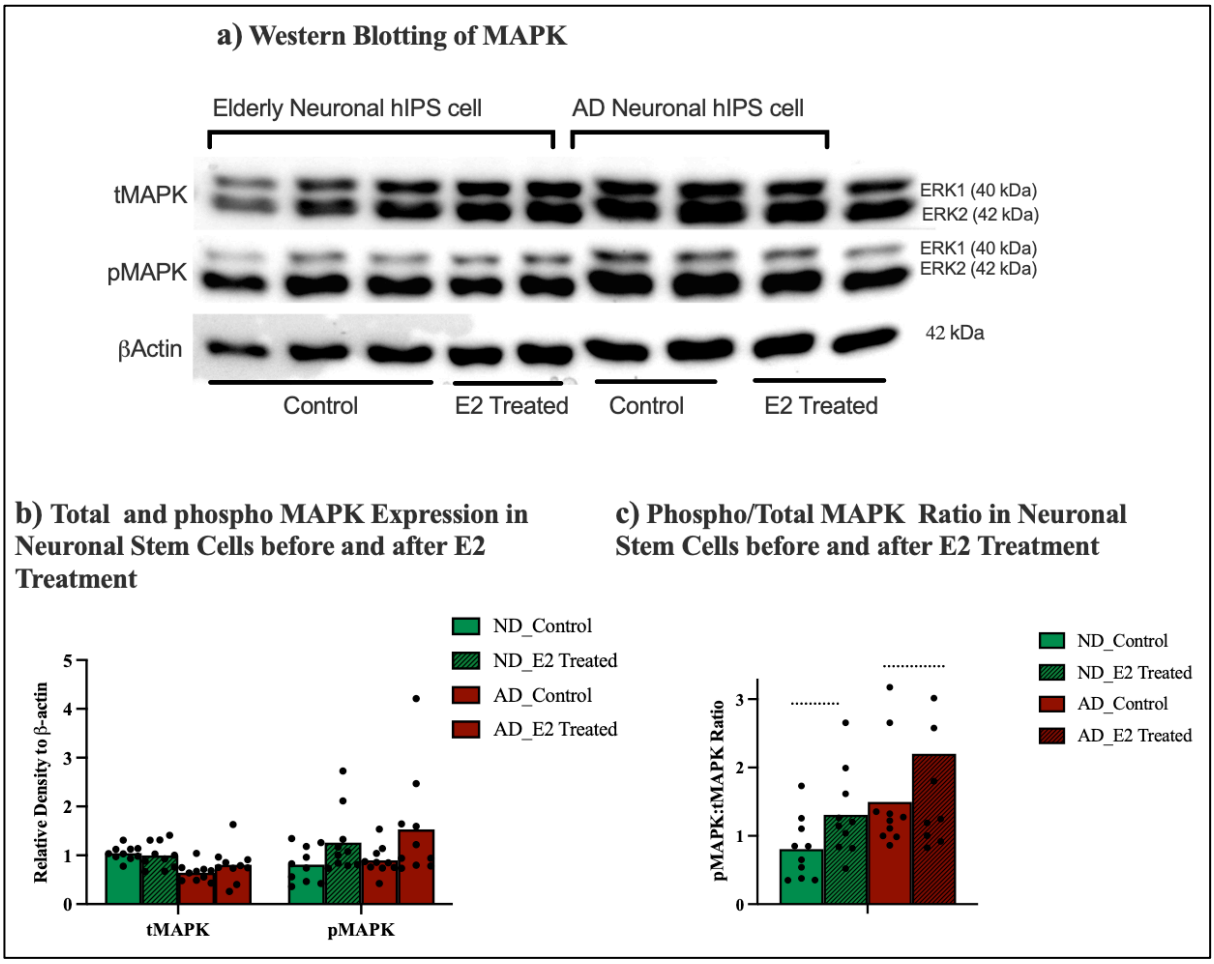


Figure 7.13 Densitometric analysis of MAPK expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours. a) Representative immunoblot for MAPK and the house keeping protein, βActin. **b & c)** an apparent higher level of phosphorylated MAPK could be seen in ND and AD cells after E2 treatment. The same pattern was also visible for the phospho/total ratio after E2 treatments. n=1 for ND and AD.

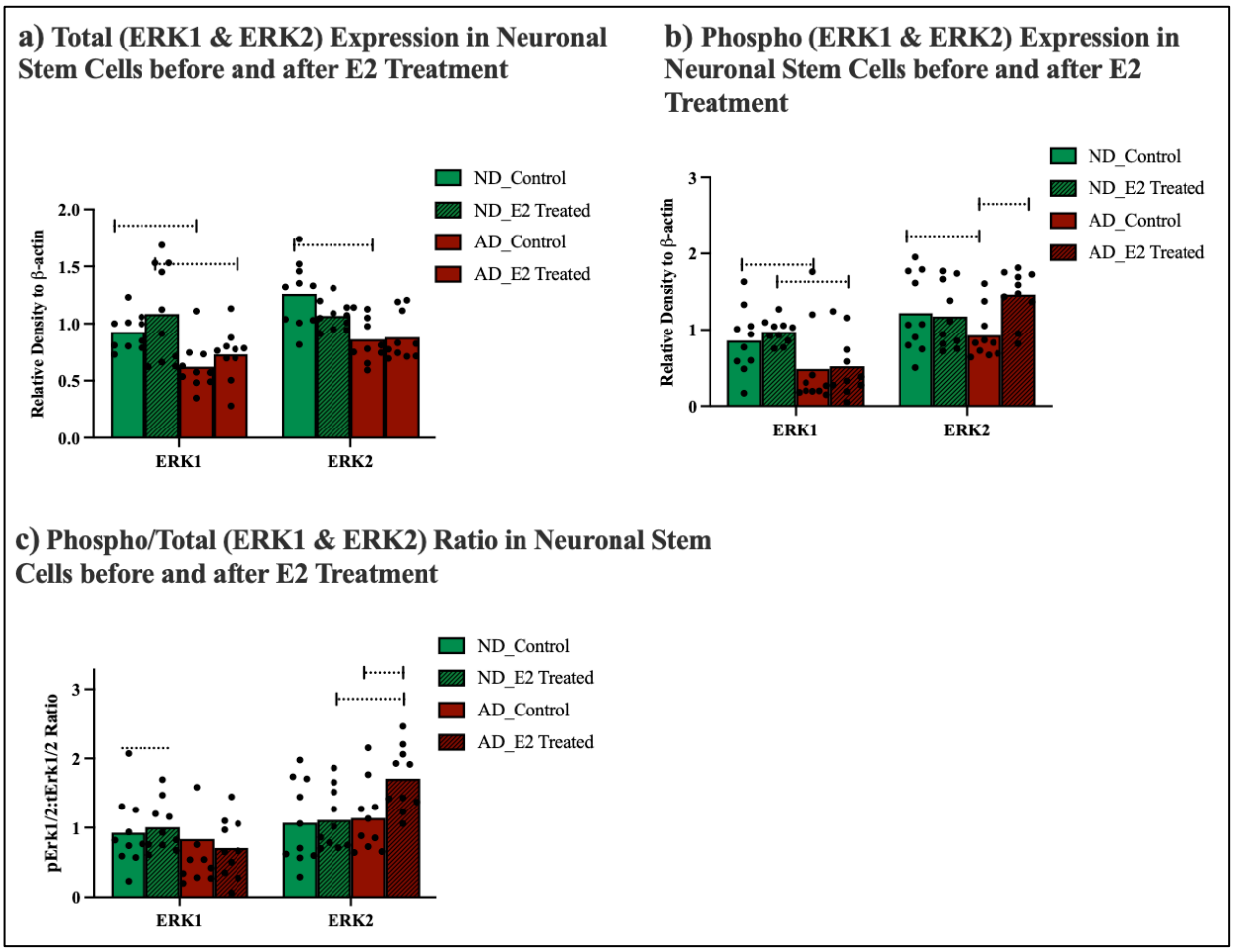


Figure 7.14 Densitometric analysis of ERK1 and ERK2 expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours, the blots are the same in Figure 7.13. a) Representative immunoblot for MAPK and the house keeping protein, βActin. **b, c & d)** an apparent higher level of ERK1 and ERK2 could be seen in ND compared to AD cells before E2 treatment. The same pattern was also visible after E2 treatments, except for ERK2 where a dramatic increase could be seen in AD compared to ND cells after E2 treatment. The latter differences in ERK2 was also apparent in the phosphorylated/total ratio of ERK2. n=1 for ND and AD.

For Akt, the expression of both its total and phosphorylated forms (at Ser473) was apparently higher in ND than in AD cells both before and after the addition of E2 (see Figure 7.15 b). There was a pattern of an apparent decline in Akt in both ND and AD stem cells after E2 treatment (Figure 7.15 b). These changes were not observed in the female brain (see Chapter 5).

While MAPK and Akt activity can be stimulated through an ER non-genomic pathway, the question is through which non-genomic pathway they are stimulated. ER could stimulate MAPK and Akt directly or through the membrane-localized ER (GPER) (Taguchi et al., 2004). E2 is a small lipid-soluble molecule and can cross the cell membrane. Hence, *in vitro* studies, when the ER localized in the cell surface are targeted, a bulkier E2 drug can be used by conjugating E2 to BSA by a 6 atom of hydrocarbon tether (E2-BSA) to increase its size and reduce its rate of diffusion through membranes. E2-BSA was shown to have high affinity in binding to ER localized on the cell membrane (Taguchi et al., 2004). As a result, future research could consider using E2-BSA as an indicator of the non-genomic impact of oestrogen on the neuronal hIPS cells.

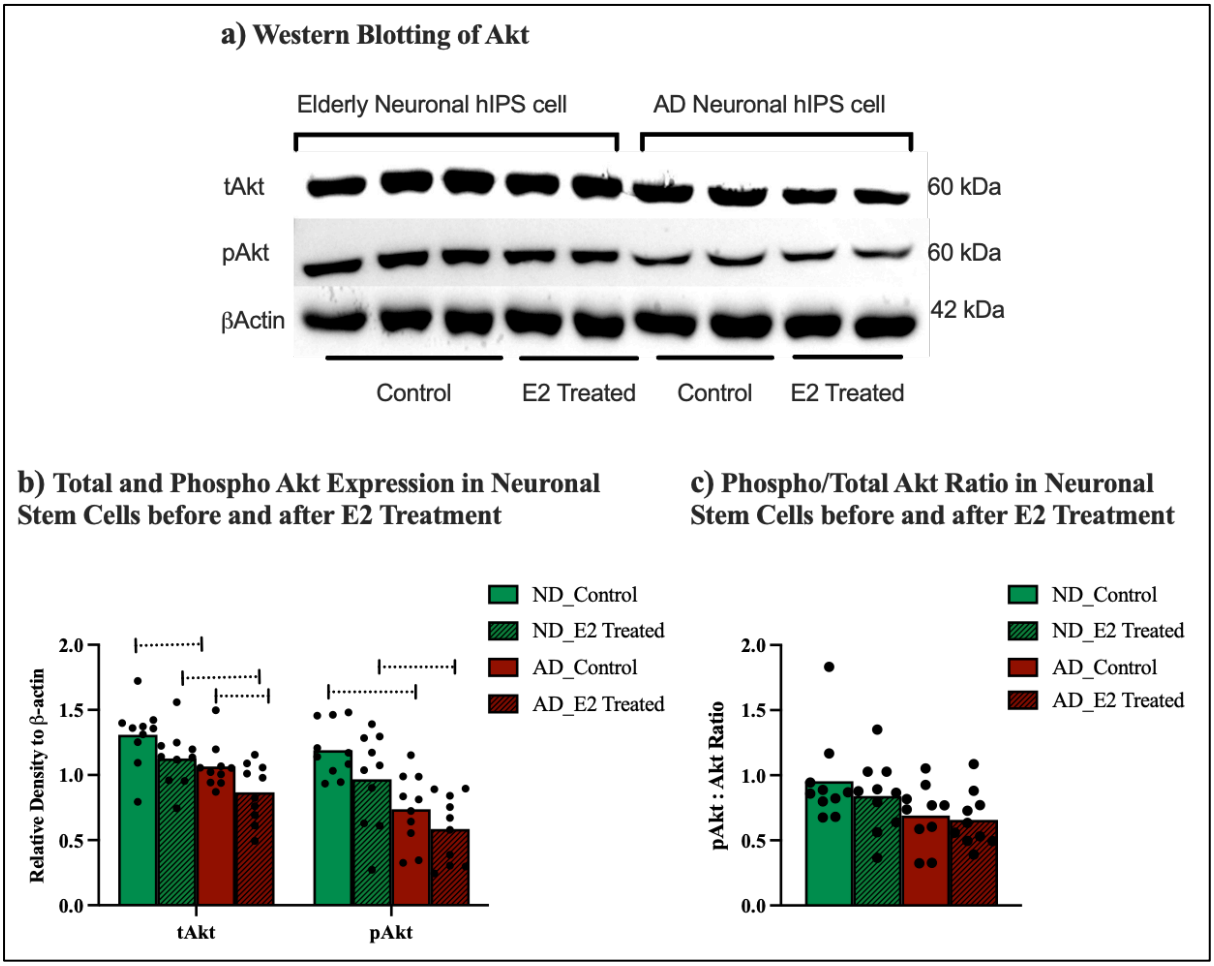


Figure 7.15 Densitometric analysis of Akt expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours. a) Representative immunoblot for Akt and the house keeping protein, βActin. **b & c)** An apparent decline in both total and Akt phosphorylated at Ser473 was identified between ND and AD cells in both control and E2 treatment conditions, but the ratio was apparently not different. n=1 for ND and AD.

7.4.4 *ELISA results for APP and soluble Aβ(1-40) and Aβ(1-42) fragments*

When APP and its amyloidogenic pathway fragmental products, $A\beta_{1-40}$ and $A\beta_{1-42,}$ were investigated in the neuronal hIPS cells, alterations in their levels were identified.

Prior to E2 treatment, the levels of APP were apparently unaltered in AD cells compared to ND cells. Conversely, soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels were clearly higher in AD cells than in ND cells (Figure 7.16). While the APP levels described in Chapter 3 did not change in female AD brains compared to age-matched ND samples, the levels of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were significantly higher in the AD brains. The same pattern in APP and its amyloidogenic metabolites was seen in the neuronal hIPS cells. Some AD transgenic animal models carry mutations that cause overexpression of APP. These animal models exhibit an overproduction of Aβ (Sasaguri et al., 2017). Thus, if neuronal hIPS cells express higher levels of $A\beta_{1-40}$ and $A\beta_{1-42}$, it might be expected that levels of APP would increase in AD hIPS cells but this was not seen. This could be that the processing of APP through the amyloidogenic pathway was predominant. This theory is supported by the apparent decline in ADAM10 seen in these cells (see Figure 7.10), thereby suggesting less processing via the non-amyloidogenic pathway. Another suggested theory is that the clearance of Aβ might be altered in AD hIPS cells, resulting in Aβ accumulation in these cells even with the no alternation in APP levels seen here. In the literature, a decrese in the levels of levels of Neprylisin and IDE were reported in AD human frontal cortex, thus stimulating Aβ accumulation in AD brain (Hellström-Lindahl et al., 2008).

When the neuronal hIPS cells were treated with E2, the apparent differences in $A\beta_{1-40}$ seen between ND and AD cells before drug treatment disappeared due to a large decline in soluble $A\beta_{1-40}$ levels in the AD cells after E2 treatment (Figure 7.16 b). APP levels were still unaltered after E2 treatment in both ND and AD cells. However, $A\beta_{1-42}$ levels remained higher in AD compared to ND cells although $A\beta_{1-42}$ levels were apparently decreased compared to their levels before the drug treatment (Figure 7.16 c).

Excitingly , the apparent decline in the levels of Aβ fragments in the neuronal hIPS cells after E2 treatment suggests stimulation of the neuroprotective effect of oestrogen against AD biomarkers, as per existing literature (Cooke and Woolley, 2005, Simpkins et al., 2010, Suzuki

et al., 2009) and not least in terms of its ability to suppress Aβ (Pike et al., 2009). In terms of *in-vitro* testing, it has been reported that oestradiol switched APP into non-amyloidogenic processing as an increase in sAPPα production was seen (Manthey et al., 2001, Gouras et al., 2000). However, here ADAM10 levels were apparently lower in the AD cells so an increase in non-amyloidogenic processing of APP may not have occurred. An in-depth investigation into sAPPα and sAPPβ levels in neuronal hIPS cells would help to clarify the activation of the amyloidogenic and non-amyloidogenic pathways after E2 treatment.

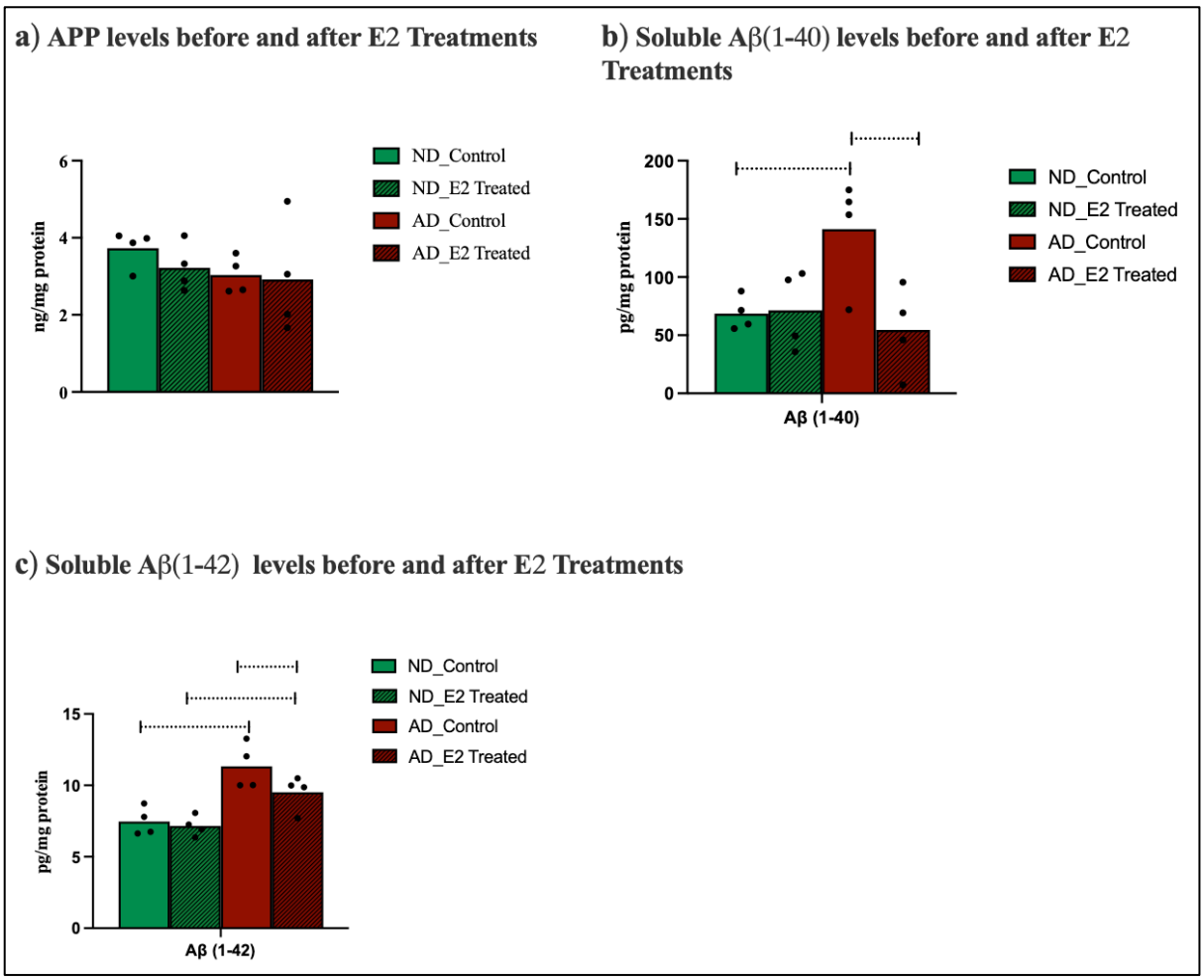


Figure 7.16 ELISA quantification of APP and Soluble $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ fragments expression in neuronal hIPS cells after 10nM E2 treatment for 24 hours. a) No change was seen in levels of APP for AD and ND cells in both control and E2 treatment conditions. **b)** Soluble $A\beta_{(1-40)}$ levels were higher in AD than in ND cells before E2 treatment, while after E2 treatment this difference was no longer apparent because of the reduction in soluble $A\beta_{(1-40)}$ levels in AD cells after treatment. **c)** Soluble $A\beta_{(1-42)}$ levels were apparently higher in AD than in ND cells before and after E2 treatment. n=1 for ND and AD.

7.4.5 Summary of E2 effect on AD biomarkers findings

This section will link the biochemical findings in this chapter together. First, while BACE1 could not investigated here, the Aβ peptide is a by-product of cleaving APP by BACE1 followed by γ-secretase (Cai et al., 2001, Wolfe et al., 1999). Even with no alteration in PS1 levels in AD cells before E2 treatment (seen in Figure 7.11 b), the overproduction of Aβ observed in AD hIPS could suggest that BACE1 and PS1 activity was higher in AD than ND hIPS cells. After E2 treatment, the levels of PS1 showed an apparent decline, especially in AD cells (Figure 7.11 b) along with a decline in Aβ production (Figure 7.16 b and c). However, this does not give a clear picture about BCAE1 activity after E2 treatment as a previous study found that inhibition of PS1 was associated with a decrease in Aβ levels but a corresponding increase in βCTF (Wolfe et al., 1999). A future study investigating BACE1 and βCTF levels in hIPS cells is needed to investigate the E2 mechanism in decreasing Aβ levels.

In the literature, E2 was reported to contribute to Aβ regulation. One of the suggested mechanisms of E2 regulating Aβ levels is through activating ER downstream signalling. This thesis focused on MAPK and Akt activity as indicators of ER downstream signalling. However, only the phospho/total MAPK ratio, had an apparent increase after E2 treatments (Figure 7.13 c), while Akt presented with a decline after E2 treatment (Figure 7.15 b and c). It has been reported in the literature that E2 shifts APP from amyloidogenic metabolism (β-secretase) to the non-amyloidogenic pathway (α-secretase) through the activation of protein kinase chain (PCK), and not via the MAPK pathway (Cisse et al., 2011). These shifting effects were associated with a reduction in Aβ production through PCK activating ADAM10 and ADAM17 (Cisse et al., 2011). In this chapter, ADAM10 was not altered after E2 treatment here, in parallel to the previous study (Cisse et al., 2011). One reason for that might be that the number of hIPS cells used in this project was not sufficient to conduct a quantitative analysis. In addition, the (Cisse et al., 2011) study used rat embryonic neurons in their study, while this Chapter used adult human stem cells. Thus there was a different in the cell types and species in the(Cisse et al., 2011) study and this thesis. Also, it has been highlighted that the stimulation of E2 proliferation in cell culture is age-dependent (Okada et al., 2008). E2 had about a 7% stimulation of proliferation in embryonic neuronal cells, but in adult cells it had no major effect on cell proliferation (Okada et al., 2008). Thus, the age of individuals from whom the neuronal cells are collected should be taken into account when studying the effect of E2. Given that, it is not clear if the apparent increase in MAPK after E2 treatment contributed to the reduction in

Aβ in the AD neuronal hIPS cells. Future work on the hIPS neuronal cells could include investigating PCK levels, and through which ER downstream signalling pathway E2 might regulate Aβ production. This could be investigated by knocking out PCK, MAPK and Akt separately and measuring Aβ after E2 treatment.

Another point which needs to be highlighted from the findings in this Chapter is related to Bcl2. While Aβ was apparently higher in AD neuronal hIPS cells compared to ND hIPS cells before E2 treatment (Figure 7.16 b and c), Bcl2 levels were not altered (Figure 7.12 b). Members of the Bcl2 family can be either pro-apoptotic or anti-apoptotic and share the Bcl2 homology domain (Youle and Strasser, 2008). These Bcl2 family members include Bim, a pro-apoptotic protein, and BclxL an anti-apoptotic protein. E2 was reported to increase the expression of BclxL *in-vitro* and that inhibited the proteolysis due to Aβ toxicity (Pike, 1999). *In vivo*, Aβ overproduction was able to decrease production of anti-apoptotic BclxL and upregulate pro-apoptotic Bim at both the mRNA and protein levels (Yao et al., 2007, Yao et al., 2005). Thus, investigating other Bcl2 family members such as Bim and BclxL could be planned with future hIPS studies.

While the findings discussed above were based on observational analysis due to the low n number, the clear decline of Aβ after E2 treatment supports the theory underlying this thesis. The thesis theorized that females are more likely to develop AD earlier than males due to the significant fall in E2 in females during the menopause transition phase. In Chapter 3, AD females had higher levels of Aβ accumulation in their brains along with a significant decrease in ER in Chapter 6. In this Chapter, the Aβ results (Figure 7.16 a and b) could indicate that the build-up of Aβ in female brains with advancing age seen in this thesis could be related to a decrease in E2 activity in their brains.

7.5 Conclusion

The first objective of this study was completed as neuronal stem cell growth was achieved via a method that can be employed in future laboratory work using an increased number of samples.

Also, this study did demonstrate that E2 has an apparent protective function against Aβ in neuronal hIPS cells. This can be seen with the apparent decreases of PS1 and Aβ along with an upregulation in the phospho/total MAPK ratio after E2 treatment especially in AD hIPS

cells. Nonetheless, more samples of neuronal hIPS cells are required for future work to conduct a quantitative analysis which would improve understanding about about how E2 regulates Aβ in the brain.

CHAPTER 8 _ Thesis Discussion

8.1 Overview

Alzheimer's disease (AD) is a progressive neurological condition and accounts for 60 - 70% of dementia cases (Alzheimer's Association, 2021). It affects mostly the elderly population, with 75% of cases reported to be 85 years old or above (Jorm and Jolley, 1998, Qiu et al., 2009). One of the most important disease risk factors is age. Due to the continuously ageing population globally, it is estimated that the number of patients with AD will triple by the year 2050 (Alzheimer's Association, 2021, Powell and Baker, 2019).

AD affects all people irrespective of sex or ethnicity. However, in some developed countries such as the UK and USA, the number of females affected with AD is higher than for males. In the USA, for every male AD patient, there are two female patients (Association et al., 2021). One of the most important biological differences between males and females is their sex steroid expression, with E2 and testosterone predominent in females and males, respectively (Sherman et al., 1976). It has been found that, during critical points of ageing, the organizational impact of the sex steroid hormones on neural growth and maturation may change in the brain (Bowers et al., 2010, Gore, 2008, Krohmer and Baum, 1989, Slob et al., 1980, Weisz and Ward, 1980). Thus, the variation between males and females in their susceptibility to developing AD may be partially a result of sexual dimorphisms that are established during development, together with disparities in the brain and circulating steroid hormone levels. In females, it is well documented that E2 and progesterone serum concentrations are reduced significantly and rapidly as a consequence of the menopause (Sherman et al., 1976), thus a decline in their supply to the brain has been postulated and a concomitant decline in their neuroprotective effects in postmenopausal women (Bonomo et al., 2009, Henderson, 2006b). In contrast, the age-dependent fall in the male primary sex steroid hormone, testosterone, is only slowly progressive (Morley et al., 1997) which is highly likely to be less problematic for neuroprotection in the brain.

A significant overarching goal in AD studies is to understand the complexity of the brain during the lifespan. This could be investigated through selecting a 'representative' sample of individuals that are presumed to generalize to the larger population. However, clinical studies in AD need to reconsider what constitutes an appropriate 'representative sample'. A major pressing problem is the under-representation of females in experimental design. For the past two centuries, neuroscience preclinical studies have focused on using male animals and excluded female animals as the later were considered to be too variable (Beery and Zucker,

2011). In clinical neuroscience studies, this problem is more subtle. While there are many studies which have enrolled both males and females to eradicate sex biases, the majority of these studies enrolled elderly populations, aged 65 and older (Taylor et al., 2019). These so-called representative samples overlooked a significant sex-specific variable point in the lifespan, the menopause transition phase. Furthermore, neuroscience was the last field to report sex disparities in its published studies, as articles generally pooled male and female findings (Garcia-Sifuentes and Maney, 2021). A recent study highlighted that approximately 70% of neuroscience publications in 2019 misreported sex disparities (Garcia-Sifuentes and Maney, 2021).

The fundamental aim of this thesis was to investigate what has been overlooked in previous studies which did not consider the female menopause transition phase and misreported sex disparities in AD (Garcia-Sifuentes and Maney, 2021). In this thesis sex disparities in AD development were explored during human brain ageing, from early adulthood to elderly people, and in AD. The main aims of this thesis were two-fold. The first was to investigate sex disparities in the expression of AD biomarkers (APP, its metabolites and secretase enzymes) in the human brain during ageing and in AD. The second was to investigate oestrogen-associated protein expression, including ER and some of their downstream signalling pathways, specifically MAPK and Akt, during brain ageing and in AD in both sexes. In this Chapter, the main finding of the thesis are summarized and discussed together and consideration is given as to how these data increase our understanding and contribute to future AD research.

8.2 Summary and Discussion of Findings

a) AD Biomarkers During Frontal Cortex Ageing in Cognitively Healthy Female and Male Individuals

Aggregation of Aβ and Tau hyperphosphorylation are the main AD biomarkers (McKhann et al., 2011). The use of these biomarkers as an indication of disease initiation has a long history. Many studies validated these biomarkers for prediction of the conversion from MCI to AD (Yuan et al., 2009, Mattsson et al., 2009, Visser et al., 2009). This thesis used them as an indication of disease initiation. Both sexes presented with a significant build up in Aβ in the frontal cortex with advancing age. One of the most novel and important findings of the

present thesis is that when APP and its metabolic enzymes were investigated, a sex disparity was observed with an increase in the levels of APP and its amyloidogenic enzymes, β-secretase and γ-secretase, and βCTF in aged female frontal cortex. However, aged male frontal cortex was associated with an increase in β-secretase only, the levels of the soluble $A\beta_{(1-42)}/A\beta_{(1-40)}$ ratio along with soluble and insoluble $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ and βCTF were significantly higher in elderly females compared to elderly males. This could indicate that β-secretase is highly activated in elderly female frontal cortex with advancing age.

The formation of NFTs is thought to be more a proximate cause of later neuronal malfunction and death in AD (Hardy and Higgins, 1992, GÛmez-Isla et al., 1997, Bennett et al., 2004). When Tau protein levels was investigated in Chapter 4, no age-related alteration was seen in both sexes. Next, the project investigated the main kinase enzymes for Tau phosphorylation, the GSK3 isoforms, reported to be associated with AD (Martin et al., 2013a). Both sexes showed a significant increase in the level of the active form of GSK3β, phosphorylated GSK3β ($Tyr_{279/216}$), in the elderly group compared to the other age groups. *In vivo,* $A\beta_{(1-42)}$ has been reported to activate GSK3β (Kirouac et al., 2017). Since soluble $A\beta_{1-42}$ was higher in the elderly subjects of both sexes (Chapter 3), it could contribute to the higher levels of GSK3β activity seen and thus, higher phosphorylation of Tau. However, phosphorylated Tau was undetectable in healthy cognitive subjects (Chapter 4). The most likely explanation for this is that a very low level of phosphorylated Tau is present in healthy human brains (Alsaqati et al., 2018, Hefti et al., 2019). Furthermore, longer PMI were reported to result in dephosphorylation of 50% of the Tau protein (Wang et al., 2015) so it is less likely that phosphorylated tau would have been seen here in these samples with long PMI. Nonetheless, elderly females had higher levels of Tau and GSK3β in their frontal cortex compared to elderly male subjects. This could be due to the higher Aβ levels in the female brains reported here.

Aβ deposition was reported in approximately 20-40% of healthy cognitively normal elderly individuals (Shaw et al., 2009, Mintun et al., 2006, Bouwman et al., 2009). It is assumed that Aβ deposition alone, even in abundant quantities, is not sufficient to initiate dementia, and that abnormalities of Aβ deposition precede AD clinical symptoms (Fagan et al., 2007, Fagan et al., 2009, Li et al., 2007, Stomrud et al., 2007). Also, the main criteria for the proper diagnosis of early AD depends on both the presence of the biomarker and memory impairment (McKhann et al., 2011). While the human subjects whose samples were used in this project were

cognitively healthy (according to the Brain Banks), sex disparities in Aβ overproduction, reported in the thesis Chapter 3, in elderly females should not be neglected. This is especially true given that Aβ deposition was reported to be initiated earlier than any manifestations of neurodegeneration and AD clinical symptoms, followed by abnormalities in Tau at later stages of the disease (Jack et al., 2010). Thus, females might have initiation of pre-symptomatic AD earlier than their male peers, and their treatment should be started before the clinical diagnosis of AD.

b) AD Biomarkers in AD Female and Male Individuals

It is not surprising that both AD female and male samples were associated with an increase in Aβ production and aggregation along with Tau hyperphosphorylation (Chapters 3 and 4), since these proteins are the main hallmarks of AD (McKhann et al., 2011). However, novel sex disparities were seen with a decrease in sAPPα, a neuroprotective fragment of APP, in AD female frontal cortex and an increase in PS1 and the ratio of soluble $A\beta_{(1-42)}/A\beta_{(1-40)}$ in males. The latter ratio is reported to be an indicator of early-stage AD (Kumar-Singh et al., 2006). Since female exhibit an age-related increase in the $A\beta_{(1-42)}/A\beta_{(1-40)}$ ratio during healthy brain ageing (Chapter 3), could this finding indicate that the theoretical so-called pre-symptomatic AD phase could be initiated earlier in females than males? This suggestion is backed up by the significantly higher levels of Aβ overproduction in elderly and AD female compared to male samples. Additionally, female AD frontal cortex was associated with significantly higher levels of Tau hyperphosphorylation than AD male samples (Chapter 4). Thus, it is possible that the disease deterioration is worse in female brains than in males. This idea is supported here by the increase in ProCaspase3 in female but not male AD subjects, which could trigger more neuronal cell death (Chapter 4).

c) Oestrogen Receptors During Frontal Cortex Ageing in Cognitively Healthy Female and Male Individuals

One of the main biological sex differences between females and males is the difference in steroid hormone expression (Sherman et al., 1976). According to the data above, it is hypothesised that the significant decline in E2 in females exhibited during the menopause transition phase, increases their susceptibility to manifest earlier abnormalities in AD biomarker proteins. Thus, the second aim of this thesis was to investigate the expression of ER

subtypes and associated signalling proteins in the ageing frontal cortex of both female and male subjects.

Male samples exhibited an age-related increase in ERβ, and increases in its downstream signalling proteins, ERK2 and Akt (Chapter 5). However, another novel finding in this thesis was that female samples were associated with an age-related decrease in ERα and a decrease in ERβ in the middle-aged group (Chapter 6). It is well documented that E2 can decrease Aβ levels, and that this function is its most significant mode of neuroprotection against AD (Carroll and Rosario, 2012, Pike et al., 2009). This neuroprotection could be due to the capability of E2 to shift APP processing towards the non-amyloidogenic pathway (Gandy, 2003) and to lower BACE levels (Singh et al., 1999). Additionally, E2 stimulates insulin degrading enzyme activity (metabolises Aβ) (Amtul et al., 2010, Zhao et al., 2011) and microglia phagocytosis of Aβ (Singh et al., 1999). E2 has also been reported to stimulate transthyretin expression (Amtul et al., 2010, Oliveira et al., 2011, Quintela et al., 2009) which attaches to, and sequesters Aβ, thus preventing the formation of neurotoxic plaques (Schwarzman et al., 1994). Further work has demonstrated that E2 stimulates the anti-apoptotic Bcl-2 protein, together with simultaneous inhibition of expression of the pro-apoptotic isoform (Pike, 1999). E2 also reduced hyperphosphorylated Tau concentrations (Zhang et al., 2008). Altogether, the sex variations in Aβ production in the elderly female and male samples reported above could be due to ER expression. In elderly females, a decline in ER expression (Chapter 6) could cause a decline in the neuroprotective function of E2 against Aβ. Hence, Aβ overproduction and accumulation would be more likely to occur in aged female brains. In males, while Aβ overproduction was visible in elderly subjects, the enhancement in E2 neuroprotection with advancing age, as seen with the upregulation in ERβ, Akt and ERK2 (Chapter 5), could be the reason for lower Aβ accumulation compared to their female peers.

d) Oestrogen Receptors in AD Female and Male Individuals

According to the findings of this thesis, the neuroprotective effect of E2 is likely to decline in the AD cortex. That was seen in Chapter 5 with the decline in ERβ in AD male frontal cortex, and in Chapter 6 with the decline in ERα and ERβ in AD female frontal cortex. However, the reduction in E2 neuroprotection in AD males could be partial, as ERα did increase in AD male cortex. Hence, Aβ overproduction and aggregation along with Tau

hyperphosphorylation were significantly higher in female AD cortex compared to male AD. samples

In addition, in the neuronal hIPS cell pilot study in Chapter 7, E2 treatment was associated with an apparent significant reduction in PS1 and Aβ levels in female AD cells. This would back up the importance of E2 in protecting the brain from the initiation of Aβ overproduction. Thus, females undergoing the menopause transition phase could be subjected to more production of Aβ in their brains due to the significant dropdown in serum E2 levels.

e) Correlation between Alzheimer's Diseases Biomarkers Data and Oestrogen Receptors Data Collected in the Thesis

Given that the same human brain samples were used throughout the thesis, a correlation test between investigated AD biomarkers (Aβ and Tau) and ER's was conducted for each age group (Table 8.1 and 8.2). Only significant results will be mentioned in this section.

When Aβ data, in chapter 3, was correlated against ER's data, in chapter 5, (Table 8.1) a positive correlation between ERα and Aβ and between ERβ and Aβ was calculated in young male samples. This positive correlation was lost with age, which might indicate a healthy regulation role of ERα and Aβ during young age in male samples. On the other hand, female presented with a positive correlation between ERβ and Aβ only in middle age. Which could be a protection function of oestrogen against Aβ during that age period. Interestingly, the positive corelation between ERβ and Aβ in young male samples switched to negative correlation in elderly and in AD, which could be translated into a drop sown of oestrogen protection in these age groups.

Next Tau data, in chapter 4, was correlated against ER's data, in chapter 5, and a negative correlation between ERα and Tau was presented in both young male and female samples. This might indicate a regulation role of ERα against Tau in healthy brains, and that regulation got lost with ageing. In AD, only male samples show a positive correlation between ERβ and the increase levels of Tau.

Finally, Aβ data, in chapter 3, was correlated against IHC ER's data of female samples, in chapter 6. Majority of significant correlation between investigated proteins was seen in GM region of female brains (Table 8.2). Specifically, middle aged and eldely female GM show a

negative correlation between ERβ and Aβ which could be translated to a loss in oestrogen neuroprotection function against Aβ during menopause transition phase. However, in AD female GM the negative correlation between ERβ and Aβ switched to a positive correlation, either if that positive correlation indicate a neuroprotection function or a burden in the disease state it's not clear yet.

8.2.1 Is Being a Female a Risk Factor for AD?

Yes, the female biological sex is a risk factor for AD. With the sex differences in AD biomarkers and ER expression reported above, it could be suggested that being female is a risk factor for manifesting an early pre-symptomatic AD phase. In addition., the decline in ER expression could contribute to a decline in the neuroprotective function of E2. The early manifestations of AD biomarkers may be masked by the relative youthfulness of female brains, as they have been shown to possess higher metabolic features compared to age-matched males (Goyal et al., 2019). However, this youthfulness in female brains is reported to decrease when the disease shifts from MCI to AD, reaching a similar pattern of glucose metabolism as in male AD brains (Beheshti et al., 2021). While the main criteria for the proper diagnosis of AD depend on both the presence of biomarkers and memory impairment (McKhann et al., 2011), tackling AD in females should be initiated much earlier before disease diagnosis, speculatively in the perimenopause phase, especially in females harbouring Aβ overproduction in their brains which could be diagnosed through Aβ-imaging (Chun, 2018). However, while the Aβ peptide has a hormesis effect; where it is beneficial at low concentrations and toxic at high ones (Puzzo et al., 2008), the exact Aβ concentration where it is harmful to the brain in periclinal AD stage has not yet been looked at.

Nonetheless, the idea suggested above of initiating AD treatment during the perimenopause phase is particularly highlighted by a 3-year longitudinal study showing that the risk of developing AD increases during the menopause transition phase (Mosconi et al., 2018). In addition, clinical findings have suggested that the likelihood of developing AD is diminished when HRT is commenced in mid-life but potentiated when HRT is prescribed late in life (Whitmer et al., 2011). However, a recent meta-analysis highlighted that these HRT clinical studies were capable of delaying the onset of AD without treating the condition itself (Song et al., 2021). Thus, it would appear that clinical studies should try using HRT along with anti-AD candidate drugs once developed and/or validated in peri-menopause females.

Table 8.1 Correlations between Alzheimer's Disease Biomarkers and ER's collected at Western Blotting Data

Correlation test (Western Blotting vs. Western Blotting)	Female Samples (P value)				Male Samples (P value)			
	Young	**Middle Age**	**Elderly**	**AD**	**Young**	**Middle Age**	**Elderly**	**AD**
tERα vs Aβ40	ns	ns	ns	ns	(+) 0.034	ns	ns	ns
pERα vs Aβ40	ns	ns	ns	ns	ns	ns	ns	ns
tERα vs Aβ42	ns	ns	ns	ns	ns	ns	ns	ns
pERα vs Aβ42	ns	ns	ns	ns	ns	ns	ns	ns
tERβ vs Aβ40	ns	ns	ns	ns	ns	ns	ns	(-) 0.05
pERβ vs Aβ40	ns	ns	ns	ns	ns	ns	ns	ns
tERβ vs Aβ42	ns	ns	ns	ns	ns	ns	(-) 0.05	ns
pERβ vs Aβ42	ns	(+) 0.045	ns	ns	(+) 0.013	ns	ns	ns
tERα vs Insoluble Aβ40	-	-	ns	ns	-	-	ns	ns
pERα vs Insoluble Aβ40	-	-	ns	ns	-	-	ns	ns
tERα vs Insoluble Aβ42	-	-	ns	ns	-	-	ns	ns
pERα vs Insoluble Aβ42	-	-	ns	ns	-	-	ns	ns
tERβ vs Insoluble Aβ40	-	-	ns	ns	-	-	ns	ns
pERβ vs Insoluble Aβ40	-	-	ns	ns	-	-	ns	ns
tERβ vs Insoluble Aβ42	-	-	ns	ns	-	-	ns	ns
pERβ vs Insoluble Aβ42	-	-	ns	ns	-	-	ns	ns
tERα vs Tau	(-) 0.05	ns	ns	ns	(-) 0.039	ns	ns	ns
pERα vs Tau	(-) 0.05	ns	ns	ns	ns	ns	ns	ns
tERβ vs Tau	ns	ns	ns	ns	ns	ns	ns	ns
pERβ vs Tau	ns	ns	ns	ns	ns	ns	ns	(+) 0.019
tERα vs pTau	-	-	-	ns	-	-	-	ns
pERα vs pTau	-	-	-	ns	-	-	-	ns
tERβ vs pTau	-	-	-	ns	-	-	-	ns
pERβ vs pTau	-	-	-	ns	-	-	-	ns

- **The Null Hypothesis (H_0) of Age:** There is no statistically significant relationship between the investigated proteins on each group. Pearson Correlation Analysis, $p < 0.05$.
- **ns** = non-significant
- **(-)** = Inverse Relationship
- **(+)** = Direct Relationship

Table 8.2 Correlations between Alzheimer's Disease Biomarkers and ER's collected at IHC Data

Correlation test (IHC vs Western Blotting)	Grey Matter (P value)				White Matter (P value)			
	Young	**Middle Age**	**Elderly**	**AD**	**Youn g**	**Middle Age**	**Elderl y**	**AD**
tERα vs Aβ40	ns	ns	ns	ns	ns	ns	ns	ns
pERα vs Aβ40	ns	ns	ns	ns	ns	ns	ns	ns
tERα vs Aβ42	ns	ns	ns	ns	ns	ns	ns	ns
pERα vs Aβ42	ns	ns	ns	ns	ns	ns	ns	(+) 0.02
tERβ vs Aβ40	ns	(-) 0.018	ns	ns	ns	ns	ns	ns
pERβ vs Aβ40	ns	(-) 0.05	ns	ns	ns	ns	ns	ns
tERβ vs Aβ42	ns	ns	ns	(+) 0.01	ns	ns	ns	ns
pERβ vs Aβ42	ns	(-) 0.045	(-) 0.014	(+) 0.036	ns	ns	ns	ns
tERα vs Tau	ns	ns	ns	ns	ns	ns	ns	ns
pERα vs Tau	ns	ns	ns	ns	ns	ns	ns	ns
tERβ vs Tau	ns	ns	ns	ns	ns	ns	ns	ns
pERβ vs Tau	ns	ns	ns	ns	ns	ns	ns	ns

- **The Null Hypothesis (H_0) of Age:** There is no statistically significant relationship between the investigated protein on each group. Pearson Correlation Analysis, $p < 0.05$.
- **ns** = non-significant
- **(-)** = Inverse Relationship
- **(+)** = Direct Relationship

Interestingly, anti-Aβ drug therapies do not have the same impact on the sexes in Tg mice (Park et al., 2003) and may even demonstrate contrasting influences, which supports the notion that sex should be considered when interpreting research that utilises models of AD (Dubal et al., 2012). Furthermore, the greater degree of amyloid genesis in females can lead to inconsistent data in anti-Aβ clinical trials (Tolar et al., 2019, Fernandez and Silva, 2021). Finally, the clinical sex disparities for the disease in this thesis, have highlighted sex variations in diagnostic and disease progression bioindicators which, if verified, would have connotations not only for diagnostic assessments in patients but also for the recruitment of subjects for clinical trials. More consideration of sex disparities may enhance diagnostic specificity and sensitivity and optimise clinical trial methodologies. Males and females should be considered a different species during clinical investigations of candidate anti-AD therapy.

8.3 Limitations of this Study

This project has some limitations, particularly relating to the cohorts under study. The first weakness is related to the wide age range in the groups of human samples used (Figure 8.1), which is likely to have a confounding effect on chronological age in disease initiation. When correlations between age and all the proteins investigated in this thesis were examined in each age group, only a few significant correlations were found but these were mainly in the elderly and AD groups (Table 8.1). This could be due to the wider age variation in these groups or the extent of AD or other disease pathology. The age range variation of samples used in this thesis was due to the availability of healthy brain samples in the UK Brain Banks and could not be avoided. Thus, age variation between human samples was a general issue in this project which is very difficult to overcome.

When the age groupings were introduced in this project, the aim was to select age ranges representing the menopause staging in females, pre-menopause (young age group), perimenopause (middle-age group) and post-menopause (elderly age group). However, perimenopause is a difficult phase that cannot be determined by age alone as there is no specific age when the perimenopause starts. This is the second limitation in this thesis, where the female middle-age group are not fully representative of the peri-menopause transition phase. The Brain Banks do not have information about E2 serum levels, so it was not possible to obtain accurate assessments of the stage of the perimenopause/menopause for any of the women whose samples were used. Also, the perimenopause phase assessment depends on clinical judgment

and cluster symptoms, including hot flushes, presence of sweatiness, insomnia, mood swings, appetite changes, cognitive problems and short-term memory complaints (Mosconi et al., 2018). These were not known for the women here.

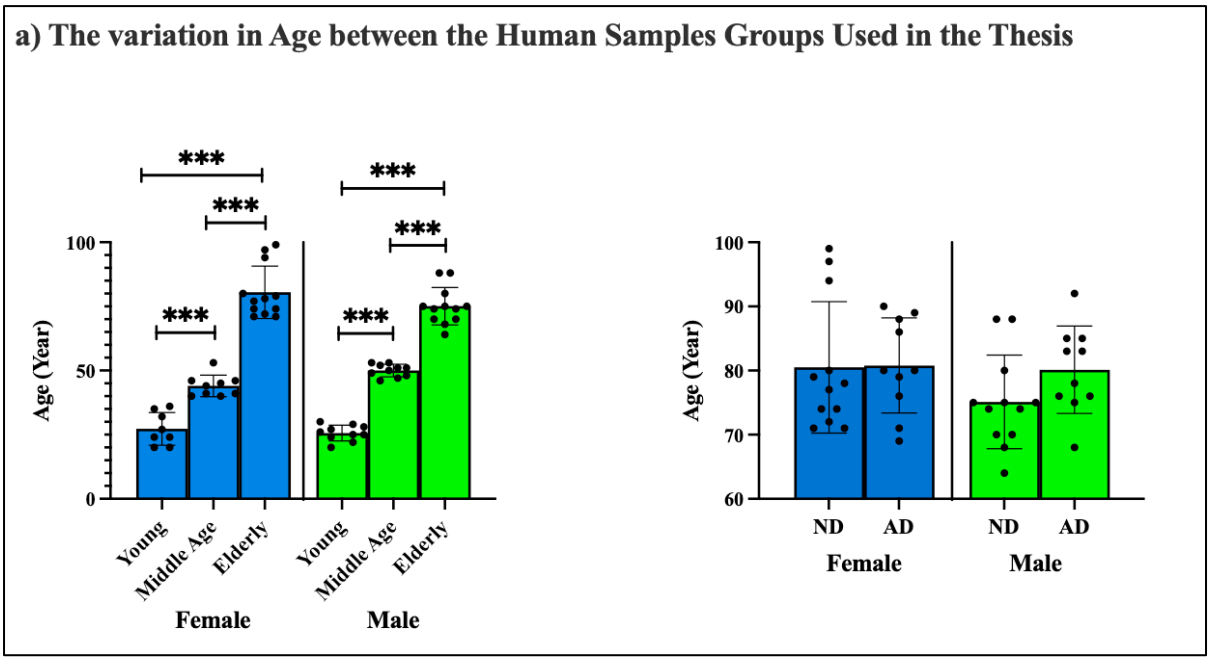


Figure 8.1 Age variation between the Human samples used in this thesis. A significant difference in age was calculated between young, middle-age and elderly age groups of both sexes. Data are presented as mean ± S.E.M. **p<0.002 and *** p<0.001. Two-way ANOVA followed by Tuky's post-hoc tests were used to compare the age ranges while Student's t-tests were used for ND vs. AD samples.

Table 8.3 Correlations between Age in each Group and Western Blotting and ELISA Protein Expression Data

Proteins	Female Samples (P value)				Male Samples (P value)			
	Young	Middle Age	Elderly	AD	Young	Middle Age	Elderly	AD
APP	ns	ns	ns	(-) 0.01	ns	ns	ns	ns
ADAM10	ns	ns	ns	ns	ns	(+) 0.03	ns	ns
BACE1	ns	ns	ns	ns	ns	ns	ns	ns
PS1	ns	ns	ns	ns	ns	ns	ns	ns
PS2	ns	ns	ns	ns	ns	ns	ns	ns
sAPPα	ns	ns	ns	ns	ns	ns	ns	ns
sAPPβ	ns	ns	ns	ns	ns	ns	ns	ns
βCTF	ns	ns	ns	(+) 0.003	ns	ns	ns	ns
Sol.Aβ$_{1-40}$	ns	ns	ns	(-) 0.01	ns	ns	ns	ns
Sol.Aβ$_{1-42}$	ns	ns	ns	ns	ns	ns	ns	ns
Tau	ns	ns	(+) 0.002	ns	ns	ns	ns	ns
tGSK3α	ns	ns	ns	ns	ns	ns	ns	ns
tGSK3β	ns	ns	ns	ns	ns	ns	ns	ns
pGSK3α	ns	ns	ns	ns	ns	ns	ns	ns
pGSK3β	ns	ns	ns	ns	ns	ns	ns	ns
PP2A	ns	ns	ns	ns	ns	ns	(-) 0.01	ns
Pro Caspase3	ns	ns	ns	ns	ns	ns	ns	ns
Bcl2	ns	ns	ns	(-) 0.01	ns	ns	ns	ns
tERα	ns	ns	(+) 0.004	ns	ns	ns	ns	ns
tERβ	ns	ns	ns	ns	ns	ns	ns	ns
pERα	ns	ns	(+) 0.002	ns	ns	ns	ns	ns
pERβ	ns	ns	ns	ns	ns	ns	ns	ns
tMAPK	ns	ns	ns	ns	ns	ns	(+) 0.03	ns
pMAPK	ns	ns	ns	(-) 0.01	ns	ns	ns	(-) 0.03
tAkt	ns	ns	ns	ns	ns	ns	ns	ns
pAkt	ns	ns	ns	ns	ns	(+) 0.03	ns	ns

- **The Null Hypothesis (H_0) of Age:** There is no statistically significant relationship between the age of each group and protein expression. Pearson Correlation Analysis, $p < 0.05$.
- **ns** = non-significant
- **(-)** = Inverse Relationship
- **(+)** = Direct Relationship

Another limitation in this thesis is that there was very little ante-mortem information on the human samples. It is well documented that the health of the brain is determined by a combination of intrinsic factors, including genetics and health behaviour, along with extrinsic factors, such as environment, lifestyle and social effects (Erickson et al., 2014). Regarding the extrinsic factors, a person's lifestyle during ageing has been linked to AD development. In fact, consistent evidence from observational studies reported that a third of AD cases worldwide are attributable to 7 common modifiable risk factors during life: smoking, low education, physical inactivity, depression, midlife hypertension, diabetes mellitus, and midlife obesity (Norton et al., 2014). The full medical history of these subjects was not provided as the Brain Banks only gave a main illness and cause of death of these patients. For instance, long-term use of psychological drugs (e.g. benzodiazepines) and anticholinergic drugs have been linked to accelerated cognitive decline and development of AD (De Gage et al., 2014, Coupland et al., 2019).

Another point that needs to be considered is that sex hormone production is chronically suppressed in approximately 100 million women worldwide through using oral contraceptives (OCs) (Christin-Maitre, 2013). OCs were reported to influence aspects of brain structure in young adults, with an increase in the grey matter volume in the amygdala and para-hippocampal gyrus and the temporal cortex relative to non-users (Lisofsky et al., 2016, Pletzer et al., 2010), and less effect in the frontal cortex but this finding was inconsistent across studies (Pletzer et al., 2010, De Bondt et al., 2013, Petersen et al., 2015). It has been highlighted with retrospective studies that OC use in mid-life had a positive effect on cognitive aging (Karim et al., 2016). However, other studies failed to find a link between OCs and healthy cognitive function (McLay et al., 2003, Tierney et al., 2013). One third of OC users start using them in early adolescence, however, there is little information about the effect of early exposure to OCs on brain structure and function (Taylor et al., 2019).. It is not possible to know whether the women in this study used OCs during their life-time and the duration of any such use. Therefore, the influence of OCs on the findings in this thesis cannot be evaluated.

Finally, the limitation faced with hIPS cells was the number of samples which was used in Chapter 7. Statistically, more than 5 samples are appropriate to conduct a quantitative analysis (Dworkin, 2012). However, this study started with one ND and one AD stem cell sample since handling stem cells was a novel method in the lab, and particular precaution measures were needed to culture them. Also, the COVID-19 breakout and lab shut down

reduced the available lab work time and budget needed to increase the number of stem cell samples. Nonetheless, culturing non-transfected neuronal hIPS cells was achieved in this project, and the established IPS culturing method could be used in future studies with more cell samples.

8.4 Future Directions

This section will include some suggested future experiments in addition to the studies mentioned in each experimental Chapter of the thesis.

Initially. it was planned to look at all steroid hormone-associated proteins during this project, including androgen receptors (AR). However, no good antibodies against AR in the brain were found, thus, future work could include exploring AR expression during brain ageing and AD in both sexes using alternative methods such as RT-PCR.

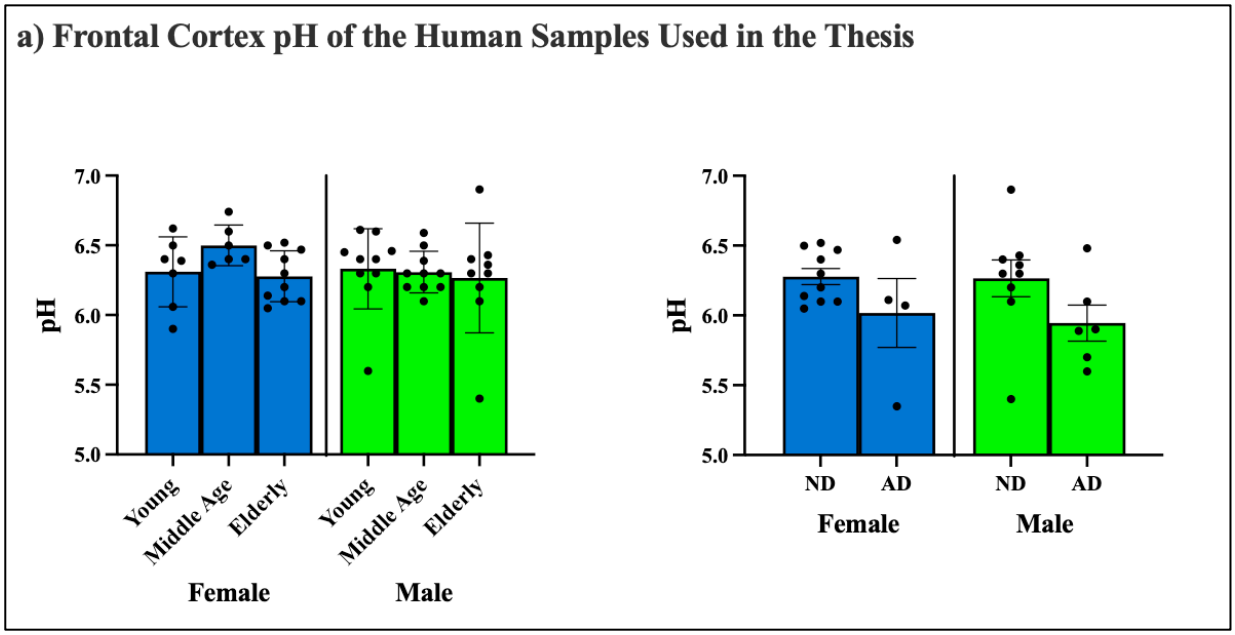
In Chapter 6, alterations in the levels of ERs in female frontal cortex were found using IHC, while in WB no alterations were identified. Future work could include investigating male frontal cortex ER expression with IHC as this would allow cellular localization to be explored, which is not possible with WB, and compared to female ER expression.

One of the most promising future studies arising from the work in this thesis is exploring the effect of E2 on neurones derived from human IPS cells. In Chapter 7, a pilot study was conducted using stem cells collected from one healthy female elderly individual and one female with AD. Thus, repeating those experiments with multiple cell expansions from the same donors and obtaining stem cells from both female and male donors would provide larger sample numbers allowing statistical analyses to be performed and would be the next step in studying the effect of E2 in male and female neuronal cells under controlled conditions.

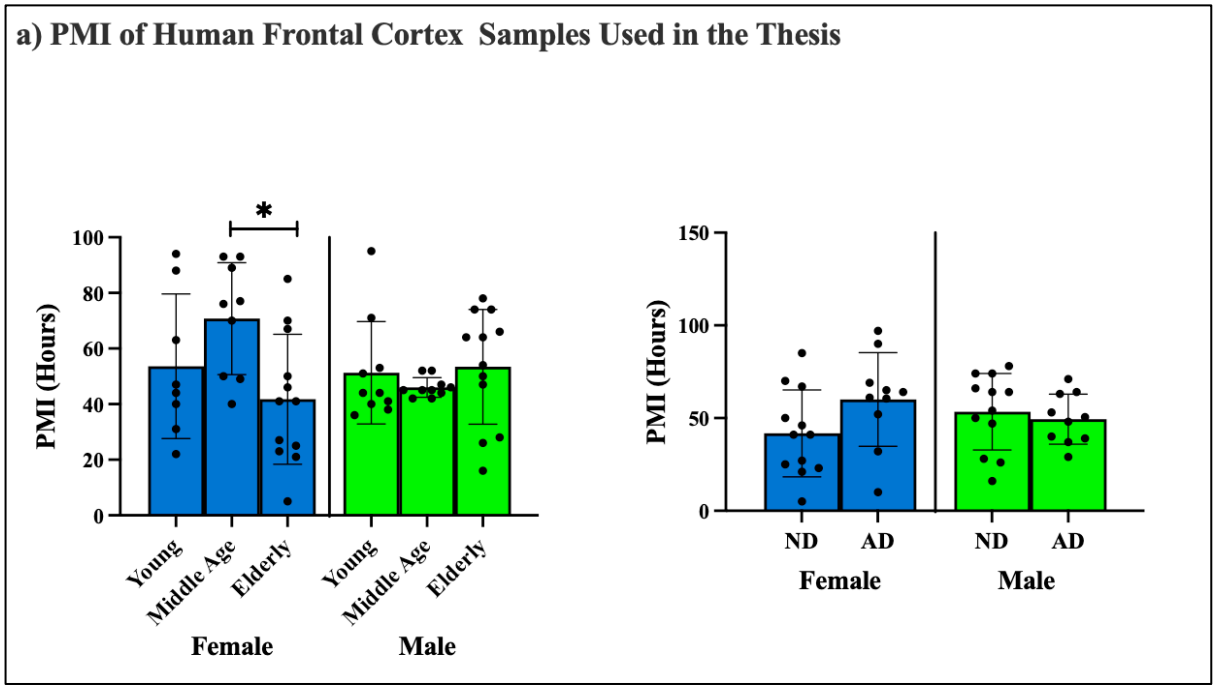
8.5 Thesis Conclusion

This thesis has shown that there is an inter-relationship between brain ageing and biological sex in AD development and suggested that females exhibit pre-symptomatic AD earlier than males. Also, the sex disparities reported in this thesis highlight how crucial it is to investigate both sexes separately, especially in reporting female findings in preclinical and clinical studies where males have previously generally been used.

Appendixes



Appendix I pH of the Human Samples used in the Thesis. There were no differences in sample pH between age groups of both sexes. Data are presented as mean ± S.E.M. Two-way ANOVA followed by Tukey's post-hoc tests were used to compare the age ranges while Student's t-tests were used for ND vs. AD samples.



Appendix II PMI of the Human Samples used in the Thesis. The PMI was significantly higher in middle-age female samples compared to elderly female individuals. However, no PMI differences were calculated between the other age groups of both sexes, nor were there sex differences in the PMI. Data are presented as mean ± S.E.M. *$p<0.05$. Two-way ANOVA followed by Tukey's post-hoc tests were used to compare the age ranges while Student's t-tests were used for ND vs. AD samples.

Appendix III Correlations between PMI and Western Blotting and ELISA Protein Expression Data

Proteins	Female Samples (P value)				Male Samples (P value)			
	Young	Middle Age	Elderly	AD	Young	Middle Age	Elderly	AD
APP	ns	ns	ns	ns	ns	ns	ns	ns
ADAM10	ns	ns	ns	ns	ns	ns	ns	ns
BACE1	0.01	0.02	0.02	ns	ns	ns	ns	ns
PS1	0.04	ns	ns	ns	ns	ns	ns	ns
PS2	ns	ns	ns	ns	ns	ns	ns	ns
sAPPα	ns	0.02	ns	0.05	ns	ns	ns	ns
sAPPβ	ns	ns	ns	ns	ns	ns	ns	ns
βCTF	ns	ns	ns	ns	ns	ns	ns	ns
Sol.$A\beta_{1-40}$	ns	ns	ns	ns	ns	ns	ns	ns
Sol.$A\beta_{1-42}$	ns	ns	ns	ns	ns	ns	ns	ns
Tau	ns	ns	ns	ns	ns	ns	ns	ns
tGSK3α	ns	0.03	ns	ns	ns	ns	ns	ns
tGSK3β	ns	ns	ns	ns	ns	ns	ns	ns
pGSK3α	ns	ns	ns	ns	0.04	ns	ns	ns
pGSK3β	ns	ns	ns	ns	ns	ns	ns	ns
PP2A	ns	ns	ns	ns	ns	ns	ns	ns
Pro Caspase3	ns	ns	ns	ns	ns	ns	ns	ns
Bcl2	ns	ns	ns	ns	ns	ns	ns	ns
tERα	ns	ns	0.01	ns	ns	ns	ns	ns
tERβ	ns	ns	ns	ns	ns	ns	ns	ns
pERα	ns	ns	ns	ns	ns	ns	ns	ns
pERβ	ns	ns	ns	ns	ns	ns	ns	ns
tMAPK	ns	ns	ns	ns	ns	ns	0.02	ns
pMAPK	ns	ns	ns	ns	ns	ns	ns	ns
tAkt	ns	ns	ns	ns	ns	ns	ns	ns
pAkt	ns	ns	ns	ns	ns	ns	ns	ns

- **The Null Hypothesis (H_0) of PMI:** There is no inverse statistically significant relationship between the PMI and protein expression. Pearson Correlation Analysis, $p < 0.05$.
- **ns** = non-significant

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