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Investigation of BACE1 as a stress-induced regulator of neuronal metabolism

Findlay, John Alexander

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Chapter 1

Introduction
1.1 Dementia

Dementia is defined by the World Health Organisation (WHO) as a syndrome comprising “deterioration in memory, thinking and the ability to perform everyday activities” and literally translates from Latin as “without mind”. As of 2010, 35.6 million people worldwide were diagnosed as having dementia, with this number increasing by 7.7 million per year. Dementia represents a huge worldwide economic burden and recent estimates put the global societal costs of dementia at 1.0% of the global domestic product (GDP; WHO; www.who.int/mediacentre/factsheets/fs362/en/). In the UK, the annual cost of dementia is £23 billion, which is more than heart disease and cancer combined (Alzheimer’s Research UK; www.alzheimersresearchuk.org/dementia-statistics/).

Currently there is no cure for dementia or indeed any treatments to slow or alter its progression. This represents a clear and unmet medical need in light of the ageing population worldwide.

Dementia causes encompass a number of diseases, four of which (Alzheimer’s disease (AD), vascular dementia (VAD), frontotemporal dementia (FTD) and dementia with Lewy bodies (DLB) represent over 90% of cases. The development of VAD was originally attributed to ischemic injury, resulting in the formation of multiple discrete lesions (Hachinski et al., 1974). Neuroimaging later revealed the correlation between these white matter abnormalities and cognitive decline, with the changes later defined as vascular cognitive impairment (Hachinski et al., 1987; Hachinski and Bowler 1993). More recently, VAD has been classified as “a syndrome with evidence of clinical stroke or subclinical vascular brain injury and
cognitive impairment affecting at least one cognitive domain (Gorelick et al., 2011). FTD encompasses three clinical syndromes, stratified by their presentation features: behavioural variant FTD or language-based subtypes marked by either language decline (impaired speech production) or impaired word comprehension (“semantic dementia”; Warren et al., 2013). The disease is also differentiated from other dementia cause on the basis of defined, selective atrophy of the frontal and/or temporal lobes (Warren et al., 2013). DLB is a classification of dementia that is particularly difficult to diagnose, as it shares symptoms common to both AD and Parkinson’s disease (PD) and current estimates suggest that it may make up as much as 10% of dementia cases (Alzheimer’s Society; www.alzheimers.org.uk/site/scripts/documents_info.php?documentID=113)

1.2 Alzheimer’s disease

1.2.1 Background

Alzheimer’s disease is the most common cause of dementia and accounts for between 60-70% of cases and affects around 400,000 people in the UK (ARUK; www.alzheimersresearchuk.org/dementia-statistics/). Disease progression is as yet irreversible and encompasses large-scale neuronal loss resulting in progressive memory deterioration and other cognitive decline. This cell death follows a characteristic spatial and temporal pattern; with the first signs of the disease presenting in layer II neurons of the entorhinal cortex (EC; Gomez-Isla et al., 1996) and spreading through the hippocampus. Selective atrophy and regional hypometabolism have also been shown to occur in the EC through the use of
functional imaging (Masdeu et al., 2005, Wu and Small 2006). This wave of cell death continues into the cerebral cortex and upon development of what is termed severe AD, extreme shrinkage is evident. It is believed that this progressive loss of neurons, and the processes underlying it, may begin decades prior to symptom presentation and diagnosis. Currently, clinical classification of AD is based on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s disease and Related Disorders Association (NINCDS-ADRDA) criteria on the basis of performance in cognitive and daily living testing (Mini Mental State Exam (MMSE), Alzheimer’s Disease Assessment Scale-cognitive subscale (ADASCog)) as well as interviews with friends and family (McKhann et al., 1984). Confirmation of this diagnosis and characterisation as AD is only fully obtained upon post mortem. At this time, two microscopic pathologies, first described by Alois Alzheimer in 1907 (Alzheimer et al., 1995) are observed: intracellular neurofibrillary tangles (NFTs) and extracellular fibrous β-amyloid plaques (See Figure 1.1) with the presence of both being required for confirmed diagnosis. While the presence of these hallmark pathologies in post mortem brains of people with dementia and AD is clear, there remains great debate with regards their relative roles in disease progression.

Figure 1.1: Image showing the hallmark plaque and tangle pathologies noted upon autopsy in AD.

(gbiomed.kuleuven.be/english/research/50000622/50525540/alzheimers-disease)
1.2.2 NFT Pathology

Tau was originally discovered as a microtubule associated protein (MAP) nearly 40 years ago and thought to have a role in actin polymerisation (Weingarten et al., 1975). Around a decade later, its presence in the NFT structures described by Alzheimer was confirmed by a number of groups (Grundke-Iqbal et al., 1986; Kondo et al., 1988; Kosik et al., 1988). Other than its role in microtubule assembly, very few functional roles for tau were then elucidated. However, more recently, it has been shown to play a role in inhibiting microtubule motor protein function in the trafficking of vesicles and organelles (Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Dixit et al., 2008). Tau is also thought to play a major role in the development of another class of neurodegenerative diseases: the non-Alzheimer tauopathies. The characterisation of these invariably comes following observation of paired helical filaments (PHFs) comprised of tau that has been hyper-phosphorylated at a number of sites (Hanger et al., 2009). Some of these phosphorylation patterns have subsequently been observed in vivo in the human AD brain and also in AD transgenic mice prior to tau filament formation.

1.2.3 Amyloid-β (Aβ) Pathology and the Cascade Hypothesis

The main component of the extracellular plaques described by Alzheimer in 1907 was subsequently found to comprise an amyloid protein as following congo red staining, a green fluorescent pattern is observed with exposure to polarized light (Swerdlow 2012). The predominant peptide within the amyloid core was determined and called β-amyloid (Aβ) protein through use of column and liquid chromatography by Glenner
and Wong around 30 years ago (Fig.1.1; Glenner and Wong 1984a). This study in vascular amyloid deposits of AD and further work identifying a similar peptide in the cerebrovascular amyloid in Down’s syndrome brains suggested a common disease precursor (Glenner and Wong 1984b). Work by Masters and colleagues later derived hydrophobic material from the core of these amyloid plaques (Masters et al., 1985). Taken together these data suggested that a common precursor (located on chromosome 21) and pathogenesis gave rise to these poorly soluble, hallmark lesions, which in turn may underlie AD and Down’s syndrome. This turned out to be true and subsequently it was confirmed that Aβ was derived from the amyloid precursor protein (APP), which resides on chromosome 21 (Kang et al., 1987). Later, mutations were discovered in the APP gene that resulted in heritable disease states that displayed severe amyloidosis and vascular plaque aggregates similar to those in the AD brain (Levy et al., 1990). The following year, a second mutation was found in a family displaying pre-senile dementia with accompanying amyloid angiopathy and cortical plaques (Goate et al., 1991). The amyloid cascade hypothesis followed and it supposes that the accumulation of these toxic Aβ fragments initiate a series of events that culminate in the large scale neuronal loss seen in AD (Hardy and Higgins 1992). This occurs via increased inflammatory and oxidative stress, the resultant formation of NFTs and amyloid plaques, all of which leads to synaptic dysfunction, neuronal cell death and dementia (for review see Mattson 2004; Agostinho et al., 2010).

1.3 Precipitating Factors in AD

Since the advent of the amyloid cascade hypothesis, the same genetic information that lies at its core has led to a rapid increase in our knowledge of the cellular and
molecular adaptations that may drive neuronal degeneration and cognitive
dysfunction seen in AD (for review see Selkoe and Schenk 2003). With this increase
in knowledge, a number of precipitating factors for AD have been proposed, with
genetic mutations (revealing a causative role for the characteristic pathologies),
mitochondrial dysfunction, synapse loss and oxidative stress all believed to play a
role. Allied to this, a number of lifestyle related risk factors have also been identified.

1.3.1 Genetic Factors in AD

AD has two forms: the rare early onset AD (presentation of symptoms before the age
of 65) and the much more common late onset (sporadic) form with symptoms
presenting after the age of 65. Early onset AD tends to cluster in families and
following the sequencing of Aβ, the gene encoding APP was located on chromosome
21. Some of these families were then screened for mutations in APP and a causative
role for a number of APP mutations discovered (for review see Hardy 1997).
Following this, linkage analysis uncovered a region on chromosome 14 that would be
shown to also underlie inherited AD in a number of lineages, with the gene later
identified as the presenilin-1 (PS1) gene (Sherrington et al., 1995). Subsequently,
mutations in a gene located on chromosome 1, sharing a high homology to PS1 (now
called presenilin-2 (PS-2)), were later also shown to cause inherited AD (Levy-Lahad
et al., 1995). While these familial forms of AD are extremely rare (<1%), molecular
genetic analyses have suggested that there may be genes that enhance an individual’s
susceptibility to AD. The best characterised example is the apolipoprotein E (ApoE)
gene. There are 3 alleles of ApoE that encode 3 different isoforms (E2, E3, and E4)
and it has been shown that individuals carrying the E4 allele are at a significantly
increased risk of developing an aggressive and early onset form of AD (Roses 1997). More recently, additional genes (e.g. Clu and Picalm) have been shown to impart an increased risk of AD (Lee et al., 2011; for review see Reitz and Mayeaux 2014).

1.3.2 Lifestyle Related Risk Factors

Currently, the greatest risk factor for AD development is age, with its prevalence roughly doubling every 5 years from around 1 in 1000 at 65 to almost 1 in 2 by the age of 85 (Puglielli et al., 2003). However the disease is not thought to be an inevitable consequence of ageing. There are also an increasing number of risk factors, which, growing evidence suggests may play individual roles in AD risk but that may also potentiate each other.

1.3.2.1 Cerebrovascular Disease/Ischemia

Recently, the strict differentiation between AD and vascular dementia (VaD) has been challenged as a result of mixed results obtained following autopsy and also the mounting evidence for a vascular component to AD pathogenesis (Sosa-Ortiz et al., 2012). Indeed it has been shown that brain hypoperfusion can increase the expression of cyclin-dependent kinase 5 (CDK5). The aberrant activity of CDK has subsequently been associated with the neuronal apoptosis (Weishaupt et al., 2003) and this serine-threonine kinase may also play a role in the hyperphosphorylation of tau resulting in NFT formation (Wen et al., 2007).
1.3.2.2 Hypertension

Hypertension, itself a risk factor for CVD, has repeatedly been identified as an age-dependent risk factor for dementia/AD in longitudinal studies, with high blood pressure (BP) in midlife linked to increased AD risk (Korf et al., 2004, Skoog et al., 1996). The data in elderly populations is more varied and this may be partly explained by recent work showing that midlife hypertension led to damage in brain areas known to play a role in BP, resulting in a progressive decline in BP after midlife (Vuorinen et al., 2013). It is thought that hypertension may play a role in damage to the blood brain barrier, leading to influx of proteins that would normally be blocked from entry to the brain, leading to synaptic dysfunction and cell death (for review see Kalaria 2010). It has also been postulated that the presence of systemic increases in BP may enhance the damage cause by subsequent adverse CVD events (Power et al., 2011).

1.3.2.3 Plasma Lipid Levels

Lipids play a number of essential physiological roles; as components of plasma membranes as well as playing various roles in intracellular signalling events and protein modifications. As with hypertension, there appears to be a correlation with high serum lipid levels in midlife and AD risk (Kivipelto et al., 2001), however the data in older cohorts is more variable (Kuo et al., 1998; for review see Michikawa et al., 2003). The potentially harmful effects of midlife dyslipidemia are supported by the findings that a number of genes conferring AD risk are physiologically involved in the transport and metabolism of cholesterol (e.g. ApoE, apolipoprotein J (APOJ))
and sortilin-related receptor (SORL1)). There are also an increasing number of studies that have shown that cholesterol can directly influence amyloidogenic APP processing (for review see Puglielli et al., 2003; Hutter-Paier et al., 2004). A number of these studies focus on plasma membrane lipid rafts, which are areas of the membrane that are enriched in cholesterol and sphingomyelin. These areas have been shown to play an important role in a number of key pathogenic events in AD progression, including APP processing, synaptic signalling and the induction of apoptosis (Ehehalt et al., 2003). There is also the compounding factor that this aberrant Aβ production can mediate the oxidation of plasma membrane cholesterol to generate \(H_2O_2\) resulting in oxidative damage (Opazo et al., 2002; Nelson et al., 2005). This data suggests that increased lipid levels may play a key role in AD progression, over and above changes to the vasculature.

### 1.3.2.4 Obesity

The rates of people classified as overweight or obese have risen dramatically over recent years. Obesity is a condition characterised by a fundamental imbalance in energy intake and energy expenditure with contributory impact on altered hormone levels, body composition and energy metabolism (Lentes et al., 1999). The resulting imbalance results in the expansion of the adipose tissue (particularly white adipose tissue (WAT)), via hypertrophy and hyperplasia of adipocytes (de Ferranti and Mazzaferri 2008). The resultant expansion leads to increased production of adipose-derived factors e.g. non-esterified fatty acids (NEFAs), proinflammatory cytokines (interleukin (IL)-6 and tumour necrosis factor (TNF)-α and insulin growth factor (IGF)-1 and hormones such as leptin, resistin and adiponectin (Schoelson et al., 2006;
de Ferranti and Mazaffarian 2008). The elevated levels of these factors then leads to the impairment of a number of signalling pathways leading to insulin and leptin resistance.

Increased body mass index (BMI) in midlife has repeatedly been shown to associate with increased AD risk, while the reverse is true in old age (Whitmer et al., 2007; Tolppanen et al., 2013). A number of complications of obesity may individually play a role in this increased AD risk, including: insulin resistance, advanced glycosylation end products (AGEs) and chronic elevations in cytokines and adipokines (Fitzpatrick et al., 2009; Luchsinger et al., 2009).

1.3.2.5 Type 2 Diabetes Mellitus (T2DM)

T2DM is a disease that presents as chronically elevated blood glucose levels (hyperglycaemia) as the result of two major pathophysiological factors: declining function of the insulin secreting beta-cells of the pancreas and the reduced action of insulin on target tissues (termed insulin resistance). While these two events present the major changes seen in T2DM, there remains debate as to their relative contributions (for review see Kahn 2003). As with obesity, the prevalence of diabetes is dramatically on the rise with current estimates suggesting T2DM comprises 90% of the 347 million people worldwide with diabetes (WHO). T2DM has previously been shown to increase the risk of AD development by around 2-fold and this increased risk is thought to encompass cerebrovascular-mediated events as well as independent effects (Luchsinger 2008). A major driver of T2DM-induced damage in the brain is chronic hyperglycaemia and the resultant development of micro-vascular disease.
Strachan et al., 2011). T2DM is also characterised by the loss of effective insulin signalling (insulin resistance). In the brain this also results in decreased levels of insulin degrading enzyme (IDE), which has previously been shown to play a role in the degradation of its competing substrates, insulin and Aβ (Farris et al., 2003). This reduction in IDE also led in the activation of GSK-3β, and a resultant increase in tau phosphorylation. This decline in IDE would also represent a change from the usual course during ageing, with increases in activity observed during ageing and in AD cases (Miners et al., 2010).

The above highlights the diverse range of conditions believed to act both individually and in concert to increase the overall risk of dementia and AD development. It is important to note that a number, if not all, of these are currently growing in incidence and severity as a result of the Westernised diet and the increasingly sedentary lifestyles. All of which means that an ageing population demographic is entering old age in generally poorer health which represents a major, currently unmet, public health need with regards effective treatments for dementia and AD.

### 1.4 Cellular Drivers of Sporadic AD

In an attempt to better understand the complex aetiology of sporadic AD, a large body of work has sought to improve our fundamental knowledge of the cellular events that drive the neurodegenerative process seen in AD progression.
1.4.1 Concerted Actions of the Hallmark Pathologies

Despite increasing scepticism regarding the role of Aβ and tau pathologies as the drivers of AD progression, there remains a great deal of work showing that they may act synergistically to impair neuronal function and cause cell death. Generally it is accepted that the Aβ pathology precedes tau changes (Jack et al., 2010) but also that the NFT pathology more closely follows the spatio-temporal progression of AD (Braak and Braak 1991). Numerous studies have also shown tau-dependent effects of Aβ in a number of models. Two such studies were published in 2001, the first of which demonstrated that in the presence of a mutated form of human tau (harbouring the P301L mutation) resulted in significantly worse tangle pathology upon injection of Aβ into the brain (Gotz et al., 2001). The reciprocal effect was also demonstrated whereby crossing of mice overexpressing human APP containing the Swedish (K670N/M671L) double mutation with tauP301L resulted in a similar plaque pathology compared to parental APPSWE mice but a far more accelerated tau pathology compared to the parental tauP301L line (Lewis et al., 2001). Further studies have also demonstrated a synergistic relationship between Aβ and tau resulting in dysfunction of learning and memory and its believed cellular correlate, long term potentiation (LTP; Roberson et al., 2007; Shipton et al., 2011). Work has also demonstrated dysfunction at the cellular level, with Aβ and tau shown to interact and cause impaired mitochondrial function and trafficking (Vossel et al., 2010; Quintanilla et al., 2011) as well as synaptic damage (Zempel et al., 2013).
1.4.2 Oxidative Stress

It is thought that ageing may itself relate closely to the appearance of damage resulting from the actions of reactive oxygen species (ROS), which in turn is indicative of increased oxidative stress. This appearance of oxidative stress may be particularly harmful in the brain due to its high oxygen consumption and relatively low antioxidant level (Floyd and Hensley 2002). In relation to AD, increased levels of oxidised proteins appear in the AD brain and these correlate well with cognitive ability (Keller et al., 2005). There is also evidence that mutations in mitochondrial DNA, which cause increased ROS production, also increase with age and in cases of neurodegenerative disease (Corral-Debrinski et al., 1992; Wang et al., 2005).

Work by Mark Smith and colleagues have demonstrated robust changes in the oxidative stress profile of the brain in AD. For instance, they have demonstrated that oxidative stress levels in the brain are detected independent of plaque pathology, with levels being lower in and around plaques than in other areas (Nunomura et al., 2001). This gave rise to the opinion that Aβ may in fact represent a compensatory mechanism to deal with enhanced oxidative stress levels in the AD brain (Smith et al., 2002; for review see Lee et al., 2006). In this model, the oxidative stress would precede the plaque deposition and consistent with this notion, it has been shown that the enzyme responsible for the rate-limiting step in Aβ production, β-secretase, is activated by oxidative stress (Tamagno et al., 2002). Further evidence of a causative role for oxidative stress in AD progression comes from the observation that early AD pathology is accompanied by the appearance of oxidative stress markers such as 4-hydroxy-nonenal (HNE; Sayre et al., 1997).
1.4.3 Mitochondrial Dysfunction

There is growing evidence to suggest that mitochondria may play a central role in the ageing process (Trifunovic et al., 2004; Kujoth et al., 2005). They also provide a potential mechanism by which ageing and AD may link and increasingly, mitochondria are being considered to play a role in AD progression. Indeed, through the use of electron microscopy, it has been shown that the number of normal-appearing mitochondria in the AD brain is decreased (Hirai et al., 2001). Accompanying this, there is also an increase in the number of degraded and degrading mitochondria. This enhancement of abnormal mitochondria may also increase the oxidative stress level of neurons. It is thought that the oxidative stress status in AD stems from an increase in reactive oxygen species (ROS) production as there is little evidence to show impairment in the levels of antioxidant or free radical scavenging enzymes in AD (Swerdlow 2012). This potential link has been demonstrated through the use of Cybrid cell lines, which are formed by depleting the mitochondrial DNA in cultured cells and then transferring in mitochondria from different sources (e.g. AD vs. controls). This resulted in a significant increase in the ROS production from the AD cybrid cell lines compared to controls (Swerdlow et al., 1997; Swerdlow, 2007). This altered mitochondrial function is believed to emanate from the dysfunction in the electron transport chain (ETC), particularly complex IV (Bennett et al., 1992; Maurer et al., 2000; Rhein et al., 2009a). Deficits in other mitochondrial enzymes have also been reported in AD, with altered activity of pyruvate dehydrogenase (PDH) and the α-ketoglutarate dehydrogenase complex (α-KGDHC) being reported (Gibson et al., 1988).
There is also a growing body of literature to suggest that Aβ may directly interact with and modulate mitochondrial function. For instance impairments in the same mitochondrial enzymes altered in AD (COXIV, PDH, and KGDHC) have been shown following Aβ treatment in isolated mitochondria and primary neurons (Casley et al., 2002a; 2002b). There is also evidence that Aβ located in mitochondria (first observed by Lustbader in 2004 (Lustbader et al., 2004) may interact directly with proteins such as cyclophilin D (CypD) and amyloid-binding alcohol dehydrogenase (ABAD). CypD functions as a component of the mitochondrial permeability transition pore (mPTP), the opening of which (following CypD translocation) results in pore formation, reduced mitochondrial membrane potential and the release of pro-apoptotic factors (e.g. cytochrome C, Smac/Diablo and apoptosis-inducing factor (AIF)). This collapse of the mitochondrial membrane potential then drives bioenergetics dysfunction and resultant cell death. It has been shown that Aβ can directly interact with CypD and that reduction in the levels of CypD can attenuate Aβ-induced mitochondrial dysfunction (Du et al., 2008). ABAD is a multifunctional enzyme that catalyses the reduction and oxidation of alcohols and ketones (for review see Muirhead et al., 2010). Following its discovery, ABAD was seen to interact with Aβ in mitochondria (Yan et al., 1997) and Aβ was noted to inhibit the activity of ABAD towards various substrates (Opperman et al 1999; Yan et al 1999).

1.4.5 Bioenergetic Changes

The altered activity and functioning of mitochondrial enzymes in AD has also been studied through investigations of changes in cellular bioenergetics. Classically, assessment of mitochondrial function is performed by measuring the oxygen
consumption of the isolated organelle or by looking at the protein level or activity of individual enzymes. While these methods provide important information about mitochondrial health, and allow the investigator to look at the function at the single complex level, assessment of oxygen consumption in intact cells has allowed a more physiological perspective for studying mitochondrial functioning. One such example is the work by Yao and colleagues, who demonstrated a significantly altered bioenergetic profile in the triple transgenic AD mouse model (3 x Tg-AD), which harbours precipitating mutations of both the Aβ (human APPsw and PS1M146V) and tau (P301L) pathologies. Primary hippocampal neurons of these animals displayed an altered metabolism, with a decrease in oxidative phosphorylation and impaired maximal respiration that they attribute to the observed impairments in PDH and COXIV activity (Yao et al., 2009).

1.4.6 Hypometabolism

Central hypometabolism represents a single, invariant change seen during the progression of AD that encompasses a number of the above predisposing factors. Over the past 50-60 years, investigators have worked to better understand the absolute requirement the brain has for efficient substrate and oxygen consumption and how this might relate to brain disease. Indeed, over this time, impairments at every level of oxidative/energy metabolism have been observed in a number of brain diseases (for review see Blass 2001). More recently, there has been growing evidence for a causative role for reduced metabolism (particularly reductions in the cerebral metabolic rate (CMR) for both glucose (CMRglu) and oxygen (CMRO2)) in AD. It has also been shown that reductions in CMR may also underlie a number of other
dementia causes (Blass and Gibson 1999; Blass 2000, 2001). Indeed, in 1959 delirium and dementia were suggested to be part of the same spectrum of disorders known as “cerebral insufficiency” (Blass 2000).

Pertaining to AD cases, this reduction in CMR was first characterised by the use of positron emission tomography and functional magnetic resonance imaging (fMRI; Ibanez et al., 1998; de Santi et al., 2001; Mosconi et al., 2009a; 2009b). Through the examination of families with inherited forms of AD, a number of studies have shown hypometabolism in healthy individuals who are at high risk of AD (autosomal AD cases) prior to the identification of brain atrophy (Kennedy et al., 1995; Reiman et al., 1996). Impaired central metabolism has also been shown in the progression towards AD for non-familial cases (de Santi et al., 2001; Mosconi et al., 2009b). More recently it has also been shown that individuals carrying the AD risk factor, APOE4, also display reduced brain metabolism prior to the onset of symptoms (Reiman et al., 2004). The potential causative role of reduced CMR in AD is also supported by studies in humans where similar symptoms akin to the cognitive decline of AD can be brought about by impairing brain metabolism (for review see Gibson et al., 1981; Blass and Gibson 1999). An association may also exist between the Aβ pathology of AD, however attempts to show this in animal models of APP overexpression have been variable (Dodart et al., 1999; Poisnel et al., 2012). This potential link is reciprocally supported by the finding that induction of brain oxidative metabolism impairments increases APP expression (Blass et al., 2000). More recently, studies have attempted to combine these lines of enquiry and connect Aβ pathology and altered CMR directly. Regional changes in the metabolic profile of the AD brain have been postulated to confer future susceptibility to cell death, with a switch to
aerobic glycolysis associating with a higher amyloid plaque burden and being suggestive of future functional impairment (Vlassenko et al., 2010; Vaishnavi et al., 2010).

1.4.7 Calcium Deregulation

Calcium ions (Ca\(^{2+}\)) play a vital role in learning and memory and have also been implicated in AD pathogenesis via induction of neuronal dysfunction and resultant cell death. Indeed, Aβ has been implicated in both the activation of receptors, leading to Ca\(^{2+}\) influx and also through the formation of non-selective cation channels in the plasma membrane (Mattson et al., 1992; Le et al., 2001; for review see Mattson and Chan 2003). The resultant increase in intracellular calcium is proposed to lead to aberrant activation of calcium-dependent proteases. One example is the calpain family of cysteine proteases, which are expressed in two major isoforms classified by their response to Ca\(^{2+}\). Calpain I (activated by low micromolar Ca\(^{2+}\) concentrations—thereby called µ-calpain) is the predominant isoform in neurons with Calpain II (or m-calpain) is activated at higher, mM, levels of Ca\(^{2+}\) (Jin et al., 2013). Calpain I has been shown to be highly expressed in neurites and synaptic terminals and becomes activated in AD (Saito et al., 1993; Trinchese et al., 2008). It has also been shown that there is a depletion of the endogenous calpain inhibitor (calpastatin) in the prefrontal cortex of the AD brain (Nixon et al., 2003). Inhibition of calpain I has also been shown to reverse both amyloid and tau pathologies as well as enhancing learning and memory task performance in the 3 x Tg AD mouse model (Medeiros et al., 2012). This synthetic inhibitor (A-705253) was also shown to attenuate cholinergic neuron
loss and cognitive deficits following direct injection of Aβ₄₂ into the hippocampus in mice (Granich et al., 2010).

1.4.8 Synapse Loss

In AD cases, one of the best correlations observed is between cognitive decline and the loss of synapses (Dekosky et al., 1996; Coleman and Yao 2003). It has therefore been postulated that this loss of synaptic integrity could constitute a key point in AD pathogenesis. It may be that the synapses are attacked by the toxic Aβ species believed to drive AD progression (Walsh et al., 2002). It may also be that the synapses fail as a result of insufficient synaptic maintenance, possibly owing to oxidative stress, impaired mitochondrial function or as a result of impaired MT trafficking of essential machinery as a result of tau dysfunction. Few however regard this as the potential cause and see it more as the manifestation of the aforementioned causes.

1.5.1 Amyloid Precursor Protein (APP)

Aβ production comes as a result of the endoproteolysis of the parental amyloid precursor protein (APP, Kang et al., 1987). APP is a large transmembrane protein encoded by a single gene transcript found on chromosome 21 (Yoshikai et al., 1990). Alternative splicing of the APP gene can yield 10 isoforms, with amino acid residue lengths ranging from 563 to 770 (Sinha and Lieberburg, 1999). The neuronal APP isoforms, APP695, differs from the other predominantly expressed variants (APP751 and 770) by lacking a Kunitz-type protease inhibitor (KPI) domain (Cordy et al.,
APP also has two paralogs, APP like protein 1 and 2 (APLP1, APLP2) (for review see Thinakaran and Koo 2008). The expression of APP and APLP2 is extremely homologous with both present in the majority of cell types while APLP1 expression mirrors these proteins but only in the brain (Heber et al., 2000). It was long supposed that there was substantial amount of redundancy between APP and its paralogs, with loss of one or two of them failing to generate an overt phenotype (von Koch et al., 1997). However ablation of APLP2 and either APP or APLP1 resulted in perinatal lethality, leading Heber and colleagues to speculate that APP and APLP2 (despite near identical sequences and expression) may have some distinct functions (Heber et al 2000).

1.5.2 APP Processing

Recent work has identified two distinct pathways involved in APP processing (Fig. 1.2) under the control of 3 major groups of enzymes or enzyme complexes termed the α-, β-, and γ-secretases. Under normal conditions in the brain, the majority of APP processing occurs down the non-amyloidogenic pathway via the actions of α- and γ-secretase. The action of α-secretase cleaves APP at the 83 amino acid residue from the carboxy (C) terminus, resulting in the release of the amino (N)-terminal ectodomain (known as soluble (s)APPα) into the extracellular environment (Kojro and Fahrenholz 2005). The remaining 83 amino acid C-terminal fragment (termed C83) is retained by the cell and subsequently cleaved by γ-secretase to yield the release of the small p3 fragment (Haas et al., 1993) and generating a cytoplasmic APP intracellular domain (AICD) polypeptide. Importantly, the action of α-secretase cleaves APP within the Aβ sequence and therefore precludes its formation. The
amyloidogenic pathway however leads directly to Aβ production via the sequential cleavage of APP by β- and γ-secretase. The action of β-secretase cleaves APP at the amino acid located 99 from the C-terminus and leads to the release of sAPPβ and the membrane retention of the 99 amino acid C-terminal portion of the protein (C99). Aβ is then released by the subsequent cleavage of C99 by γ-secretase. The precise amino acid residue where γ-secretase cleaves C99 can vary (resulting in Aβ fragments ranging from 36-43 amino acids in length), with the majority of activity producing a 40 amino acid long form of Aβ under normal conditions. The remaining Aβ produced (around 10%) however is of the 42 amino acid form, with this longer structure conferring a more hydrophobic structure which is believed to play a role in fibril and eventually plaque formation (Younkin, 1998). As in the case of non-amyloidogenic APP processing, γ-secretase activity here also yields AICD as an end product.

Figure 1.2: Schematic diagram showing the amyloidogenic and non-amyloidogenic pathways of APP cleavage (www.molecularneurodegeneration.com)
1.6 Characterisation of the Secretase Enzymes

The potential for α-secretase dependent cleavage was first described by Buxbaum and colleagues in 1990, whereby they observed protein kinase C-dependent APP cleavage (Buxbaum et al., 1990). This activity was later attributed to a group of enzymes comprising the “a-disintegrin and metalloproteinase” (ADAM) family: ADAM 9, ADAM10 and TACE/ADAM17 and ADAM 19. Targeted disruption of the genes for each of the family members did not result in the loss of α-secretase-mediated processing of APP therefore it is thought that the concerted activity of numerous family members is required (Buxbaum et al., 1998; Merlos-Suarez et al., 1998; Hartmann et al., 2002). The use of proteinase inhibitors has also suggested that the activity of three family members; ADAM 9, 10 and 17 may provide the major α-secretase component of APP processing (Roberts et al., 1994; Allinson et al., 2003). A little more is known in neurons where it is thought that ADAM10 provides the predominant α-secretase activity (Kuhn et al., 2010).

Meanwhile, γ-secretase has also been identified and was shown to be an enzyme complex made up of presenilin 1 or 2 (PS1/2) nicastrin, anterior pharynx defective and presenilin enhancer 2 (Wolfe et al., 1999; Zhang et al., 2000; Arawaka et al., 2002; Francis et al., 2002).

With the knowledge that β-secretase is the rate-limiting step in the production of Aβ, around a decade ago, a number of groups attempted to characterise the protein responsible. One of these groups was Vassar and colleagues, who utilised expression cloning and Aβ-elisa in APP overexpressing human embryonic kidney (HEK 293)
cells to identify the β-site APP cleaving enzyme 1 (BACE1) as β-secretase (Vassar et al., 1999). A second group utilised affinity purification followed by cloning methods to elucidate the amino acid sequence of β-secretase (Sinha et al., 1999). Finally, a number of groups utilised genomic techniques to scan for novel aspartic proteases with the potential for β-secretase activity (Hussain et al. 1999, Lin et al. 2000). Taken together, the identical amino acid sequence generated by multiple methods provides a robust argument for BACE1 (alternatively Asp2 or Memapsin) being responsible for the observed β-secretase activity.

This argument is enhanced by observation that their properties mirror each other and that BACE1 displays many of the classic hallmarks of eukaryotic aspartic proteases comparable to the pepsin family. For instance, BACE contains two active site motifs: PTGS (amino acid 93-96) and DGST (289-292) and work by Bennett and colleagues demonstrated that site directed mutagenesis (of these sites) led to inactivation of the protein (Bennett et al., 2000). BACE1 is also a type-1 transmembrane protein harbouring a luminal active site, which is predicted of β-secretase. This arrangement is the correct orientation for the cleavage of APP at the β-secretase site (Vassar et al., 2001). β-secretase and BACE1 also both display optimal activity in acidic environments (pH around 4.5) and this fits with their predominant localisation in acidic subcellular compartments of the secretory pathway like the golgi and endosomes. It has also been shown that BACE1 has high expression in neurons with relatively low expression in the glial cells, as is the case for β-secretase (Vassar et al., 1999).
Groups have also utilised APP expressing cell lines to look at the interaction between β-secretase and α-secretase activity. Upon transfection of BACE1 into cells, there was an increase in β-secretase mediated cleavage products (sAPPβ and C99) compared to untransfected cells (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999). Following this transfection, there was also an observed decrease in sAPPα, suggesting the activity of α- and β-secretase may be competitive. This antagonistic nature of APP processing was also seen in the study by Vassar and colleagues as a decrease in β-secretase resulted in enhanced α-secretase activity following antisense inhibition of BACE1. This phenomenon has also been observed via the overexpression of ADAM10 in neurons resulting in increased α-secretase activity and decreased BACE1-mediated processing (Postina et al., 2004).

Further work in animal models has also shown the key role of BACE1 in amyloid plaque pathology formation. Following the development of mice deficient in BACE1, a complete absence of Aβ production was observed in the brain and crossing these animals with those overexpressing human APP_{SWE} resulted in the eradication of their characteristic plaque pathology (Luo et al., 2001; Roberds et al., 2001). The reverse relationship has also been observed, with BACE1 activity being increased in AD, resulting in increased Aβ and plaque deposition (Stockley and O’Neill 2007). Furthermore, it has also been shown that BACE1 elevation correlates with the appearance of amyloid pathology in AD animal models (Zhao et al 2007). The above findings strongly implicated BACE1 as the key enzyme in producing the Aβ and resulting amyloid plaque pathology of AD and provided groups with a potential therapeutic target in AD.
1.7 BACE1 Structure, Trafficking and Regulation

Following its identification and characterisation as the protein responsible for the observed β-secretase activity, BACE1 provided researchers with a molecular target by which they could potentially slow Aβ production. This led a number of groups to begin looking at the structure, transcriptional and translational regulation as well as molecular trafficking of BACE1.

The BACE1 gene is located on chromosome 11, and encodes a protein of 501 amino acids (Capell et al., 2000). Alternative splicing of BACE1 mRNA has previously been observed to occur in the brain (Bodendorf et al., 2001). These result in the deletion of 25, 44 or 69 amino acids (yielding BACE1-476, 457 and 432), all of which have been observed in the brain and pancreas, with BACE1-501 and -476 displaying the greatest activity (Tanahashi and Tabira, 2001).

BACE1 activity is almost universally noted as being increased in pathology laden areas of the AD brain, with this change directly driving the increased Aβ deposition (Fukumoto et al., 2002; Tyler et al., 2002; Stockley et al., 2006). There is also a generally accepted increase in BACE1 protein levels in AD (Holsinger et al., 2002; Zhao et al., 2007) but these findings are not without contradiction (Tyler et al., 2002). One possible reason for this discrepancy is that BACE1 proteins levels may not be increased in all neurons so no overall change is observed in whole brain lysate. This proximity of BACE1 elevation around pathological plaques has also been noted in the brains of AD animal models (Zhao et al., 2007). Despite these changes in BACE1 protein level, it is generally accepted that they do not account for the observed activity
changes and this opinion is strengthened by the finding that rarely do BACE1 mRNA levels change in AD (Holsinger et al., 2002; Preece et al., 2003).

While no consistent changes in BACE1 mRNA levels have been observed in AD, there does appear to be a role for altered BACE1 transcription in response to cellular stress. Indeed, cell stressors have been shown to regulate total BACE1 mRNA, protein and activity levels. In terms of transcription, the BACE1 promoter has been shown to contain numerous sites that regulate its expression (for review see Rossner et al., 2006). These include sp-1, YinYang-1, STAT3 and 6, NFκB and HIF1α (Christensen et al., 2004; Sambamurti et al., 2004; Nowak et al., 2006; Sun et al., 2006; Buggia-Prevot et al., 2008; Wen et al., 2008) that have been shown to induce BACE1 expression, while peroxisome proliferator-activated receptor γ (PPAR-γ) has been shown to repress expression (Sastre et al., 2006). In neuronal tissue and cells, these factors have also been shown to be regulated by a number of precipitating factors of AD including: pro-inflammatory cytokines, mitochondrial dysfunction, oxidative stress, altered calcium homeostasis, hypoxia and Aβ (Zhang et al., 2002; Buggia-Prevot et al., 2008; Choi et al., 2008; Xiong et al., 2008).

With the knowledge that BACE1 protein level can be altered with no change in mRNA, this led groups to examine the untranslated regions (UTRs) of the coding sequence. It has been proposed that unique features of the BACE1 5’ UTR such as its length, high GC content (around 77%) and the presence of upstream AUGs impart a key regulatory role for this region (Mihailovich et al., 2007). Indeed, it has been demonstrated that the presence of the 5’ UTR could impart around a 90% repression in BACE1 translation (Lammich et al., 2004). Northern blot analysis of HEK-293,
COS and H4 cells demonstrated that all 4 BACE1 mRNA species contain the 5’ UTR (Lammich et al., 2004), a finding later confirmed for all BACE1 mRNA in the brain and pancreas (Mihailovich et al., 2007). This region also contains three upstream open reading frames (uORFs), however these were found to be unlikely to play a role in translational repression as determined by mutagenesis experiments (Rogers et al., 2004). It was instead concluded that the long nature of the region, along with the high GC content lead to a robust secondary structure that impedes the progress of the ribosome during translation (Lammich et al., 2004). While the presence of this region has been shown to regulate BACE1 translation, it remains to be fully investigated whether its presence or function are altered by physiological or pathophysiological stimuli. In this regard, it has been shown that the translational repression imparted by this region is attenuated in activated astrocytes (De Pietri Tonelli et al., 2004) and that such cells are found near to amyloid plaques in AD and AD animal models (Rossner et al., 2001; Leuba et al., 2005).

The observation that BACE1 activity may be regulated in a transcription independent manner led to a great deal of research attempting to better understand the trafficking and posttranslational modification of BACE1. The BACE1 protein is made up of an N-terminal signal sequence (amino acids 1-21), a propeptide domain (amino acids 22-45), a catalytic domain, a single transmembrane domain near the C-terminal and finally a cytoplasmic C-terminal tail. The protein is produced as a zymogen in the endoplasmic reticulum (ER) and its trafficking and activity are influenced by further post-translational protein modifications. Initially, the signal domain of the protein is removed by signal peptidase in the ER, with subsequent removal of the remaining prodomain occurring in the trans-golgi network (TGN) either via autocatalysis (Sidera
et al., 2002) or through the action of furin or a furin-like convertase for example PC-5 (Bennet et al., 2000). It has been proposed by Haniu and colleagues that this removal of the prodomain occurs at the same time as removal and addition of carbohydrate residues to the N-terminal (Haniu et al., 2000). While the 501 amino acid structure of BACE1 confers a molecular weight of around 58 kDA, the protein will run on SDS-PAGE at a weight closer to 66-70 kDA and this is thought to be due to post-translational glycosylation (Haniu et al., 2000).

BACE1 contains 4 potential N-linked glycosylation sites and it is thought that the close proximity of these sites (at Asn 153, 172, 223 and 354) to both the three intermolecular disulfide linkages of BACE1 and its catalytic site may have a pronounced effect upon the molecules physico-chemical properties (Charlwood et al., 2000). Indeed this group used Chinese hamster ovary (CHO) cells to show that with the use of tunicamycin to generate an unglycosylated form of BACE1 there was a resulting 40% decrease in activity. They then went on to look at the effect of mutating two of the four sites (generating 3 different mutants) and again saw a significant drop in the proteolytic activity of BACE1. Yan and colleagues whose data found that the four sites are occupied by a heterogeneous mix of carbohydrates have also looked at the glycosylation pattern of BACE1. The group suggests that due to their varying proximities to the active site and the need for all of them to be filled that they may be involved in maintaining the correct folding of the protein, which in turn may modulate intracellular trafficking (Yan et al., 1999).

Work has also been undertaken to look at the impact of acetylation upon BACE1 trafficking. Costantini and colleagues demonstrated that BACE1 can be acetylated on
seven lysine residues and that this allows the protein to be trafficked from the ER to the golgi. They demonstrated the importance of these lysine residues by mutating them first to alanine (a non-acetylated mutant) and found impaired transport from the ER to the TGN and plasma membrane. This mutant also displayed physical retention to the ER and a reduced half-life. In contrast, a constitutively acetylated form of BACE1 (lysine to glutamine mutant) displayed enhanced efficiency of transport from the ER along the secretory pathway and increased half-life (Costantini et al., 2007).

Further work conducted by Jonas et al. in 2008 saw that acetylated intermediates were trafficked from the ER to the golgi while non-acetylated forms were retained or degraded. This information suggests that lysine acetylation may act as a quality control check-point for the protein marking them either for transport or earmarking them for degradation. This group also found that these acetylated forms of BACE1 were protected from degradation by the serine protease PC subtilisin kexin type 9 (PCSK9) (Jonas et al., 2008). This role was demonstrated through the use of a PCSK9 knock out mouse which displayed increased levels of both BACE1 and Aβ, suggesting impaired BACE1 degradation.

Protein modification of the last few amino acids of the C-terminus of BACE1 (DISLLK) has also been shown to modulate trafficking. For instance, work by Pastorino and colleagues looked at the relative contributions of the dileucine motif (amino acids 499 and 500) on the trafficking of BACE1 in the TGN and the endocytosis from the plasma membrane. Through the use of site-directed mutagenesis they observed that removal of this dileucine motif led to a higher level of mature BACE1 protein (i.e. lacking its prodomain) and also increased cell surface expression (Pastorino et al., 2002). The group also looked at the ability of the
adjacent serine residue to modulate the activity of this sorting signal via its phosphorylation. Again utilising site-directed mutagenesis they found that loss of the phosphorylation site resulted in no major change in BACE1 activity (as measured by level of secreted Aβ). More recently, work by Wahle and colleagues as well as Tesco et al. have highlighted a role for the γ-ear containing ADP ribosylation factor-binding (GGA) family of adaptor proteins in BACE1 trafficking. They have shown that the phosphorylation at serine 498 precludes BACE1 interaction with GGA proteins resulting in enhanced recycling to the cell surface (Wahle et al., 2005, Tesco et al., 2007). Another interesting observation from these groups is that the levels of GGA 1 and 3 are reduced in AD, which may suggest impaired BACE1 trafficking as part of the disease aetiology.

A further protein modification which has been studied with a view to its impact on BACE1 trafficking is palmitoylation. BACE1 contains three cysteine residues within its transmembrane domain and C-terminal tail (at amino acids 478, 482 and 485). Work by Benjannet saw that removal of these cysteine residues (via site-directed mutagenesis) led to a slowed exit from the ER to the TGN and that by preventing palmitoylation they observed increased shedding of soluble BACE1 into the media and suggest that these residues may provide a secondary anchor for the BACE1 protein to the plasma membrane (Benjannet et al., 2001).

The above highlights the important role that protein trafficking may play in BACE1 levels and activity and hence Aβ production and groups are now beginning to look at how these mechanisms may be altered under stress conditions. These post-translational modifications provide an intriguing option to explain observations that a
rapid increase in BACE1 levels following stress is not coupled with increased mRNA levels. This would suggest that post-translational changes in the structure or half-life of BACE 1 might impact on APP processing.

1.8 Physiological Roles of BACE1 and APP

1.8.1 APP

While much of the work towards our current understanding of APP has focussed on its role as the precursor of Aβ plaques, there have also been other physiological roles proposed for the protein. Three key functions that have been proposed for APP are as a trophic factor, involvement in cell adhesion and as a receptor.

Some of the first information for the physiological role of APP came from knockout mice, which displayed weakness in the extremities and brain gliosis (Zheng et al., 1995). Further investigation yielded the finding that in the brain these animals also showed a significant reduction in synapse number (Dawson et al., 1999). There were also changes observed in the development of the peripheral nervous system (PNS) with APP knockout resulting in reductions in pre- and postsynaptic elements of the neuromuscular junction (NMJ; Wang et al., 2005) as well as reduced synaptic vesicle densities (Yang et al., 2005).
1.8.1.1 Trophic Effects

Of all the proposed roles for APP, the best characterised and most often cited is its role as a trophic growth/differentiation factor. This effect was initially seen as fibroblasts in which APP was knocked down by antisense APP expression displayed reduced growth rates but this could be attenuated by addition of sAPP (Saitoh et al., 1989). This trophic effect was later found to be dependent upon an extracellular pentapeptide region (RERMS; positions 403-407) of sAPPα (Ninomiya et al., 1993). It was later shown that this region shares a similar structure to cysteine rich growth factors (Rossjohn et al., 1999). A central effect for this peptide was also displayed with infusion into the brain resulting in increased synapse density and memory retention (Roch et al., 1994, Meziane et al., 1998). This stimulatory effect on synapse formation can also be suggested by work showing that APP expression in the brain is highest during neuronal maturation (Hung et al., 1992). A region upstream of this sequence, the N-terminal heparin binding domain (residues 28-123) has also been shown to stimulate neurite outgrowth and synapse formation (Small et al., 1994). This region has recently also been shown to play a role in the neuroprotective role of APP in a model of traumatic brain injury (TBI; Corrigan et al., 2014). In cell models, acute application of sAPPα has also been shown to stimulate glucose and glutamate uptake into synaptosomes and as well as protecting against Aβ-induced oxidative damage (Mattson et al., 1999).
1.8.1.2 Cell Adhesion

The evidence for this potential role for APP is more limited but centres on the RDHS motif located near the C-terminal, within the Aβ sequence, which has been shown to promote cell adhesion (Ghiso et al., 1992). This proposed role is supported by finding that the APP colocalises with integrins at axonal sites of cell adhesion (Storey et al., 1996; Yamazaki et al., 1997). The potential involvement of APP in cell adhesion also makes sense considering that a number of the above trophic effects, i.e. neurite outgrowth and synaptogenesis, would require some level of cell adhesion.

1.8.1.3 Receptor Function

It has previously also been suggested that APP may also act as a receptor with evidence that the glycoprotein F-spondin, which is secreted by neurons can bind to the extracellular domain of APP, APLP1 and APLP2 (Ho et al., 2004).

1.8.2 BACE1

The potential role of BACE in physiology was first suggested by phenotypic traits present in the BACE1 knockout mice. These animals were seen to be anxious and timid and these changes were reasoned to be due to alterations in central dopamine and serotonin systems (Harrison et al., 2003). Later, further cognitive deficits were observed, with changes in their ability to perform learning and memory tasks (Ohno et al., 2004; 2006), with these changes being thought to be due to a loss of APP processing by BACE1 (Laird et al., 2005). Second commonly reported phenotype for
the BACE knockout mice is nervous system hypomyelination. These changes have since been shown to be due to the loss of neuregulin (NRG) signalling, which plays a role in myelination, peripheral nervous system development as well as synapse formation and synaptic plasticity (for review see Falls 2003; Michailov et al., 2004). The resulting changes are thought to be the result of impaired NRG-protein kinase b, ErB4 and PSD-95 signalling (Hu et al., 2006; 2008; Savonenko et al., 2008). Work has also demonstrated that the β2-4 subunits of the voltage-gated sodium channel (VGSC) are substrates for BACE1. These subunits of the channel are not required for channel function but perform auxiliary roles and in modifying the channel and altering intracellular trafficking (Wong et al., 2005). In this study, the group found that the β subunit of the VGSC was a substrate for both BACE1 and γ-secretase and underwent sequential cleavage by these enzymes. Work by Dominguez and colleagues demonstrated that BACE1 KO mice display altered inactivation of the VGSC (Dominguez et al., 2005). It has also been shown that increased levels of BACE and also in AD brains, there is an increase in the processing of the VGSC β-subunit, leading to diminished sodium current densities (Kim et al., 2007).

More recently further substrates have been postulated for BACE1, including those involved in the inflammatory system: α2, 6-sialyltransferase, P-selectin glycoprotein ligand-1, inter-leukin receptor 11 and the low density lipoprotein receptor-related protein 1 (LRP1) (Kitazume et al., 2003; Lichtenthaler et al., 2003; von Einem et al., 2010). There have also been further screens to find novel BACE1 substrates (Gruninger-Leitch et al., 2002; Hemming et al., 2009) and while many of these substrates may be of physiological relevance, often they are found using
overexpression systems and as yet have not been investigated as to their physiological impact in vivo.

1.9 Modulation of the BACE1-APP Axis by Cellular Stress

There is increasing knowledge that factors mentioned above, which act as risk factors for AD i.e. increased age, stroke, T2DM and obesity also act to increase BACE1 (De la Torre 2006; Hassing et al., 2009; Luchsinger and Gustafson 2009). This shift in APP metabolism would result in increased Aβ production that, as mentioned above, can induce a number of factors which can drive AD risk: oxidative stress, impaired mitochondrial metabolism, synaptic dysfunction and neuronal cell death. Therefore how this vicious cycle of events begins is of great interest, with a number of groups working to gain a better understanding of how stress, at the cellular level, can impact APP metabolism.

1.9.1 Oxidative Stress

As mentioned above, a key pathway in the pathogenesis of AD is believed to be the generation of chronic oxidative stress in the brain. Groups have therefore been keen to identify how these events may act to alter APP metabolism, in favour of Aβ production. A key source of oxidative stress in the brain is following ischaemic injury (e.g. during a stroke or chronic hypoperfusion). At the cellular level, groups have investigated the effect of hypoxic stress on APP metabolism. One such study was the work Wen and colleagues, who demonstrated increases in BACE1 protein levels and activity following transient (1hr) cerebral ischaemia in ovariectomised
(OVX) female Sprague Dawley rats (Wen et al., 2004). A subsequent study by Zhang et al. furthered these observations by demonstrating a role for hypoxia-inducible factor 1α (HIF1α) in alterations to BACE1 following hypoxia. In N2A cells stably expressing APP, they saw that exposure to hypoxia (1% O₂) led to significant increases in BACE1 gene, protein and activity levels and also pushed APP metabolism down the amyloidogenic pathway, with observed increases in β-CTF and Aβ₄₀ and ₄₂. This work also showed a HIF binding site on the BACE1 promoter and that transient overexpression and conditional knockout of HIF could induce and repress BACE1 protein levels respectively (Zhang et al., 2007). Following this, work by Gugliemotto and colleagues showed that prolonged (up to 72h) hypoxia (3% O₂) brought about a biphasic response in BACE1 gene expression and protein levels. They showed that this early (6h) peak in BACE1 protein expression was brought about by hypoxia-induced ROS production and altered JNK signalling pathway activity, while the later (48h) increase was driven by HIF1α (Guglielmotto et al., 2009). This finding of early post-hypoxic effects on BACE1 being mediated by JNK/c-jun signalling confirmed earlier work observing similar effects in vitro and in vivo following exposure to oxidative stress inducing agents and 4-hydroxynonetal (4-HNE; Tamagno et al., 2005; 2008).

Further work in this area has also revealed a key role for γ-secretase in the oxidative stress-induced changes in BACE1. The study by Tamagno and colleagues for instance demonstrated that treatment of SK-N-BE cells with hydrogen peroxide (H₂O₂) or 4-HNE led to significant increases in BACE1 and γ-secretase gene, protein and activity levels, resulting in increased intracellular and extracellular Aβ₄₂. Subsequently they saw that these changes in BACE1 could be alleviated following
treatment with a γ-secretase transition analogue (L685, 458) or RNAi directed towards PS1/2. The effect was also lost following exposure of PS1/2 double knock out mouse embryonic fibroblast (MEF) cells to the oxidant agents. Finally, the group modelled cerebral ischaemic/reperfusion and observed ROS-induced changes in BACE1 and γ-secretase as before and that these effects were mediated through the JNK/c-jun pathway (Tamagno et al., 2008). A more recent study has also observed this for γ-secretase in the induction of oxidative stress-induced change in BACE1 (Jo et al., 2010). Here they demonstrated that BACE1 and γ-secretase activity levels are correlated in AD brain areas where neuronal loss is observed, but not in the cerebellum. They also observed that exposure of MEF cells to 4-HNE induced significant change in BACE1 protein levels and that, in primary cortical/hippocampal neurons this 4-HNE driven change could be attenuated by γ-secretase inhibition. This relationship was not reciprocal, as treatment with a BACE1 inhibitor failed to stop the increase in γ-secretase activity induced by 4-HNE treatment. Finally, the group observed a significantly increased level of BACE1 in both the 3xTgAD mouse model and also mice harbouring the PS1 (M146L) mutation when compared to age-matched controls and that this increase could be significantly attenuated following treatment with a γ-secretase inhibitor.

1.9.2 Metabolic Stress

With the knowledge that hypometabolism is an invariant feature of both AD but may also drive the progression from MCI to AD, groups have investigated that effect of metabolic and energy challenges on APP processing. One such study is the work by Veliquette et al who observed the effect of energy inhibition on BACE1. They
observed that a single intraperitoneal injection of agents to inhibit energy status (insulin, 2-deoxyglucose (2-DG), 3-nitropropionic acid (3NP) and kainic acid) resulted in significant increases in BACE1 and sAPPβ levels as well as a significant increase in amyloid plaque load in the brain 7 days post injection (Velliquette et al., 2005). A second study, carried out in HEK-293 cells demonstrated differential effects of graded energy failure on APP processing. In this model, mild energy decline, correlated with the increased intracellular Aβ levels while more severe energy decline correlated with reduced release of all APP processing metabolites. This work also demonstrated that during energy failure, the level of full length APP correlates well with the levels of adenosine triphosphate (ATP; Hoyer et al., 2005). This link between perturbations in energy production and BACE1 was also investigated by Xiong and colleague who showed that intraocular administration of mitochondrial inhibitors (3NP, rotenone and sodium azide (NaN₃)) resulted in significant increases in BACE1 protein level and β-CTF expression. They also showed that this treatment resulted in increased Aβ load and induced similar changes (glial cell activation and synapse loss) as injection of Aβ itself (Xiong et al., 2007). The effect of energy depletion also formed the basis of work by O’Connor and colleagues who examined the effect of glucose removal on BACE1 (O’Connor et al., 2008). Here, the group showed that in HEK 293 cells, removal of glucose induced a time-dependent increase in BACE1 protein levels (with a peak being observed after around 6 hours). They hypothesised that this was via a post-translational modification (due to an observed reduction in BACE1 mRNA) brought about by the increased phosphorylation of the translation initiation factor, eukaryotic initiation factor 2α (eIF2α). The same effect was observed in primary neurons following prolonged glucose removal (36 hours) and could also be induced by inhibition of the eIF2α phosphatase inhibitor salubrinal
(Sal003). Finally, the group observed increased levels of p-eIF2α and BACE1 following chronic treatment with 2-DG and 3NP in mice and that p-eIF2α levels were observed in an AD mouse model and in AD brain tissue, correlating with regions of increased BACE1 and Aβ load.

With the advance in our knowledge of how these stressors impact BACE1, has come investigations into alterations in subcellular localisation of BACE1 and APP during stress. One such example is the work by Domingues and colleagues who utilised COS-7 cell expressing an APP-green fluorescent protein (GFP) construct and monitored changes in APP localisation following treatment with 2-DG and NaN₃ (Domingues et al., 2007). Under mild energy challenge, they observed a redistribution of APP₆₉₅ from the plasma membrane to the ER and following increased challenge, they observed a reduction in sAPPβ release and hypothesised that this was due to the retention of APP metabolites. This study compares favourably with a recent study showing that following mild oxidative stress (H₂O₂) in primary neurons, there was a preferential redistribution of BACE1 from low density structures (e.g. membrane vesicles and lipid rafts) to denser areas, thought to be the TGN and ER and a subsequent increase in β-CTF release, indicative of increased BACE1-APP association (Tan et al., 2013).

1.9.3 Lipid Stress

As noted above, increased plasma lipid and cholesterol levels can result in changes in BACE1-driven APP processing, but at the cellular level there is also believed to be a role for the lipid second messenger, ceramide. Ceramide is produced by either being
synthesised at the plasma membrane or via the hydrolysis of sphingomyelin (Costantini et al., 2005). Under physiological conditions, ceramide can induce a number of cellular processes including cell growth and apoptosis as well as vesicular trafficking and neuronal differentiation while playing a key role in cellular senescence (Venable et al., 1995; 1999). It has also been demonstrated that exogenously applied ceramide can inhibit or activate apoptosis in a concentration dependent manner in motorneurons and that the levels of ceramide in the brain of AD patients have been found to be 3 times higher than age-matched controls (Ariga et al., 1998; Irie et al., 1998; Han et al., 2002; Cutler et al., 2004). Studies have also shown that exogenously applied ceramide can induce Aβ production via the stabilisation of BACE1 in Chinese hamster ovary and H4 cells and that this effect was not observed when using the inactive analogue dihydroceramide (DHC; Puglielli et al., 2003). This work also showed that treatment with the ceramide synthase inhibitor FB1 could reverse this change via the depletion of ceramide and sphingomyelin levels and could be restored with additional ceramide supplementation. This finding that the inhibition of ceramide synthase could reduce Aβ was also observed in another study using the human neuroblastoma, SH-SY5Y cell line (Tamboli et al., 2005). A subsequent study observed three lipid groups that were able to stimulate the activity of BACE1 purified from insect cells: natural glycosphingolipids (cerebrosides), anionic glycosphingolipids and sterols (cholesterol; Kalvodova et al., 2005). A further study made use of synthetic ceramide analogues to demonstrate that these could impair Aβ production but that this was likely due to a γ-secretase-mediated effect as for two of the analogues (PDMP and PPMP) they observed a significant increase in β-CTF production (Li et al., 2010). These data suggest that in addition to the
lipid/cholesterol-induced changes to APP processing, changes in the lipid signalling pathways may also play a role.

Taken together, these studies show that cellular stress can fundamentally drive APP metabolism towards Aβ production through the modulation of secretase enzymes involved in its processing but also through redistribution of their subcellular location. This enhanced Aβ can itself then initiate further oxidative stress events, impair mitochondrial function to enhance energy deficits as well as impact on neuronal synapse functioning, all of which could constitute key events in the progression of late-onset sporadic AD.

1.10 Activity-induced Change in APP Processing

Allied to this increase in amyloidogenic APP processing at times of cellular stress, the same phenomenon has been noted to occur during neuronal activity, which may also drive AD progression. Among the first demonstrations of this relationship was the finding that patients with temporal lobe dementia display Aβ plaque pathologies and this is thought to be due to the increased neuronal activity in these cases (Mackenzie and Miller 1994, Gouras et al., 1997). It has also been noted that areas of the brain that constitute the “default network” i.e. posterior singulate cortex and the frontal and parietal lobes display the most severe Aβ pathology and this is thought to come as a result of the increased basal metabolic activity resulting from high neuronal activity (Gusnard et al., 2001, Buckner et al., 2005). These observations have also been modelled in animals and cells in an attempt to better understand the relationship between neuronal activity and APP processing. One such study was the work by
Kamenetz and colleagues which monitored the production of Aβ in hippocampal slices from mice overexpressing APP$_{SWE}$ treated with agents to increase and decrease neuronal activity. The group observed that increasing neuronal activity significantly increased the production of both Aβ$_{40}$ and Aβ$_{42}$. Further analysis revealed increased production of sAPPβ, β-CTF, revealing that neuronal activity had the ability to induce BACE1 activity. They also demonstrated that this Aβ could in turn alter synaptic transmission and this came as a result of a reduction in the number of functional synapses on surrounding neurons (Kamenetz et al., 2003). Further studies have also suggested Aβ-mediated reductions in the spine density and synapse formation in hippocampal CA1 neurons and that these effects can be driven by axonal or dendritic Aβ production (Hsieh et al., 2006, Wei et al., 2010). This enhanced appearance of Aβ during neuronal activity has been hypothesised to come as a result of the acute release of pools of Aβ rather than an increase in the half-life of produced Aβ (Cirrito et al., 2005, 2008). There is also evidence that the reverse relationship also exists, whereby chronic inhibition of neuronal activity in mouse models of Aβ production resulted in reduced plaque pathologies (Tampellini et al., 2010). Another study has also displayed a relationship between neuronal activity and interstitial fluid (ISF) Aβ and lactate levels in the AD Tg2576 mouse model (Bero et al., 2011). More recently, it has been shown that neuronal activity can drive the colocalisation of BACE1 and APP in neuronal cultures and that this pattern of redistribution to denser structures when exposed to a sucrose gradient was also observed in AD cases and following H$_2$O$_2$ exposure (Das et al., 2013, Tan et al., 2013).
1.11 Neuronal Metabolism and Activity

Under basal conditions glucose oxidation in neurons is near maximal, with activity-dependent (increased firing and synaptic activity) raised glucose utilization correlated with increased glycolysis. Although glycolysis is less efficient than oxidative phosphorylation (5-7 vs. 30-33 ATP per mole of glucose), it can generate sufficient ATP for the neuron’s needs by producing ATP at a faster rate, which is useful to meet high demand but requires an abundant supply of glucose. The importance of maintaining glucose uptake and metabolism in central neurons can be seen from rodent studies showing that brain glucose levels (notably in hippocampus) decline with age and are associated with memory deficits, which are prevented by injections of glucose (M'Nay and Gold 2001; Morris and Gold 2013). Although such an age-dependent decline in brain glucose metabolism is still contentious in healthy humans, in AD patients brain glucose metabolism is 20-25% lower (Cunnane et al., 2011). It is thought that memories are encoded by changes in synaptic strength, with hippocampal LTP widely accepted as a cellular correlate of learning and memory (for review see Stuchlik 2014). Indeed, synaptic efficacy and plasticity (notably LTP) are strongly influenced by the sufficiency of energy metabolism. For example, limiting glucose uptake and usage with 2-deoxyglucose for a short period (e.g. 20 min) induces LTP following return to glucose (Godfraind and Yu 2006) whereas chronic inhibition of glucose metabolism depresses late-phase LTP (Potter et al., 2010). Furthermore, hypoglycaemia inhibits LTP induction and maintenance in acute hippocampal slices (Sadgrove et al., 2007) and repetitive insulin-induced hypoglycaemia in rats prevented induction of LTP in hippocampal slices (Yamada et al., 2004). Numerous investigators have demonstrated that exogenously applied Aβ
disrupts synaptic plasticity and inhibits hippocampal LTP (Ondrejcak et al., 2010). Although this latter action has been attributed to factors such as oxidative/inflammatory stress and/or signalling pathways downstream of the NMDA receptor (Rowan et al., 2005; Yamin 2009), we hypothesise that an important primary event is the availability of energy from glucose.

1.12 Role for BACE1 in Metabolism

Recent work by our group has suggested that modulation of the BACE-APP axis can fundamentally alter metabolism and glucose homeostasis. Previously we have shown that BACE1 KO mice are leaner than age matched controls and that they display improved insulin sensitivity and glucose use. We also saw that when challenged, these animals display resistance to diet-induced obesity (DIO; Meakin et al., 2012). More recently we have also seen that treatment with BACE inhibitors can reverse high fat feeding-induced weight gain and removal/suppression of BACE1 activity can alter obesity-mediated inflammatory drive (Meakin et al. in preparation). Finally, we recently published that altering APP cleavage can have significant effects on glucose uptake and metabolic flexibility in C2C12 myotubes (Hamilton et al., 2014).

1.13 Project Aims

Taken together, the above implicates BACE1-mediated processing of APP as a potential sensor of not only neuronal activity but also cellular stress status. It is therefore of particular importance to further investigate a potential role for BACE in the regulation of neuronal metabolism as impairments in these systems are strongly
implicated in the development and progression of aberrant Aβ production that may then underlie a number of processes leading to sporadic AD.

With this in mind, the principal objective of the present study was to characterise the response of BACE1-mediated APP processing following stimulation with a number of cellular stressors in the human, neuroblastoma SH-SY5Y cell line and the mouse brain. In addition, the effect of chronic elevation in BACE1 protein and/or activity was to be investigated with regards to alterations in cellular substrate uptake and use.
Chapter 2

Materials and Methods
2.1 General

2.1.1 Chemicals

Ammonium persulfate (APS), benzamidine, bovine serum albumin, calcium chloride, dimethyl sulfoxide, ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), ethidium bromide, glycerol, β-mercaptoethanol, phenylmethylsulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), sodium orthovanadate, tetrathylethylene diamine (TEMED), TrisAcetate, Triton X-100 and Tween®20 were purchased from Sigma Aldrich. Ethanol, glycine, hydrochloric acid, methanol, sodium chloride, sodium fluoride, sodium hydroxide and sucrose from VWR. Bromophenol blue, D-glucose, magnesium sulfate and tris(hydroxymethyl)aminomethane (TRIS) were from Fisher.

2.1.2 Statistical Analysis

Data are presented as mean ± the standard error of the mean (SEM). Comparisons between groups were made using an unpaired two-tailed Student’s t test, one sample Student’s t test or one way ANOVA and Tukey’s multiple comparison test as appropriate, using GraphPad (Prism 5) software (GraphPad Software). Significance was categorized as the following p-values; * ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001.

2.2 Cell Culture

All immortalized cell line work was carried out under aseptic conditions and cells were cultured in a 37 °C incubator with an atmospheric O₂:CO₂ of 95:5 %. All cell culture plastic ware was purchased from Nunc.
2.2.1 SH-SY5Y Cells

SH-SY5Y cells are a neuroblastoma cell line commonly used in neurodegenerative research and were originally derived from a metastatic bone tumour biopsy. The resulting SK-N-SH cells were then sub-cloned three times; to yield SH-SY, SH-SY5 and finally SH-SY5Y.

Wild type SH-SY5Y cells were maintained in DMEM F-12 media (Gibco; 21331) supplemented with 10% fetal bovine serum (Sera Lab; EU-000-S), 4 mM L-glutamine (Gibco; 250-30), penicillin streptomycin (100 units/ml, Gibco; 15070). Cells were grown to 70-90% confluency and typically passaged 1:5, 2-3 times a week. To do this, T75 flasks of cells were washed with 37 °C phosphate-buffered saline (PBS) and 2ml of 0.05% trypsin (Gibco; 25300-054) was added. Flasks were then placed back into the culture incubator for 5-10 minutes. The cells were then fully washed off the flasks and rinsed in 5ml of media before being dispersed to awaiting T75 flasks containing 10 ml of culture media.

Throughout these studies, a number of different stable cell lines were also utilised. The culture of which was as above, with the addition of G418 sulfate to act as a selection antibiotic. The cells overexpressing the wild type form of APP were a kind gift from Dr Ritchie Williamson and those overexpressing APP containing the Swedish mutation (KM670/671NL) were generated by GlaxoSmithKline as part of a previous PhD project. SH-SY5Y cells overexpressing BACE1 (construct generated by GSK) and a secretase-dead form of BACE1 (mBACE1) (kindly provided by Professor M. Wolfe) were generated by the following protocol.
1x10^6 cells were seeded into a 10 cm² cell culture plate and incubated for 3 hours, 1 per transfection plus 1 to act as a cell death control. Prior to the end of this period the following reagent mixes were prepared in 15ml falcons tubes:

<table>
<thead>
<tr>
<th>Tube A</th>
<th>Tube B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>Volume</td>
</tr>
<tr>
<td>DNA (0.5ug/ul)</td>
<td>24ul</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimem</td>
<td>1435ul</td>
</tr>
</tbody>
</table>

Table 2.1: Components of then cell transfection mixes used to generate stable BACE1 and mBACE1 overexpressing cell lines.

These mixes (plus one lacking the DNA) were left at room temperature for 5 minutes before the 2 tubes were mixed and allowed to stand at room temperature for 30 minutes. During this time, the plates housing the cells were washed twice with warm (37 °C) PBS and 6ml of serum and antibiotic-free media was applied. Following the 30-minute incubation period, the reagent mix for each transfection was applied to the cells. The following morning, the cells were washed twice with warm PBS and the reagent mix was replaced with normal culture media. The cells were then left to recover prior to antibiotic selection. For this, culture media containing 1mg/ml G418 was applied and the cells were left for 3-4 days (with media changed every 2 days) until the cell death control cells had died. The remaining transfected cells were then exchanged into media containing a lower, maintenance G418 concentration of 0.5 mg/ml. For these experiments, a minimum of two, individually generated stable cell lines with appropriate empty vector controls were used.
2.2.2 Additional Culture Media Supplements

As indicated in the results section, the cell culture media was additionally supplemented with (R)-(+)-%lipoic acid (Sigma; 07039) or β-hydroxybutyrate (Sigma; 54965), both obtained from Sigma.

2.2.3 Cell Treatments

2.2.3.1 Stress Treatments

Prior to exposure to cellular stressors, cell monolayers (of 60-70 % confluency) were switched into low serum Optimem media (Gibco; 31985) overnight. Cells were then washed and fresh Optimem applied containing the relevant agent (3-nitropropionic acid; N5636, anisomycin; A9789, antimycin-A; A8674, oligomycin; 75351, palmitic acid; P5585, rotenone; R8875 were purchased from Sigma Aldrich. Ceramide and dihydro-ceramide (DHC) were purchased from Calbiochem. Following incubation for the time indicated in the results section, the Optimem (Gibco) was retained for analysis of the released APP cleavage products.

2.2.3.2 Inhibitor and Other Treatments

During these studies, a number of inhibitors of the enzymes involved in APP metabolism were used. Cells were typically treated overnight (18-20 hours) following a wash with PBS with the inhibitors applied in Optimem (BACEi IV (CAS
797035-11-1); 565788, DAPT (γ-secretase inhibitor IX – CAS 208255-80-5); 565770 and TAPI-1 (CAS 171235-71-5); 579051 all Calbiochem).

Prior to glucose oxidation assay, cells were treated overnight with the pyruvate dehydrogenase inhibitor, dichloroacetate (DCA – Sigma; 347795), which was applied in culture media. Prior to glucose uptake measurement, cells were incubated with purified human sAPPα (Sigma; S0865).

2.3 Lysate preparation

2.3.1 Cell Lysates

Following incubation of cells with the relevant treatments, cells were washed twice with ice-cold PBS to halt cellular activity. The appropriate volume of ice-cold lysis buffer (solutions table) was added to the cell culture dish (150 μl, 300 μl and 500 μl for 6-well, 60mm² and 10cm² respectively). Cells was then scraped and transferred to a labeled eppendorf, vortexed and left on ice for 15 minutes. The lysate was again vortexed for 30 seconds before being centrifuged at 4 °C for 15 minutes at 16,060 g. The supernatant was then transferred to a new eppendorf.

2.3.2 Tissue Lysates

Hippocampal tissue was obtained as part of a previous PhD project as previously described (Meakin et al., 2012). For lysate preparation, tissue was placed in a tight fit glass homogenizer (Fisher) and 500 μl of lysis buffer (solutions table) was rapidly
added. The tissue was homogenized on ice for 30 strokes and the lysate was pipetted into a labeled eppendorf and placed on ice for 15 minutes. Lysate samples were then vortexed and centrifuged at 4 °C for 15 minutes at 16,060 g. The supernatant was then transferred to a new eppendorf.

2.3.3 Protein Content Determination

The protein content of cell and tissue Lysates was quantified by use of the Bradford assay (Bradford 1976). This is based on the change in absorbance from 465 nm to 695 nm of Coomassie Blue G-250 upon binding to protein. 1 µl of cell lysate was added to 9 µl of distilled H2O and 250 µl of Bradford reagent (Sigma; B6916) were added to a 96-well culture plate and the colour allowed to develop for 15-20 minutes at room temperature before the absorbance at 595 nm was measured using an EnVision 2104 Multilabel plate reader (Perkin Elmer). Serial dilutions of a Bovine Serum Albumin (BSA) standard were also made up in the plate so that a standard curve of absorbance was plotted against protein content, allowing the lysate concentration to be calculated.

2.3.4 Supernatant Lysates

Upon completion of the various cell treatments, the Optimem on the cells was removed and placed in a 15ml falcon tube and stored at -80°C. 5ml of this was then concentrated through an Amicon® 30K centrifugal filter device (Merck Millipore) at 4000 g for 15 minutes. This protocol yielded 50 µl of concentrated supernatant. 5 µl of this was then combined with 10 µl of dH2O and 5 µl of sample buffer (solutions
table) before being run on an SDS gel. Resulting expression was then normalised to total protein content in the cell culture plate as determined by the Bradford method.

2.4 Western Blotting

2.4.1 SDS Page

SDS is an anionic detergent with the ability to dissolve hydrophobic molecules and imparts a net negative charge on the resulting protein complexes. This disruption of the hydrophobic regions reduces globular proteins to linear molecules that display a constant charge-to-mass ratio. The proteins comprising the cells or tissue lysate can therefore be separated solely on the basis of their molecular weight. The polyacrylamide gel represents a cross-linked matrix with pores of a given size (determined by the acrylamide percentage present in the gel), which acts to impede the progress of molecules when they are moved by the application of an electric current. During the running (SDS-polyacrylamide gel electrophoresis (PAGE)), the negatively charged proteins are pulled through this matrix towards the anode, with the smaller proteins progressing at a faster rate and therefore move further down the gel. The concomitant running of marker proteins of known molecular weight allow for the comparison to our proteins of interest to ensure correct identification.

Protein lysates (10-50 μg) were separated by SDS-PAGE using Hoefer™ gel casting equipment (Amersham Bioscience). To facilitate the movement of protein lysates through the gel, samples were prepared by the addition of SDS-containing sample buffer (solutions table). This contains glycerol to add weight to the samples to aid
loading and movement, 20 % SDS that imparts a negative charge to the protein, ß-mercaptoethanol as a reducing agent to further disrupt secondary and tertiary structure formation and bromo-phenol blue to ease loading and give a clear indication of the gel front to observe the progression during running.

2.4.2 Hofester™ self-poured SDS-PAGE gels system

Self-poured gels were created by mixing the relevant constituents (e.g. 3 gels, table 2.2) and allowing them polymerase (15-30 minutes) in the cast formed of 1 aluminium back plate and 1 glass front plate separated by 2 plastic spacers. Once this was set, a 4 % acrylamide stacking gel (table 2.2) was applied on top, housing the 10 or 15 space well combs and allowed to set (15-30 minutes). To ensure straight tops to the lower gels, a 50:50 (v/v) mix of dH$_2$O and n-butanol (Fisher; BP505) was placed above the gel mix during setting. Electrophoresis was performed using a Tris/Glycine running buffer (solutions table) at a constant voltage of 150V for 1-2 hours.

<table>
<thead>
<tr>
<th>Component</th>
<th>Lower gel percentage</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7%</td>
<td>10%</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>7.9ml</td>
<td>6.2ml</td>
</tr>
<tr>
<td>0.5M Tris, pH 6.8</td>
<td>4.7ml</td>
<td>4.7ml</td>
</tr>
<tr>
<td>Bis-Acrylamide</td>
<td>3.9ml</td>
<td>5.6ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>165µl</td>
<td>165µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>16.5µl</td>
<td>16.5µl</td>
</tr>
<tr>
<td>20% APS</td>
<td>83µl</td>
<td>83µl</td>
</tr>
</tbody>
</table>

Table 2.2: Relative composition of SDS-PAGE gel to give required acrylamide percentages.
2.4.3 Transfer to Nitrocellulose Membranes

After vertical-plane electrophoresis, the now separated proteins were transferred from the gel to a nitrocellulose membrane (Amersham Bioscience) to provide a stronger backbone of support for viewing the proteins. Application of another electric current, now in the horizontal direction, acts to drive the proteins from the gel and onto the membrane. This was done using the BioRAD mini Trans-Blot® Cell system.

To do this, the gel casting apparatus was gently prized apart by a metal spatula and the well-containing portion of the gel scraped off. The remaining section of the gel (containing any protein) was then removed from the aluminium plate and placed on the glass plate. A presoaked piece of nitrocellulose was then placed on top of the gel before assembling the transfer sandwich. To do this, 2 pre-soaked fibre blotting pads were placed on the cathode (colourless) side of the transfer cassette. Onto this was placed a pre-soaked piece of blotting paper (Whatman) before the nitrocellulose and gel were turned and placed on top of this. A second piece of blotting paper was placed on top and then the metal spatula run over the top to remove any air bubbles. The sandwich was completed by the addition of two further blotting pads were added and the transfer cassette closed and placed into the transfer cell. The cell was then filled with the remaining transfer buffer (Table 2.4) and proteins transferred by horizontal electrophoresis at 100V for 1 hour.

Upon completion of the transfer, the membranes were stained with Ponceau S stain (0.1% Ponceau S (w/v) in 5% acetic acid (v/v); Sigma). This negatively charged red dye binds to positive amino acid residues present in the proteins and allows bands to
be visualised on the membranes to confirm even transfer. Following this, the membranes were washed in Tris-buffered saline-Tween (TBS-T; Table 2.4) to remove any staining.

2.4.4 Immuno-blotting

Non-specific binding of the primary antibody was minimised by first blocking the membranes with 10% (w/v) dried skimmed milk (Marvel™) in Tris-buffered saline plus Tween (TBS-T) for 1 hour at room temperature. The membranes were then washed briefly with TBS-T to remove excess milk prior to the addition of relevant primary antibodies (Table 2.3). Membranes were incubated overnight at 4ºC with gentle rocking to allow antibody binding. The following day, the primary antibody was removed and the membranes given three 10-minute washes in TBS-T. Following these, the blots were blocked for 20 minutes in 5% (w/v) Marvel. Secondary antibodies were then applied in TBS-T for 1 hour at room temperature. The membranes were then washed and blocked as prior to secondary addition and left in TBS-T prior to imaging and analysis.

2.4.5 Analysis

Membranes were scanned using a Licor Odyssey infrared scanner and densitometric analysis carried out as per the manufacturer’s instructions using the ImageStudio Lite programme.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Species</th>
<th>Concentration</th>
<th>Blocking Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Sigma</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Total AMPK</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Phospho AMPK (thr172)</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>APP</td>
<td>GSK</td>
<td>Rabbit</td>
<td>1:4000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>sAPPα</td>
<td>Covance</td>
<td>Mouse</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>Covance</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>sAPPβ (APP_swe)</td>
<td>GSK</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>BACE1</td>
<td>Sigma</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>BAD</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>PGC1</td>
<td>Santa Cruz</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Total JNK</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Phospho JNK (thr183/tyr185)</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Total p44-42 MAPK</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Phospho p44-42 MAPK</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Total PDH</td>
<td>DDU</td>
<td>Sheep</td>
<td>1 mg/ml</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Phospho PDHe1α</td>
<td>DDU</td>
<td>Sheep</td>
<td>1 mg/ml</td>
<td>5% BSA</td>
</tr>
<tr>
<td>PDK4</td>
<td>DDU</td>
<td>Sheep</td>
<td>1 mg/ml</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Total PKB</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Phospho PKB (ser473)</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Phospho PKB (thr308)</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Santa Cruz</td>
<td>Rabbit</td>
<td>1 mg/ml</td>
<td>5% BSA</td>
</tr>
</tbody>
</table>

Table 2.3: Summary of the primary antibodies, incubation concentration and blocking agents used in the present studies.

2.6 2-Deoxyglucose Uptake Assay

2-deoxyglucose (2-DG) uptake assays were performed at room temperature as previously described (Tsakiridis et al., 1994). Cell mono-layers were plated into 12-well cell culture plates and relevant treatments were carried out as described in the results section. Cells were washed twice with pre-warmed hepes-buffered saline [(HBS); 140 mM NaCl, 20 mM Heps, 5 mM KCl, 2.5 mM MgSO₄ and 1 mM CaCl₂ (pH 7.4)] and incubated for between 10-12 minutes in HBS containing 10 μM 2-
deoxy-D-[3H]glucose (1 µCi/ml; PerkinElmer) at room temperature (20°C). Following this incubation period, cells were washed twice with ice-cold 0.9 % NaCl. Cells were subsequently lysed using 1 ml of 50 mM NaOH and 850 µl of this volume was taken to quantify the cell-associated radioactivity using a Beckman LS6000IC scintillation counter. Protein content of the wells was determined using the Bradford method on the remaining 150 µl of cell lysate. These values, along with the specific activity of the incubation buffer (quantified via scintillation counting of 10 µl of the assay buffer) were used to normalise the rate of 2-DG uptake.

2.7 14C-Glucose Oxidation Assays

Cell mono-layers were plated into 6-well cell culture plates and relevant pre-treatments carried out as described in the results section. To begin the assay, cells were washed twice with HBS and incubated in HBS containing 2.5 mM glucose and 2 µCi/ml [U-14C-D-glucose] along with any relevant inhibitors or treatments indicated in the results section for between 2 and 3 hours at 37 °C. Following this incubation period, 1ml of the culture medium was carefully transferred to a sealable tube, the cap of which housed a Whatman (GF/B) filter paper pre-soaked in 1M KOH. The 14C-labelled CO2 that had been released into the media was then liberated via the acidification of the media using 60 % (v/v) perchloric acid and this assembly was left overnight. The filter papers were then removed from the lid of the tubes and soaked in scintillation fluid before the radioactivity that had been absorbed was quantified by liquid scintillation counting using a Beckman LS6000IC scintillation counter. The cells from the assay were also washed twice with ice-cold 0.9 % NaCl and lysed by the addition of 1 ml of 50 mM NaOH. The radioactivity contained within this lysate
was then quantified (by scintillation counting) and this served as a measure of the $^{14}$C incorporation into the cell during the assay period. Remaining lysate was also used to quantify protein content via the Bradford method and this, along with the specific radioactivity associated with the assay buffer, was used to normalize the glucose incorporation and oxidation rates for each sample.

2.8 Seahorse XF24 Analyser

Actively respiring mitochondria consume oxygen and therefore oxygen consumption rate (OCR) can be taken as a measure of substrate flux through the oxidative phosphorylation pathway while extracellular acidification rate (ECAR) reflects the release of lactate (lactic acid) converted from pyruvate following glycolysis. Cell monolayers were plated into Seahorse XF culture plates the day prior to the treatment or assay as indicated in the results section. Optimal cell number was determined following a cell titration assay taking into account oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) readings, oxygen tension values during each reading and appearance of the cell monolayers in the well and was determined to be 30,000 cells (See Appendix I Figure 1.2). On the day of the assay, cells were changed to unbuffered DMEM media with relevant inhibitors/treatments as indicated and placed in a non-CO$_2$ incubator for 1 hour prior to beginning the assay. During this time, the XF cartridge was loaded with drug additions to be made during the assay and placed in the instrument to calibrate. Once the cells were de-gassed (CO$_2$ released from solutions and plasticware) the cell plate was placed in the instrument to begin the assay. Standard 3 minute mix, 2 minute wait and 3 minute measure cycles
(See Appendix I Figure 1.1) were used during these assays with 5 baseline points taken before and a subsequent 3 measurements made following any drug additions.

### 2.8.1 Cellular Respiratory Control (CRC) Experiments

These assays were carried out in an attempt to observe how mitochondria handle the application of specific inhibitors and taken together, give a strong general measure of mitochondrial efficiency. During the cell titration experiment, sequential additions of 0.2 µM FCCP were added via the ports of the XF cartridge to determine the optimal concentration to induce maximal uncoupling of the mitochondria (See Appendix I Figure 1.3A). Optimal oligomycin concentration was determined as the highest dose to completely inhibit ATP synthase oxygen consumption while still allowing for uncoupling of the mitochondria and this was found to be 1 µM. Finally, a standard dose of 2 µM was used for the final addition of the electron transport chain (ETC) inhibitors rotenone and antimycin A (See Appendix 1.3B).

### 2.8.2 Glycolysis Stress Test

This assay was carried out to look further at glycolytic function and capacity. During these assays, cells are switched into assay media that contained mitochondrial substrates (pyruvate and L-glutamine) and zero glucose for 1-2 hours to deplete glucose stores and reduce their ECAR rate to minimal levels. That the cells were at their lowest ECAR rate (termed non-mitochondrial acidification) was observed during baseline measurements. The first addition made was glucose, to stimulate basal glycolysis, followed by oligomycin to make the cells wholly reliant on glycolysis for
ATP generation and induce their maximal glycolytic rate. Finally, a high competing dose of 2-DG (25 mM) was added to completely inhibit glucose uptake and glycolysis.

2.9 Activity Assays

2.9.1 BACE1 Activity Determination

BACE1 activity was determined by use of the SensiZyme BACE1 Activity Assay Kit (Sigma), which was performed as per the manufacturer’s instructions. 100 µg of whole cell lysate from the different SH-SY5Y cell treatment groups were made up in duplicate to a final volume of 100 µl in assay buffer and pipetted into the antibody coated assay plate, covered and incubated at 2-8 °C for 2 hours. The plate was then washed 4 times with assay buffer, before 50 µl of substrate A was added to the wells and the plate was incubated overnight at room temperature. During this incubation, Substrate A, a proenzyme containing a BACE1 cleavage site fused to a second, is activated upon cleavage by BACE1 in the cell lysate. 50 µl of substrate B (a peptide that is cleaved by the now active substrate A peptide to produce a colour change) and incubated at room temperature for an initial 3 hours before the absorbance at 405 nm was measured. Regular readings were made for a further 20 hours. The duplicate values were then averaged and the background absorbance subtracted before control values were compared to those following cell stress treatments.
2.9.2 Cellular Metabolism Assays

Throughout the project, a number of enzyme assay kits were utilised, all were carried out as per the manufacturer’s instructions (pyruvate dehydrogenase (ab109902), fumarase (ab110043) and NAD⁺/NADH (ab63548); Abcam, isocitrate dehydrogenase (MAK062); Sigma Aldrich and oxoglutarate dehydrogenase (OGDH, alternatively α-ketoglutarate dehydrogenase (α-KGDH); E96187Hu); USCN Life Sciences).

2.9.2.1 Pyruvate Dehydrogenase Activity

Pyruvate dehydrogenase activity was determined by an indirect assay method. This assay works by coupling the reduction of NAD⁺ to NADH to the production of a colour (yellow) reporter. Briefly, 70-80 % confluent 10 cm cell culture dishes were trypsinised and pelleted (at 1000 g for 4 minutes) before being resuspended in 200 µl of PBS. Protein content was then determined using the Bradford method and all samples were made to 10 mg/ml concentrations. The cells were then solubilised by the addition of detergent (1 part in 10) and incubated on ice for 10 minutes. Cells lysates were then centrifuged at 1000 g for 10 minutes at 4°C and diluted to a concentration of 500 µg in 200 µl in duplicate. These were then loaded to the 96-well assay plate and incubated at room temperature for 3 hours. The wells were then emptied and washed twice with 1x stabiliser solution before adding 200 µl assay buffer. The plate was then read at 450 nm at room temperature using a kinetic program reading the plate every minute for 15 minutes.
2.9.2.2 Isocitrate Dehydrogenase Activity

Isocitrate dehydrogenase (IDH) was determined through the use of a specific activity assay. This assay monitors the NAD$^+$-dependent, NADP$^+$-dependent or NAD$^+$- and NADP$^+$-dependent enzyme reaction of the substrate isocitrate to produce a colorimetric product proportional to enzyme activity. One unit of IDH is determined as the amount of enzyme needed to generate 1 µM of NADH or NADP per minute at pH 8.0 at 37 °C. 1 million cells were homogenised in 200 µl of ice-cold IDH activity assay buffer and centrifuged at 16, 100 g for 10 minutes and the supernatant retained. 50 µl of the supernatant was then added to a 96-well plate along with 50 µl of master reaction mix containing the appropriate co-factor (NAD$^+$, NADP$^+$ or both). The reaction proceeded for 3 minutes at 37 °C before taking the first reading of absorbance at 450 nm ($A_{450}$ initial). The microplate was then incubated for 5 minutes and read at 450 nm again, with these steps repeated until the sample absorbance values were above the highest standard curve value (10 nmole/well). The reading prior to any samples being above the standard curve was then designated $A_{450}$ final. The change in absorbance ($A_{450}$ initial – $A_{450}$ final) for each sample was then compared to the standard curve to reveal how much NADH or NADP was generated during the assay (B). The final IDH activity for each sample was then determined as follows:

$$\text{IDH activity} = \frac{B \times \text{Sample Dilution Factor}}{(\text{Reaction time}) \times \text{Volume}}$$
2.9.2.3 Alpha-ketoglutarate Dehydrogenase

α-KDH activity was measured through the use of a specific sandwich ELISA. 10 cm² dishes of cells were grown to confluency and trypsinised before being counted using a Moxy automatic cell counter (Orflo). 1 x 10⁶ cells were then pelleted in an eppendorf and washed three times with ice cold PBS before being collected in 300 µl PBS and ultrasonicated for 5-10 seconds 4 times. Samples were then centrifuged at 4 °C and the supernatant retained for use during the assay. 100 µl of cell supernatant was applied to the assay plate (each sample run in duplicate) along with the standard curve and blank wells before being covered and incubated at 37 °C for 2 hours. The plate was emptied and 100 µl of detection reagent A added and the plate was covered and incubated for 1hr at 37 °C. This solution was removed and the plate washed 3 times by the addition of 350 µl of wash solution, sitting for 1-2 minutes before being emptied by snapping the plate onto absorbent paper. 100 µl of reagent B was then added and the plate incubated for 30 minutes at 37 °C before being washed as above for another 5 repetitions. 90 µl of substrate solution was added to each well and the plate was covered and incubated for 20 minutes at 37 °C while protecting from light. 50 µl of stop solution was then added and the plate read at 450 nm. A standard curve was prepared by averaging the duplicates of the standard absorbance values (OD) and subtracting the blank value. These corrected optical density readings are plotted against α-KDH concentration and the samples optical density compared to this standard curve to give the α-KDH concentration in each well.
2.9.2.4 NAD⁺/NADH Ratio Assay

Measurement of nicotinamide nucleotides was carried out using a specific NADH/NAD quantification kit as per the manufacturer’s instructions (Abcam). Cell monolayers, in 60 mm cell culture dishes, were washed with PBS before being trypsinised and pelleted (at 1000 g for 4 minutes). These were then resuspended in a volume of 10 ml of media and counted using an Oxy automatic cell counter. 2 x 10⁵ cells were then placed in an eppendorf and re-pelleted before being washed and pelleted in ice-cold PBS. This was removed before the addition of 400 µl of ice cold NADH/NAD extraction buffer and the cells were ruptured by 2 freeze thaw cycles (20 minutes on dry ice followed by 15 minutes at room temperature). Cell samples were then vortexed for 10 seconds before being centrifuged at 17, 400 g for 5 min. 150 µl of the resultant supernatant was retained as it was to provide a total NAD level (i.e. NAD⁺ and NADH, NAD_total) while a second 150 µl eppendorf was prepared for decomposition. To do this, the sample was heated at 60 °C, resulting in the loss of all NAD⁺ in the sample, while retaining the NADH. In order to measure total NAD nucleotide levels, 50uL of the NAD_total sample were loaded (in duplicate) to wells of a 96-well plate and a corresponding well for the relevant decomposed sample added to the latter half of the plate. 100 µl of Master Mix was then added to each NADH standard and sample well and mixed for 5 minutes. 10 µl of NADH Developer was then added and the reaction was allowed to cycle at room temperature for 3.5 hours with readings every 30-60 minutes. 10 µl of stop solution was added after the incubation time and a final reading taken 30 minutes later. The NADH standard curve was plotted and the absorbance values for the average of each sample duplicate
compared to this to give the NAD$_{\text{total}}$ and NADH concentrations (in pmol/well).

Finally, the NAD$^+$:NADH was calculated by:

$$\frac{\text{NAD}_{\text{total}} - \text{NADH}}{\text{NADH}}$$

2.9.2.5 Fumarase Specific Activity Assay

This assay utilises immunocapture in each assay well to ensure its specificity for fumarase. Fumarase activity was measured by following a fumarase-catalysed reaction:

$$\text{Fumarase} + \text{H}_2\text{O} \rightarrow \text{S-malate}$$

This production of malate is subsequently coupled to colorimetric detection by means of the following reactions:

$$\text{S-malate} + \text{NAD}^+ \rightarrow \text{oxaloacetate} + \text{NADH}$$

$$\text{NADH} + \text{tetrezoium salt} \rightarrow \text{NAD}^+ + \text{formazan dye}$$

($\uparrow$ Abs at 450 nm)

Following this measurement of fumarase activity, the addition of a fumarase specific antibody means that the quantity of fumarase in a well can then be measured using a colorimetric horseradish peroxidase (HRP) label.

Prior to running this assay in an experimental manner, a protein tritration was performed whereby quantity and activity was measured with increasing amounts of protein (0, 5, 10, 25, 50, 100, 250, 500 μg). Cell monolayers in 10 cm$^2$ cell culture plates were washed with PBS and trypsinised before being pelleted (at 900 g for 4 minutes). The cells were then washed with ice-cold PBS and re-pelleted before the
PBS was removed and replaced with 9 volumes of sample extraction buffer and briefly ultrasonicated on ice. Cells were then placed on ice for 20 minutes before being centrifuged at 15,000 g for 20 minutes at 4 °C. The supernatant was then moved to a fresh eppendorf and the protein content determined by BCA assay (BioRad; 500-014). The relevant protein concentrations were then prepared in incubation buffer and loaded (in triplicate) into the assay plate, covered and incubated at room temperature for 3 hours. The plate was then emptied and washed with 300 µl of wash buffer before 200 µl of activity solution was added. Fumarase activity was determined by reading the plate at 450 nm at 1-minute intervals for 30 minutes (with the activity remaining linear for 24 minutes). The above wash steps were then repeated and 100 µl detector antibody added before incubating at room temperature with gentle shaking. The 3 wash steps were again repeated before the addition of 100 µl HRP label and the plate was incubated at room temperature for 1 hour with gentle shaking. The previous wash steps were repeated, this time for 4 washes before 100 µl of HRP development solution was added and the plate incubated at room temperature for 10 minutes. The development was stopped by the addition of 50 µl stop solution before the fumarase quantity was determined by reading the plate at 450 nm. The kinetic plot of Abs at 450 nm vs. time was then prepared for the fumarase activity for each protein amount as well as a plot of the Abs at 450 nm versus protein amount for the fumarase quantity measurement. From these, a twenty minute activity measurement using 100 µg of protein was determined as optimum assay conditions for the SH-SY5Y cell line. Subsequently, the fumarase activity was measured in stable cell lines under these conditions as stated in the results.
2.9.3 Cell Death Assay

Cell death was monitored by the cytoplasmic enrichment of DNA mono- and oligonucleosome fragments using a kit as per the manufacturer’s instructions (Roche Bioscience – 11 774 425 001). A test 96-well plate was seeded prior to use of the assay kit and 7500 cells was selected as the optimum for overnight growth. On the day of assay, cells were incubated with varying concentrations of the mitochondrial uncoupler dinitrophenol (DNP) for 20 minutes. The microplate was then centrifuged for 10 minutes at 200 g before the removal of the supernatant (to remove any necrotic material). The microplate was washed twice with PBS before the addition of 200 μl of lysis buffer. The plate was then incubated at room temperature for 30 minutes before being centrifuged at 200 g for 10 minutes. 20 μl of the lysate supernatant was transferred to the streptavidin coated microplate for analysis. 80 μl of immunoreagent was added to each well and the plate covered and shaken gently for 2 hours at room temperature. The assay solution was removed prior to 3 washes with 300 μl of incubation buffer before the addition of 100 μl of ABTS solution. The plate was gently shaken for 20 minutes to allow for colour development, then 100 μl of ABTS stop solution was added and the absorbance at 450 nm measured.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer (5 x Stock)</td>
<td>Tris HCl</td>
<td>125mM</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>500mM</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>5mM</td>
</tr>
<tr>
<td></td>
<td>EGTA</td>
<td>25mM</td>
</tr>
<tr>
<td></td>
<td>NaF</td>
<td>250mM</td>
</tr>
<tr>
<td></td>
<td>NaP Pi</td>
<td>50mM</td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td>5% (v/v)</td>
</tr>
</tbody>
</table>
Table 2.4: Composition of solutions used during the present studies.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>1 x Lysis Buffer Supplements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Added at time of use)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>92mg/ml</td>
</tr>
<tr>
<td></td>
<td>(\beta)-mercaptoethanol</td>
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<tr>
<td></td>
<td>Benzamidine</td>
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</tr>
<tr>
<td></td>
<td>PMSF</td>
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<tr>
<td><strong>Additional for acetylation studies</strong></td>
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<tr>
<td></td>
<td>NAD</td>
<td>10mM</td>
</tr>
<tr>
<td></td>
<td>Trichostatin A</td>
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</tr>
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<td><strong>Sample Buffer</strong></td>
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<tr>
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<td>(\beta)-mercaptoethanol</td>
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</tr>
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<td>Bromophenol blue</td>
<td>Pinch</td>
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<td><strong>SDS-PAGE Running Buffer</strong></td>
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<tr>
<td></td>
<td>dH₂O</td>
<td>900ml</td>
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<td>10 x Running Buffer (National Diagnostics: 25mM Tris, 192mM Glycine, 0.1% SDS)</td>
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<td><strong>SDS-PAGE Transfer Buffer (1L)</strong></td>
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<td>Methanol</td>
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<td></td>
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<tr>
<td><strong>Tris-buffered Saline-tween (TBS-T)</strong></td>
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<tr>
<td></td>
<td>Tris HCl (pH 7.4)</td>
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<tr>
<td></td>
<td>NaCl</td>
<td>150mM</td>
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<tr>
<td></td>
<td>Tween-20</td>
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<tr>
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<td></td>
<td>NaCl</td>
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</tr>
<tr>
<td></td>
<td>HEPES (pH 7.4)</td>
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</tr>
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</tr>
<tr>
<td></td>
<td>MgSO₄</td>
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</tr>
<tr>
<td></td>
<td>CaCl</td>
<td>147mg (1.3mM)</td>
</tr>
</tbody>
</table>
Chapter 3

Effect of Cellular Stress on BACE1 and APP Processing
3.1 Introduction

3.1.1 Effect of Acute Cellular Stress on BACE1 and APP Processing

As previously mentioned (Section 1.9), there are thought to be a number of lifestyle-related risk factors for late-onset, so called sporadic AD (for review see Mayeaux and Retiz 2014). In the brain, a number of these factors can act individually or in concert to increase BACE1 levels and/or activity, evidenced by the presence of increased BACE1 activity observed in the brain regions displaying AD pathology (Stockley and O’Neill 2007). Indeed, stroke, T2DM and obesity have all previously been demonstrated to increase AD risk (for review see De la Torre JC 2006; Hassing et al., 2009; for review see Luchsinger and Gustafson 2009). Enhanced transcription of BACE1 has also been previously demonstrated in response to a number of factors at the cellular level that act to increase AD risk including cytokine production, mitochondrial dysfunction, oxidative stress and hypoxia (Zhang et al 2002; Buggia-Prevot et al 2007; Cho et al 2008; Xiong et al 2008). These changes culminate in aberrant processing of APP by BACE1, leading to over production of Aβ. This in turn induces a number of deleterious effects including: further oxidative damage and mitochondrial function, synaptic dysfunction and cell death. With this in mind, interest has grown in gaining a better understanding of the mechanisms that underlie this initial shift towards BACE1 cleavage.

A number of studies have reported increases in BACE1 mRNA, protein and activity as a response to oxidative stress-inducing agents such as HNE and H₂O₂ across a variety cell line and primary culture models (Tamagno et al., 2008; Jo et al., 2010).
There have also been investigations demonstrating a stimulatory effect of metabolic challenges (e.g. mitochondrial inhibition and substrate removal) on BACE1 (Veliquette et al., 2005; Xiong et al., 2007; O’Connor et al., 2008). Additionally, lipid stress conditions, such as hypercholesterolemia and obesity as well as application of the lipid second messenger ceramide enhance BACE1 (Puglielli et al., 2003; Wang et al., 2013). Further evidence for this intimate relationship between lipid excess and BACE1 comes from the findings that BACE1 levels are enriched in lipid rafts regions of the cell membranes and it has been shown that targeting BACE1 to these regions, enhances APP processing by BACE1 and amyloid production (Cordy et al., 2003).

The present studies aimed to recapitulate and extend the previous work on cell stress-induced changes in the BACE1-APP axis. Initially, mitochondrial inhibitors were utilised to confirm the previous findings as well as incorporating both acute and prolonged incubations times to observe alterations in BACE1 protein levels and activity. While many of these previous studies have identified enhanced BACE1-driven APP processing as a result of these stressors, few, if any have looked at the effect of these interventions on APP itself. With this in mind, APP protein expression was also monitored during these stress exposures in an attempt to investigate any changes in the enzyme-substrate relationship between BACE1 and APP. Metabolic disturbance will also be modelled in a more physiological context by utilising the glucose-sensing, hypothalamic, GT1-7 cell line through the use of recurrent low glucose exposures. This experimental protocol has been used previously to model recurrent hypoglycemic episodes associated with type-1 diabetes. This fluctuating, or impaired substrate delivery to the brain may also form part of the
hypoperfusion/ischemia models that have been shown to elicit increases in BACE1 expression in the brain (Wen et al., 2004; Zhang et al., 2007). Allied to this, the impact of a single acute bout of oxidative stress (induced via the application of H$_2$O$_2$) will also be assessed. The impact of fatty acid excess was also modelled in a number of ways, initially through exposure of Swedish AD mutation bearing APP overexpressing SH-SY5Y cells to acute and chronic palmitate oversupply or acute treatment with the lipid second messenger (and palmitate metabolite), ceramide. Subsequently, the brains from mice fed on a high fat diet were investigated for changes in the BACE1 and APP.

These interventions were hypothesised to increase BACE1 protein and activity as well as fundamentally altering APP processing towards sAPPβ (and presumably Aβ). It was of interest to know how these changes may be impacted by length and nature of stressor exposure and how they may act to drive pathological and potentially physiological change in the early stages of AD progression.
3.2 Results

3.2.1.1 Acute Cellular Stressor Treatment Increases BACE1 and APP Protein and BACE1 Activity Levels

Cellular stress has previously been shown to increase BACE1 mRNA, protein and activity. In an attempt to better understand conditions that may induce changes in both BACE1 and APP protein levels and activity, a number of cellular stress paradigms (both acute and chronic) were attempted and the effect on BACE1 (protein level and activity) and APP (protein level and processing) assessed. Initially, the effect of the protein synthesis inhibitor anisomycin (1 μM) and the lipid second messenger ceramide (50 μM) was examined in SH-SY5Y cells overexpressing APP harbouring the familial AD Swedish double mutation (SH-SY5Y APPswe). Acute (1 and 4 hour) treatment with anisomycin, ceramide did not elicit any significant changes in BACE1 protein levels with dihydroceramide (DHC) acting as an inactive control for ceramide and ethanol (EtOH) as a vehicle (Fig. 3.1). BACE1 activity was then assessed in two ways, first through the use of a BACE1 activity assay and also via western blot analysis of the relative abundance of the BACE1-mediated cleavage product sAPPβ released into the media. A 1-hour treatment with these stressors elicited a trend toward an increase in BACE1 activity, as measured by the BACE1 activity assay (Fig. 3.2A), but there was a significant increase in sAPPβ following treatment with anisomycin and ceramide (Ctl; 1.00 vs. Anis; 1.95 ± 0.05 and Ceramide; 2.68 ± 0.45, p < 0.05, n = 4-5, Fig.3.2B, C). Following the 4-hour incubation, both active agents brought about significant increases in BACE1 activity
Figure 3.1: Acute treatment of SH-SY5Y<sub>APPsw</sub> cells with the protein synthesis inhibitor anisomycin or the lipid second messenger ceramide does not alter BACE1 protein levels.

(A) Representative Western blot showing BACE1 protein levels in response to 1- and 4-hour treatments with anisomycin (1 μM) and ceramide (50 μM) with a 50 μM dihydroceramide (DHC) and ethanol (EtOH) serving as negative and vehicle controls respectively. (B, C) Densitometric analysis showing no changes in BACE1 protein expression in response to any of the treatments following 1- (B) or 4- (C) hour treatments.
Figure 3.2: Acute (1-hour) treatment of SH-SY5Y<sub>APP</sub> <sub>Are</sub> cells with anisomycin and ceramide significantly increases BACE1 activity.

(A) BACE1 activity assay data showing a trend towards an increase following incubation of SH-SY5Y<sub>APP</sub> <sub>Are</sub> cells with anisomycin (1 μM) and ceramide (50 μM), with no change elicited by DHC (50 μM) or EtOH (n = 5-6). (B) Representative Western blot showing the increased abundance of the BACE1-dependent cleavage product, sAPPβ, released into the conditioned media during a 1-hour incubation with anisomycin (1 μM) and ceramide (50 μM) but not DHC (50 μM). (C) Densitometric analysis showing the significant increases in sAPPβ release following cell stress treatment (p < 0.05, n = 4-5).
These increases were accompanied by a trend towards an increase in sAPPβ abundance following anisomycin and a significant increase after ceramide treatment (Ctl; 1.00 vs. Anis; 2.28 ± 0.53 and Ceramide; 1.75 ± 0.16, p < 0.05, n = 5-6, Fig. 3.3B, C). Taken together, these suggest that acute treatment of SH-SY5Y cells with the protein synthesis inhibitor anisomycin and the lipid second messenger, ceramide results in marked increases in BACE1 activity without any significant change in BACE1 protein levels.

Previous studies have shown a profound effect of mitochondrial inhibition and oxidative stress agents in altering APP processing. Here, the effect of mitochondrial inhibition (via treatment with the complex I inhibitor rotenone (1 μM) or complex II inhibitor 3-nitropropionic acid (3NP; 1 mM) or induction of oxidative stress with 100 μM H2O2, on BACE1 was assessed as above. All of these treatments resulted in rapid, significant increases in BACE1 protein levels, following either a 1 (Ctl; 1.00 vs. 3NP; 1.68 ± 0.09, p < 0.001, Rotenone; 1.86 ± 0.08, p < 0.05 and H2O2; 1.94 ± 0.14, p < 0.01, n = 6-7, Fig. 3.4) or 4-hour incubation (Ctl; 1.00 ± 0.01 vs. 3NP; 2.19 ± 0.25, Rotenone; 2.26 ± 0.23, and H2O2; 2.17 ± 0.33, p < 0.01, n = 7, Fig. 3.4).

BACE1 activity, as assessed by looking at sAPPβ levels in the media, was significantly increased following treatment with each of these stressors for 1 (Ctl; 1.00 vs. 3NP; 2.12 ± 0.33, Rot; 2.26 ± 0.33 and H2O2; 2.28 ± 0.4, p < 0.05, n = 6, Fig. 3.5) or 4 hours (Ctl; 1.00 vs. 3NP; 2 ± 0.19, p < 0.01, Rot; 2.06 ± 0.37 and H2O2; 2.21 ± 0.38, p < 0.05, n = 5, Fig. 3.5). Observation of BACE1 activity using the specific activity assay also yielded significant increases following 4-hour treatment with 3NP.
Figure 3.3: Acute (4-hour) treatment of SH-SY5Y_{APP\_ave} cells with anisomycin and ceramide significantly increases BACE1 activity.

(A) BACE1 activity assay data showing a significant increase following incubation of SH-SY5Y_{APP\_ave} cells with anisomycin (1 \mu M) and ceramide (50 \mu M), with no change elicited by DHC (50 \mu M) or EtOH (p < 0.05, n = 6).

(B) Representative Western blot showing the increased abundance of the BACE1-dependent cleavage product, sAPP\_\beta, released into the conditioned media during a 4-hour incubation with anisomycin (1 \mu M) and ceramide (50 \mu M) but not DHC (50 \mu M).

(C) Densitometric analysis showing the significant increases in sAPP\_\beta release following ceramide (50 \mu M) treatment (p < 0.01, n = 5-6). There was also a trend towards an increase following anisomycin treatment (n = 5-6).
Figure 3.4: Acute mitochondrial inhibition and oxidative stress significantly increase BACE1 protein levels in SH-SY5YAPPswc.
(A, C) Representative Western blots showing enhanced BACE1 protein levels following 1- (A) and 4- (C) hour treatment with 3NP (1 mM), rotenone (1 μM) and H2O2 (100 μM). (B, D) Densitometric analyses showing the significant increases in BACE1 protein levels following 1- (3NP; p < 0.001, rotenone; p < 0.001 and H2O2; p < 0.01, n = 6-7) and 4-hour (3NP; p < 0.01, rotenone; p < 0.01 and H2O2; p < 0.05, n = 7) treatment with the cell stressors.
Figure 3.5: Acute mitochondrial inhibition and oxidative stress significantly increases sAPPβ release from SH-SY5Y APPsw cells.
(A) Representative Western blots showing enhanced sAPPβ release into the culture media during 1- and 4-hour treatment with 3NP (1 mM), rotenone (1 μM) and H2O2 (100 μM). (B, C) Densitometric analyses showing the significant increases in sAPPβ release following 1- (3NP, rotenone and H2O2; p < 0.05, n = 6) and 4-hour (3NP; p < 0.01, rotenone and H2O2; p < 0.05, n = 5) treatment with the cell stressors.
3.2.1.2 Effect on Protein APP Level

Having observed changes in BACE1 protein level and activity as a result of treatment with a range of cellular stress-inducing compounds, the effect of these exposures on APP protein level was then assessed. Acute stress exposure, be it mitochondrial (3NP (1 mM), rotenone (1 μM)), lipid (ceramide (50 μM)) or oxidative (H₂O₂ (100 μM)) or more general (anisomycin (1 μM)) in nature all acted over a 1 or 4-hour exposure to induce significant or a trend towards decrease in APP protein expression (3.7A). The 1-hour incubation yielded a significant reduction in APP expression in the anisomycin group (Ctl; 1.00 vs. 1 μM Anis; 0.13 ± 0.06, p < 0.001, n = 4, Fig. 3.7B). The 4-hour treatment period yielded strong trends towards significance in the rotenone and H₂O₂ groups (Ctl; 1.00 vs. 1 μM Rot; 0.74 ± 0.1 and 100 μM H₂O₂; 0.52 ± 0.18, p = 0.06, n = 5, Fig. 3.7C) and an almost complete removal of APP expression in the anisomycin treated cells (Ctl; 1.00 vs. 1 μM Anis; 0.03 ± 0.01, p < 0.001, n = 5, Fig. 3.7C).

3.2.2 Acute Stress Treatment Results in Prolonged Increases in BACE1 Protein Level and Activity

To assess the long lasting effects of an acute exposure of cells to either mitochondrial dysfunction or oxidative damage, SH-SY5Y APPswe cells were treated with 3NP, rotenone or H₂O₂ for 1 hour before these treatments were removed and the cells left
Figure 3.6: 4-hour but not 1-hour incubation with mitochondrial inhibitors and H2O2 increases BACE1 activity in SH-SY5Y APPsw cells.

(A) 1-hour treatment with 3NP (1 mM), rotenone (1 μM) and H2O2 (100 μM) did not significantly alter BACE1 activity as measured by activity assay. (B) 4-hour treatment with 3NP (1 mM) and H2O2 (100 μM) induced significant increases in BACE1 activity (p < 0.05, n = 3) while rotenone (1μM) resulted in a trend towards an increase (n = 3).
Figure 3.7: Acute stress treatment results in lowered APP protein expression in SH-SY5Y_{APPswc} cells.

(A) Representative Western blots showing APP expression in response to treatment with 3NP (1 mM), rotenone (1 μM), anisomycin (1 μM), ceramide (50 μM) and H₂O₂ (100 μM) for 1 and 4 hours. (B, C) Densitometric analyses showing a significant reduction in APP protein expression following 1- (B) and 4-hour (C) treatments with anisomycin (1 μM; p < 0.001, n = 4).
for between 8 and 24 hours. Following this protocol, the significant increases in BACE1 protein level elicited by these compounds previously (see fig. 3.4 and 3.5) were prolonged throughout the wash out time-course with significantly increased BACE1 protein levels being observed 8 (Ctl; 1.00 vs. 3NP; 1.90 ± 0.18, p < 0.01, Rotenone; 1.92 ± 0.11 and H₂O₂; 1.72 ± 0.07, p < 0.001, n = 6, Fig. 3.8A/B), 16 (Ctl; 1.00 vs. 3NP; 1.81 ± 0.1, p < 0.001, Rotenone; 1.54 ± 0.11 and H₂O₂; 1.9 ± 0.18, p < 0.01, n = 6, Fig. 3.8A, B) and 24 hours (Ctl; 1.00 vs. 3NP; 1.69 ± 0.1, Rotenone; 1.72 ± 0.16 and H₂O₂; 1.72 ± 0.16, p < 0.01, n = 6, Fig. 3.8A, B) following treatment washout. This trend was also observed with the significant increase in sAPPβ release brought about by these compounds, being maintained following 8 (Ctl; 1.00 vs. 3NP; 2.00 ± 0.11, p < 0.001, Rotenone; 1.89 ± 0.14, p < 0.01 and H₂O₂; 1.82 ± 0.08, p < 0.001, n = 6, Fig. 3.8C/D), 16 (Ctl; 1.00 vs. 3NP; 1.78 ± 0.11, Rotenone; 1.88 ± 0.13, p < 0.001 and H₂O₂; 1.90 ± 0.18, p < 0.01, n = 6, Fig. 3.8C, D) and 24 hours (Ctl; 1.00 vs. 3NP; 1.73 ± 0.13, p < 0.01, Rotenone; 1.80 ± 0.08 and H₂O₂; 1.78 ± 0.09, p < 0.001, n = 6, Fig. 3.8C, D). These data suggest that the significant increases in BACE1 protein level and activity elicited by an acute period of mitochondrial inhibition or oxidative stress are prolonged for up to 24 hours after stressor removal.

### 3.2.3 Chronic Mitochondrial Inhibition Results in Concerted Increases in BACE1 and APP Protein Expression

Evidence suggests that prolonged mitochondrial dysfunction may underlie AD progression. In an attempt to model this, the ETC of the mitochondria was inhibited via treatment with rotenone and the complex III inhibitor antimycin A (each 0.5 μM). Application of these drugs results in a rapid reduction in cellular respiration (appendix...
Figure 3.8: Increases in BACE1 protein levels and sAPPβ release following acute mitochondrial inhibition and oxidative stress in SH-SY5Y APPsw mice are prolonged upon washout. (A, C) Representative Western blots showing BACE1 protein expression and sAPPβ levels in the culture media following 8, 16 and 24 hours washout after a 1-hour treatment with 3NP (1 mM), rotenone (1 μM) and H2O2 (100 μM). (B, D) Densitometric analyses showing that the significant increase in BACE1 protein levels and sAPPβ after acute stress treatment is prolonged throughout 8, 16 and 24-hour recovery periods (**, p < 0.01 and ***, p < 0.001).
I Fig. 3B) and prolonged exposure of cells to these agents resulted in a significant increase in BACE1 protein levels by 4 hours (Ctl; 1.00 ± 0.03 vs. Rot/Ant; 1.57 ± 0.12, p < 0.05, n = 5, Fig. 3.9B), reaching a peak at 8 hours (Ctl; 1.00 ± 0.05 vs. Rot/Ant; 2.21 ± 0.31, p < 0.05, n = 5, Fig. 3.9B) and remained significantly elevated until 16 hours (Ctl; 1.00 ± 0.09 vs. Rot/Ant; 1.91 ± 0.14, p < 0.01, n = 5, Fig. 3.9B). APP protein levels were also significantly increased by this treatment with significantly enhanced levels being observed in the 4, 8 and 16-hour treatment groups (4hr; 1.54 ± 0.07, p < 0.01, 8hr; 1.8 ± 0.03, p < 0.001 and 16hr; 1.3 ± 0.09, p < 0.05 vs. control, n = 5, Fig. 3.9C). The significantly elevated protein levels both returned to control levels following 24-hour incubation with rotenone and antimycin A. This cellular model of chronic mitochondrial inhibition was also attempted through treatment with oligomycin (500 nM). Application of oligomycin to SH-SY5Y cells significantly lowers and increases their oxygen consumption rate and extracellular acidification rate (indicative of glycolysis) respectively, indicating an enhanced reliance upon glycolysis for ATP generation (See Appendix I Fig. 3C). Prolonged exposure to oligomycin resulted in a significant increase in BACE1 protein levels relative to controls (4hr Oligomycin; 1.94 ± 0.65, 8hr Oligomycin; 1.9 ± 0.16, 16hr Oligomycin; 1.7 ± 0.12 and 24hr Oligomycin; 2.4 ± 0.21, all p < 0.01, n = 4-5, Fig. 3.10B). Significant increases in APP protein levels were also induced by oligomycin after 16 and 24 hours relative to control (16hr Oligomycin; 1.46 ± 0.05, p < 0.001 and 24hr Oligomycin; 2.4 ± 0.28, p < 0.01, n = 4-5, Fig. 3.10C).
Figure 3.9: Prolonged mitochondrial inhibition of SH-SY5Y AppRw cells results in significant increases in BACE1 and APP protein expression. (A) Representative Western blots showing BACE1 and APP protein levels in response to combined treatment with the complex I rotenone and complex III inhibitor antimycin A (each 1 μM) for between 4 and 24 hours. (B, C) Densitometric analyses showing significant increases in BACE1 and APP protein levels after 4 (BACE1; p < 0.05 and APP; p < 0.01), 8 (BACE1; p < 0.05, APP; p < 0.001) and 16 (BACE1; p < 0.01 and APP; p < 0.05) with rotenone and antimycin A (n = 3).
Figure 3.10: Prolonged stimulation of aerobic glycolysis significantly increases BACE1 and APP protein levels in SH-SY5Y APPswp cells.

(A) Representative Western blots showing changes in BACE1 and APP following treatment with the ATP synthase inhibitor oligomycin (500 nM) for between 4 and 24 hours. (B, C) Densitometric analyses showing significantly increased BACE1 protein expression following 8, 16 and 24 (p < 0.01, n = 5) and APP following 16 (p < 0.001) and 24 (p < 0.01) hour incubation with oligomycin (n = 4-5).
3.2.4 Recurrent Low Glucose Exposure Increases BACE1 and Decreases APP

**Protein Levels**

Previous work by O’Connor and colleagues showed that amyloidogenic processing can be significantly enhanced following prolonged glucose removal (6 hours in HEK293 cells and up to 36 hours in primary neuronal cultures (O’Connor *et al.*, 2008). In an attempt to place this observation in a more physiological context, the effect of recurrent hypoglycemia (RH) on BACE1 and APP was investigated. RH is a major therapeutic complication for people with type 1 diabetes and presents as the result of supraphysiological insulin supplementation. Under physiological conditions, a period of hypoglycemia will elicit the counter regulatory response (CRR), whereby glucagon and adrenaline are produced by the pancreas and adrenal glands respectively and act on a number of tissues to increase blood glucose levels. In people with type 1 diabetes, this response is habituated over the course of the disease leading to the prevailing theory that “hypoglycemia begets hypoglycemia”, predisposing individuals to resultant bouts of recurrent hypoglycemia (Cryer 1993). It has also been demonstrated that cognition can be impaired following severe hypoglycemic episodes with patients consistently showing impaired cognitive testing results and altered mood in the days following such an event (Strachan *et al.*, 2000). To examine the effect of recurrent hypoglycemia on BACE1 and APP, the mouse hypothalamic GT1-7 cell line was utilised. Previously, these cells have been shown to possess all the machinery to respond to glucose removal and do so by reducing their action potential firing (Beall *et al.*, 2012). For these experiments, cells were treated for 3 hours with either 0.1mM (RH) or 2.5mM (control) glucose containing normal saline on 3 consecutive days with recovery periods between each and following the final low
glucose exposure (in 2.5mM glucose containing media). Cell lysates obtained at the conclusion of this protocol displayed a significant increase in BACE1 protein levels (Ctl; 1.00 ± 0.14 vs. RH; 2.5 ± 0.4, p < 0.4, n = 5, Fig. 3.11B) and this was accompanied by a significant decrease in APP protein expression (Ctl; 1.00 ± 0.08 vs. RH; 0.71 ± 0.06, p < 0.01, n = 5, Fig. 3.11C).

3.2.5 Fatty Acid Treatment and High Fat Diet Significantly Increase BACE1 and APP Protein Levels

Having previously observed a significant impact of the lipid second messenger, ceramide, on BACE1 and APP processing, SH-SY5Y<sub>APPswe</sub> cells were then treated with increased levels of the ceramide precursor, palmitate. Previous work from our lab has shown that palmitate treatment significantly enhances both BACE1 and APP protein levels in muscle cells (Hamilton et al., 2014). Following a 24-hour incubation with palmitate (125 and 250µM), significant increases in both BACE1 (Ctl; 1.00 ± 0.06 vs. 125 µM Palmitate; 1.79 ± 0.12 and 250 µM Palmitate; 1.53 ± 0.11, p < 0.01, n = 5-6, Fig. 3.12B) and APP (Ctl; 1.00 ± 0.01 vs. 125 µM Palmitate; 1.98 ± 0.23 and 250 µM Palmitate; 2.13 ± 0.23, p < 0.01, n = 6, Fig. 3.12C) were detected. Having observed this effect of chronic fatty acid oversupply, an acute model was attempted, whereby SH-SY5Y<sub>APPswe</sub> cells were treated for 4 hours with palmitate and then this was washed off and the cells left for 20 hours to mirror the prolonged (24-hour) treatment group. A second group was also treated in this way, however these cells were also treated a second time for 4 hours before recovering for 20 hours as before. Following a single 4-hour treatment with 250µM palmitate, significant increases in both BACE1 (Ctl; 1 ± 0.06 vs. 250 µM Palmitate; 1.34 ± 0.12, p < 0.05, n = 6, Fig.
Figure 3.11: Recurrent low glucose exposures result in significant changes in BACE1 and APP protein levels in GT1-7 cells.

(A) Representative Western blot showing changes in BACE1 and APP following 3 bouts of low glucose (0.1 mM) on consecutive days (recurrent hypoglycemia, RH) followed by a 20-hour recovery period. (B, C) Densitometric analyses showing a significant increase in BACE1 (B, p < 0.05, n = 5) and decrease in APP (C, p < 0.01, n = 5) following the RH protocol in glucose-sensing neurons.
Figure 3.12: Chronic fatty acid treatment significantly increases BACE1 and APP protein levels in SH-SY5Y_{APPswc} cells.

(A) Representative Western blots showing BACE1 and APP protein expression in response to 24-hour treatment with a BSA loading control (Ctl) or palmitate (125 and 250 μM). (B, C) Densitometric analyses showing significant increases in BACE1 (B) and APP (C) in response to 24-hour treatment with 125 and 250 μM palmitate (p < 0.01, n = 5-6).
3.13B) and APP (Ctl; 1 ± 0.05 vs. 250 μM Palmitate; 1.35 ± 0.11, p < 0.05, n = 6, Fig. 3.13C) were observed. The addition of a second 4-hour palmitate exposure yielded significant increases in BACE1 in response to both concentrations (Ctl; 1 ± 0.05 vs. 125 μM Palmitate; 1.69 ± 0.04, p < 0.001 and 250 μM Palmitate; 1.84 ± 0.11, p < 0.01, n = 4, Fig. 3.13E) while APP protein levels were significantly increased by the two treatments with 125 μM palmitate (Ctl; 1 ± 0.0.05 vs. 125 μM palmitate; 1.28 ± 0.07, p < 0.05, n = 4, Fig. 3.13F). Taken together these data show that BACE1 and APP protein expression can be significantly enhanced by palmitate oversupply and also suggest that the changes in BACE1 may be induced more quickly, in response to acute exposure, with the APP changes developing more during the chronic treatment.

High fat diet (HFD) feeding has long been used as a paradigm for the induction of a number of metabolic disturbances such as obesity, hyperinsulinemia, hyperglycaemia and hyperlipidemia as well as diabetes and metabolic syndrome in experimental animals. Metabolic syndrome is in turn defined clinically by the presence of several CVD risk factors, including; abdominal obesity, low high-density lipoprotein (HDL), hypertriglyceridemia, hypertension and hyperglycemia. A number of these factors have previously been, both independently and in concert, shown to increase cognitive decline and AD risk. Indeed, longitudinal studies have demonstrated that the presence of metabolic syndrome enhances cognitive decline during ageing (Taylor et al., 2007), with the presence of obesity and diabetes also shown to increase dementia risk (Whitmer et al., 2005). Evidence also exists that in non-aged individuals, obesity results in increased levels of brain atrophy (Ward et al., 2005).
Figure 3.13: Acute fatty acid exposure stimulates BACE1 and APP protein expression in SH-SY5Y<sub>APP<sub>sw</sub></sub> cells.
(A) Representative Western blots showing BACE1 (A) and APP protein levels in response to a 4-hour treatment with palmitate (125 and 250 μM) followed by a 20-hour washout period. (B, C) Densitometric analyses showing a significant induction of BACE1 (B, p < 0.05) and APP (D, p < 0.05) protein levels following treatment with 250 μM palmitate (n = 6). (D) Western blots showing changes in BACE1 (E) and APP following 2, 4-hour palmitate treatments separated by a 24-hour washout period and with a 20-hour recovery period before lysis. (E, F) Densitometric analyses showing a significant increase in BACE1 (F, 125 μM; p < 0.001, 250 μM, p < 0.01) and APP (F, 125 μM, p < 0.05) following 2, 4-hour palmitate treatments (n = 4).
In experimental animal models, diet-induce obesity (DIO) has also been shown to result in cognitive impairment, through the actions of hypertriglyceridemia, increased glucocorticoids and resultant oxidative stress (Jurdak et al., 2008; Stranahan et al., 2008a; 2008b). With respect to AD, previous studies have also shown that high fat/sucrose diets induce increases in amyloid pathology in the brain of experimental animals (Levin-Allerhand et al., 2002; Cao et al., 2007). With this in mind, the hippocampus of C57bl/6 fed on a high fat diet for 20 weeks were analysed for changes in BACE1 and APP against normal chow (NC) fed animals. Following high fat feeding there was a significant increase in both BACE1 (NC; 1.00 ± 0.18 vs. HFD; 2.58 ± 0.32, p < 0.01, n = 5-7, Fig. 3.14B) and APP (NC; 1.00 ± 0.09 vs. HFD; 1.52 ± 0.19, p < 0.05, n = 5-7, Fig. 3.14C). A number of studies have implicated the JNK/c-Jun family of proteins as potential mediators of oxidative stress-induced changes in BACE1 (Tamagno et al., 2005; 2008). Following HFD feeding, a significant increase in the phosphorylation status of both the 54 (at threonine 183) (NC; 1.00 ± 0.16 vs. HFD; 1.57 ± 0.21, p < 0.05, n = 5-7, Fig. 3.15B) and 46 (tyrosine 185) (NC; 1.00 ± 0.2 vs. HFD; 1.69 ± 0.21, p < 0.05, n = 5-7, Fig. 3.15C) SAPK isoforms was observed.

Herein, a number of cell stress-inducing agents have been tested for their ability to induce changes in BACE1 and APP protein expression as well as BACE1 activity and subsequent change in APP processing. The results have shown significant effects of metabolic, oxidative and lipid stressors can all significantly modulate these outputs and in the case of HFD, the JNK/c-Jun signalling pathway may mediate these effects.
Figure 3.14: High fat diet (HFD) feeding in mice significantly enhances BACE1 and APP protein expression in the hippocampus

(A) Representative Western blots of hippocampal lysates showing BACE1 and APP in response to normal chow (NC) and HFD feeding. (B, C) Densitometric analyses showing a significant increase in protein expression of both BACE1 (B; p < 0.01, n = 5-7) and APP (C; p < 0.05, n = 5-7) following HFD feeding.
Figure 3.15: High fat diet (HFD) feeding of mice results in increased stress-activated protein kinase (SAPK) activation in the hippocampus.

(A) Representative Western blots showing the phosphorylated and total SAPK (54 and 46) protein levels in the hippocampus following HFD feeding. (B, C, D, E) Densitometric analyses showing a significant increase in the phospho- to total protein ratio in both SAPK 54 (B) and SAPK 46 (C) following HFD feeding (p < 0.05, n = 5-7) with no changes elicited in total SAPK54 or 46 protein levels (D, E).
3.3 Discussion

3.3.1 Acute Cellular Stress Fundamentally Enhances BACE1-mediated APP Cleavage via Different Mechanisms

Herein, it has been shown that a variety of challenges to cellular homeostasis, including: metabolic and protein synthesis inhibition, fatty acid excess and pro-oxidant compounds all act to fundamentally increase BACE1-dependent APP processing. These findings are in broad agreement with the consensus in the literature on BACE1, which has long been known to function as a stress-induced aspartyl protease. Interestingly, at least in terms of acute treatment with these agents, there do appear to be differences in how they mediate this altered APP cleavage. For instance, treatment with the protein synthesis inhibitor, anisomycin and the lipid second messenger resulted in increases in the activity of BACE1 (as observed via activity assay; Fig. 3.2 and 3.3). Whereas, acute (1 and 4 hours) with mitochondrial inhibitors (3NP/rotenone) and \( \text{H}_2\text{O}_2 \) resulted in more rapid and pronounced increases in BACE1 protein levels (Fig. 3.4), with less response observed via the activity assay (Fig. 3.6). It is interesting to note however that the sum effect of these changes were the same, with all treatments enhancing the cleavage of APP, yielding greater abundances of sAPP\( \beta \) being released into the media in response to all treatments (Figs. 3.2 and 3B and 3.5). Finally, treatment with all the agents induced a trend towards reduced APP expression (with anisomycin reaching significance during both 1- and 4-hour incubations (Figs. 3.7). This pronounced effect of anisomycin is to counter the stimulatory effect on APP protein expression observed previously (Guo et al., 2011). This previous study showed increases in APP protein levels following 1- and 72-hour
treatments with 7.5 nM anisomycin, with BACE1 expression also being increased at the latter time point. These observations suggest a dose dependent difference in the effect of anisomycin in SH-SY5Y. Hence, low doses (i.e. 7.5 nM) lead to a reduction in the DNA methylation status of the APP and BACE1 promoter, and enhancing transcription, as suggested by Guo and colleagues, while higher concentrations stimulate a rapid increase in BACE1 activity (with no changes in protein level observed) and a subsequent decrease in APP. It may however be difficult to draw similar conclusions in the current studies as the overexpression of APP in this work is no longer under the control of its endogenous promoter. At this higher (1 μM) concentration, anisomycin will exert protein synthesis inhibition (Zinck et al., 1995) and therefore the change observed may indicate a rapid turnover rate for APP, which is blocked during treatment, leading to the reduced protein expression.

3.3.2 Differential Mitochondrial Inhibition Induces Altered Temporal Induction of BACE1 and APP Protein Expression

To date, the mitochondrial inhibitors that have been used to investigate BACE1 changes typically act at the level of individual electron transport chain complexes (Veliquette et al., 2005; Xiong et al., 2008). The present study observed similar stimulatory effects on BACE1 protein and a smaller effect on BACE1 activity (Fig. 3.4 and 3.6) following acute treatment with mitochondrial complex inhibitors (3NP and rotenone). In an attempt to delineate if there may be different mechanisms underlying the effects on BACE1 and APP protein expression, time course experiments were carried out. It was hypothesised that there may also be different mechanisms driving altered BACE1/APP protein levels between direct inhibition of
the ETC or ATP synthase of the oxidative phosphorylation pathway of the mitochondria. With respect to BACE1, direct inhibition of complex I and III (via the combined actions of rotenone and antimycin A) elicited a more rapid peak increase in BACE1 (after 8 hours), with this dying away over the subsequent 2 time points (16 and 24 hours; Fig. 3.9). This is in contrast to oligomycin (which acts to inhibit ATP synthase and force the cells to utilise glycolysis for ATP generation – see appendix I). Chronic exposure of cells to oligomycin results in a slower development of BACE1 expression with the peak response not being elicited until the 24-hour time point (Fig. 3.10). This trend was also present with respect to changes in APP protein levels, which may be suggestive of a coupled mechanism to pair transcription of both enzyme and substrate. These potential mechanisms would however need to be investigated in cells expressing endogenous APP to better understand the transcriptional events. More investigation would be required but this may implicate differential mechanisms in the regulation of BACE1. For instance, inhibition of the ETC complexes is associated with an increased production of ROS, and such production has been shown previously to enhance BACE1 mRNA and protein levels in hypoxia and ischemia models (Rosenstock et al., 2004; Zhang et al., 2007; Guglielmotto et al., 2009; Moreno-Sanchez et al., 2013) while oligomycin may more closely mirror energy/ATP depletion as a mechanism of enhanced BACE1/APP protein expression.
3.3.3 Increased BACE1 Expression Following Cellular Stressors may Involve a Number of Mechanistic Alterations

The BACE1 protein expression and activity changes following acute exposure of SH-SY5Y\textsubscript{APP\textsubscript{swe}} to cells either mitochondrial inhibition (rotenone and 3NP) or \textsubscript{H}_2\textsubscript{O}_2 were also prolonged for up to 24 hours following a washout of the relevant drugs (Fig. 3.8). Over this increased time frame, there may be an additional role for changes in BACE1 transcription. Indeed, it has been shown previously that stimulation of PC12 cells with \textsubscript{H}_2\textsubscript{O}_2 results in significant increases in the activity of the BACE1 promoter (Tong \textit{et al.}, 2005). In contrast, following prolonged treatment with mitochondrial inhibitors, BACE1 mRNA levels have been shown to be decreased in a model of chronic 3NP treatment in mice (O’Connor \textit{et al.}, 2008). These findings suggest the possibility that BACE1 protein expression levels may be regulated via different mechanisms dependent upon the nature and duration of the stress stimulus. Indeed, O’Connor and colleagues postulate that the changes they observed in BACE1 protein expression, in response to glucose deprivation or chronic energy impairment (via 2-DG or 3NP treatment) induces a post-translational modification on BACE1 to increase its expression (O’Connor \textit{et al.}, 2008). Previous work by Puglielli and colleagues also demonstrated that BACE1 protein level and activity could be increased via a post-translational modification that acted to stabilise BACE1 and increase its half-life in response to ceramide (Puglielli \textit{et al.}, 2003). A subsequent study then showed that in CHO and neuroglioma cells, increased acetylation of lysine residues on BACE1 by the actions of acetyltransferases (ATase1 and ATase2) results in diminished BACE1 degradation (Ko and Puglielli 2009).
Recent work has also implicated palmitoylation as another potential post-translational mechanism that may alter BACE1 expression or activity. This is the addition of a palmitic acid group to cysteine residues and has been shown previously to play a role in membrane targeting of a variety of proteins, with the main function being an increase in protein hydrophobicity (for review see Charollais and Van der Goot 2009).

To date, four C-terminal cysteine residues of BACE1 have been shown to be palmitoylated (C472, C478, C482 and C485; Vetrivel et al., 2009). This study by Vetrivel and colleagues demonstrated that mutation of these sites resulted in impaired localization of BACE1 to lipid raft area of the plasma membrane in MEF and N2A cells (Vetrivel et al., 2009). It should however be noted that mutation of these sites did not induce any significant changes in BACE1 trafficking or in Aβ production, suggesting that lipid raft localisation is not required for BACE1 activity towards APP (Vetrivel et al., 2009). Work by Motoi and colleagues reached a similar conclusion, this time carrying out the experiments in SH-SY5Y neuroblastoma cell and primary neuronal cultures (Motoki et al., 2012).

While there does not appear to be a significant effect of removing the BACE1 C-terminal palmitoylation sites on trafficking of BACE1, there may still be an effect on protein turnover. As observed here, treatment with palmitate significantly elevates BACE1 and APP protein levels, with recurrent, acute treatments also inducing significant increases (Fig. 3.12 and 3.13). This increased presence of palmitic acid may in turn increase the level of palmitoylation of not only BACE1, but also APP. Recently, a study by Bhattacharyya and colleagues across CHO cells and primary neurons identified two palmitoylated cysteine residues on APP (C186 and C187; Bhattacharyya et al., 2013). This work showed that mutation of these sites resulted in
retention of APP within the ER and that palmitoylated APP proved to be a better substrate for BACE1 than α-secretase. This work also demonstrated that inhibition of palmitoylation (through use of acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitors) leads to decreased APP processing (Bhattacharyya et al., 2013). The reversible nature of protein palmitoylation is modulated by the action of palmitoyl acyl-transferase (PAT) enzymes, with a number of these belonging to the DHHC (so called as a result of their conserved Asp-His-His-Cys motifs) family of proteins. Using CHO and PC12 cells, Bhattacharyya et al demonstrated the dominant actions of DHHC-7 and 21 in the palmitoylation of APP (Bhattacharyya et al., 2013). Interestingly, DHHC-7 was among those observed to significantly enhance the palmitoylation of immature BACE1 in N2A cells (along with DHHC-3, 4, 15 and 20; Vetrivel et al., 2009). These data strongly suggest that any factors, such as palmitate excess, which may alter the palmitoylation status of BACE1 and/or APP, may in turn fundamentally alter subsequent processing, pushing it towards Aβ production.

The potential for these post-translational modifications to alter BACE1 and APP trafficking provide an intriguing line of enquiry to explain the enhanced Aβ production seen in AD progression. Indeed, recently it has been noted that mild oxidative/metabolic stress induction (through treatment of primary neuronal cultures with H2O2 or COS-7 cells with 2-DG or NaN3) resulted in a redistribution of BACE1 and APP (Domingues et al., 2007; Tan et al., 2013). These changes may come about as a result of altered rate of trafficking of these proteins, effects that could be monitored through the use of cell surface biotinylation or pulse chase studies. Combination of these techniques would allow for analysis of the prevalence of BACE1 and APP at the cell surface (with colocalisation of which underlies the
enhanced Aβ production from lipid raft domains) and also the turn over time of the proteins, which may underlie the enhanced expression following stress exposure.

### 3.3.4 Impact of Oxidant Treatment of SH-SY5Y APPsw Cells on BACE1 Protein Expression

The present studies also agree with the previous literature with regards to the impact of oxidant molecules, in this case H₂O₂, on BACE1 expression (Tamagno et al., 2008, Jo et al., 2010). Herein, acute (1- and 4-hour) treatments with H₂O₂ resulted in significant increases in BACE1 expression. These results match the work of Tamagno and colleague who demonstrated significant increases in BACE1 protein levels following a 6-hour treatment with H₂O₂ in SK-N-BE cells (Tamagno et al., 2008). Interestingly, these two previous studies demonstrated a fundamental requirement for PS1/PS2 in these observed effects of both H₂O₂ and HNE on BACE1. This stimulatory effect on BACE1 expression following H₂O₂ treatment was also observed in work by Mouton-Liger et al., who demonstrated acute stimulation of BACE1 protein expression in response to H₂O₂ in SH-SY5Y cells (Mouton-Liger et al., 2012). This work also demonstrated that the observed BACE1 induction came as a result of activation of the protein kinase R (PKR) – eukaryotic initiation factor (eIF) 2α pathway via PKR activating protein (PACT)-mediated phosphorylation. Indeed, oxidative stress-induced changes in BACE1 could be blocked via inhibition of either PACT (via shRNA) or PKR (through inhibition or siRNA) activation could prevent the subsequent phosphorylation of eIF2α phosphorylation (at serine 51) and depression of the 5’UTR of BACE1 (Mouton-Liger et al., 2013). This study is one of
a number that have noted the association between BACE1 activation and eIF2α signalling.

3.3.5 Control of BACE1 by eIF2α Kinases and their Role in AD pathogenesis

The process of mRNA translation is a rapid and effective regulator of protein abundance in mammalian cells (Schwanhausser et al., 2011). At times of cellular stress, a widespread down regulation of protein translation is mediated by activation of eIF2α (via phosphorylation at serine 51), which acts to conserve cellular resources and stimulate gene expression aimed to attenuate damage. During these periods, the selective activation of activating transcription factor (ATF) 4, a transcriptional activator of nutrient uptake, and redox status controlling genes (Harding et al., 2000). These findings place ATF4 as a nodal checkpoint for the integration of signals from multiple eIF2α kinases, with the eIF2α-ATF4 pathway being dubbed the integrated stress response (ISR; Harding et al., 2003).

Four upstream kinases have to date been shown to phosphorylate eIF2α in response to a host of different stress signals, three of which are expressed in the brain (Donnelly et al., 2013). General control non-derepressible kinase 2 (GCN2) is known to be activated in response to nutritional stress, the N-terminal of the previously mentioned PKR responds to the presence double stranded RNA during viral replication and PKR-like ER kinase (PERK), which responds to ER stress, as observed during glucose deprivation and high fat diet conditions (for review see Baird and Wek 2012). The fourth eIF2α-activating kinase is heme-regulated eIF2α kinase (HR1) that is activated by heme depletion in peripheral erythroid cells (Han et al., 2001). With
respect to the present findings, induction of eIF2α phosphorylation has been shown previously to result in enhanced BACE1 translation (O’Connor et al., 2008; Devi and Ohno 2010a; Mouton-Liger et al., 2012). Indeed, BACE1 is amongst a subset of protein whose translation is increased by eIF2α activation, on account of its long (greater than 200 nucleotide), GC-rich (above 70%) 5’UTR (see section; ILL-Raga et al., 2011). PERK-mediated phosphorylation of eIF2α was also noted in conditions of insulin-deficient diabetes and behavioural stress in young 5 x FAD animals (Devi and Ohno 2010b; 2012). This strongly suggests that changes in the ISR may underlie the enhanced BACE1 and APP expression observed following any of the present stress treatments.

This kinase-induced phosphorylation of eIF2α and resultant activation of ATF4 have both been shown to result in impaired LTP and performance in learning and memory tasks (Costa-Mattioli et al., 2007; Jiang et al., 2010). In the work by Costa-Mattioli and colleagues, point mutation of the eIF2α phosphorylation site (S51A) resulted in restoration of contextual and spatial task performance, mediated through attenuated ATF4 levels and improved CREB functioning (Costa-Matiolli et al., 2007). Allied to this, recent findings have observed increased levels of phosphorylated eIF2α and increased ATF4 expression in AD (Chang et al., 2002; O’Connor et al., 2008; Lewerenz et al., 2009). This work by Chang and colleagues also postulated that increased levels of phosphorylated eIF2α were required for AD-associated neurodegenration (Chang et al., 2002a). A number of subsequent studies have therefore looked at the relative contributions of the brain-expressed eIF2α kinases (GCN2, PERK and PKR) in AD progression (for review see Ohno 2014).
3.3.6 Potential Role of GCN2 in AD

GCN2 is activated in the presence of nutritional deficits, whereby amino acid depletion results in increased cytoplasmic levels of uncharged transfer RNA (tRNA), which binds to GCN2 and induces a conformational change and subsequent autophosphorylation (Padyana et al., 2005; Garriz et al., 2009). Activation of GCN2 has also been observed during glucose deprivation (O’Connor et al., 2008). Work by Ma and colleagues also implicated GCN2 as a major driver of synaptic and behavioural changes in the APP/PS1 AD mouse model (Ma et al., 2013). In this study, conditional, forebrain specific, knockout of GCN2 stimulated a trend toward enhanced LTP responses in comparison to slices from wild type animals and conferred resistance to Aβ-induced synaptic deficits (Ma et al., 2013). This removal of GCN2 also completely reversed the aberrant eIF2α phosphorylation and increased ATF4 protein expression in the APP/PS1 brain and alleviated the impaired performance in Y-maze and Morris water maze (MWM) learning and memory paradigms (Ma et al., 2013). Devi and Ohno reported an interesting and contradictory observation, that GCN2 removal worsened amyloid burden, further enhanced ATF4 expression and CREB dysfunction in the 5 x FAD animal model of AD and that this came as a result of PERK overactivation following GCN knock out (Devi and Ohno 2013). The authors propose that GCN2 may therefore act as a negative regulator of PERK-mediated eIF2α.
3.3.7 PERK Activity in AD progression

The aforementioned work of Ma and colleagues also demonstrated that the dephosphorylation of eIF2α that occurs during the induction of LTP (Trinh et al., 2012) can be blocked by Aβ and that this effect can be alleviated via the conditional knock out of PERK (Ma et al., 2013). It was also noted that impaired performance in Y-maze and MWM paradigms in APP/PS1 mice were reversed by PERK knock out, as with GCN2 removal (Ma et al., 2013). A subsequent study by Devi and Ohno also observed this causative role for PERK-mediated eIF2α phosphorylation (Devi and Ohno 2014). Here, reduced levels of PERK (brought about by heterozygous expression) reversed the increased phosphorylation of PERK and eIF2α as well as the memory deficits and cholinergic neuron degeneration of the 5 x FAD model (Devi and Ohno 2014). This intervention also prevented BACE1 induction and resultant Aβ pathology. Allied to this, ATF4-driven CREB dysfunction was also averted (Devi and Ohno 2014).

3.3.8 Linking Oxidative Stress and PKR Signalling as Mediators of AD Dysfunction

The final eIF2α kinase that has been looked at with regards a causative role in AD progression is PKR. Phosphorylated PKR has been noted in and around the site of senile plaque formations in both AD and AD animal models (Chang et al., 2002b; Peel et al., 2003) and may induce abnormal tau phosphorylation (Bose et al., 2011). A potent activator of PKR is the herpes simplex virus (HSV1), which has also been noted to induce the herpes simplex encephalitis brain disorder that presents in the
same areas as AD (i.e. frontal and temporal cortices; Ball 1982). HSV1 has also been noted in the brains of elderly individuals (Jamieson et al., 1991) and AD cases (Wozniak et al., 2005). Work by ILL-Raga and colleagues showed that HSV1 infected SH-SY5Y cells, or those treated acutely (3 and 6 hours) with the polyinosonic:polycytidylic acid (Poly I:C) virus analog resulted in phosphorylation of PKR (at threonine 446), which in turn stimulated eIF2α phosphorylation, BACE1 protein expression and a subsequent increase in Aβ40 production (ILL-Raga et al., 2011). Inhibitor studies revealed that this stimulation of BACE1 translation was dependent upon activation of PKR and eIF2α, which together led to de-repression of the 5’UTR of BACE1. Finally, this work showed that phosphorylated PKR colocalised with BACE1 in AD brain neurons (ILL-Raga et al., 2011).

Subsequent work by Mouton-Liger and colleagues showed that oxidative stress (induced by treatment with H2O2) induced a rapid (peak at 2 hours) increase in BACE1 protein expression. This increase in BACE1 protein and eIF2α phosphorylation was also shown to be independent of PERK and knock down of PKR blocked either change (Mouton-Liger et al., 2012). PKR activation by both double stranded RNA and oxidative stress can also activate JNK (SAPK) signalling (Mouton-Liger et al., 2012). Indeed, activation of this pathway has previously been shown to increase BACE1 mRNA levels under conditions of oxidative stress (Tamagno et al., 2008). This in agreement with the present findings that HFD feeding leads to increased SAPK (JNK) activation in the hippocampus, and resultant increases in both BACE1 and APP (Fig. 3.14 and 3.15).
Taken together, the data presented herein and those of the previous studies described above strongly implicate the ISR pathway as a key modulator of BACE1 and APP protein expression during times of both acute and prolonged cellular stress (See Fig. 3.16). The ISR also provides a central pathway whereby changes in a number of stress pathways may be translated into robust increases in both AD pathological hallmarks. With the knowledge that a wide range of stress signals (from nutrient deprivation to viral infection) can mediate enhanced BACE1-mediated cleavage of APP, it remained to be seen what effect chronically elevated BACE1 expression/activity may have on cellular function.
Figure 3.16: Schematic diagram showing the possible integration of stress signals at the level of eIF2α in the brain, via the actions of 3 upstream kinases (GCN2, PKR and PERK) and the resultant modulation of protein translation. With relevance to AD, these signals activate BACE1, resulting in enhanced Aβ production and down regulate CREB transcriptional activity (via ATF4) thereby impairing cognitive functioning.
Chapter 4

Effect of APP Processing Manipulation on Glucose Uptake in SH-SY5Y Cells
4.1 Introduction

4.1.1 Glucose Transport to the Brain

The human brain has an absolute requirement for glucose as a fuel for various functions, in particular, maintenance of ionic gradients and synaptic transmission events. Glucose delivery to the brain is mediated via the actions of 2 predominant glucose transporter (GLUT) isoforms (GLUT1 and 3). As a consequence of the tight junctions formed between the endothelial cells of the BBB, glucose requires to be transported across the luminal and abluminal membranes. This movement is mediated via the 55kDa isoform of GLUT1 (Gerhart et al., 1989). Once across the endothelial cells of the BBB, glucose uptake into the end feet of astrocytes occurs through the 45kDa GLUT1 isoform. Under normal conditions, glucose is taken up by neurons via GLUT3, with widespread, neuron-specific patterns of mRNA expression having been observed in the brain (Bondy et al., 1992; Nagamatsu et al., 1992). Previously, reduced glucose uptake and use have been noted in the brains of people with AD (Jagust et al., 1991; Minoshima et al., 1995). This reduced uptake has also been correlated with diminished GLUT3 expression levels (Simpson et al., 1994a; 1994b).

There may also be a role for GLUT4-mediated glucose uptake into neurons, with low level expression having been observed (Brant et al., 1993). It has also been shown that translocation of GLUT4 in central neurons and neuronal cell lines can be stimulated by insulin and leptin in a PI3K-dependent mechanism as in peripheral cell types (Apelt et al., 1999; Benomar et al., 2006; Grillo et al., 2009). With particular relevance to AD, GLUT4 expression has been noted in discrete neuronal populations...
of the hippocampus, a site of major neuronal loss in the disease (Kar et al., 1993; Dore et al., 1997).

4.1.2 Insulin-Stimulated Glucose Uptake

The insulin receptor consists of 2 extracellular α-subunits that facilitate insulin binding and the transmembrane spanning β-subunits (Jones and Clemmons 1995). Upon insulin binding, the tyrosine kinase activity of the β-subunit leads to the phosphorylation and subsequent activation of the insulin receptor substrate proteins (IRS1 and 2) and the resultant activation of phosphatidylinositol 3-kinase (PI-3K). This leads to the stimulus-dependent production of phosphatidylinositol (3,4,5)-triphosphate (PIP3), which then binds to the pleckstrin homology (PH) domain of protein kinase B (PKB; also known as Akt) resulting in a conformational change and its translocation to the plasma membrane where it undergoes phosphorylation. This can occur at two distinct sites, at threonine 308 located within the catalytic domain via the action of 3-phosphatidylinositol dependent kinase 1 (PDK1) and at serine 473 by PDK2. Recently, work has shown that mammalian target of rapamycin complex 2 (mTORC2) may mediate phosphorylation at this site in HEK and HELA cells (Glidden et al., 2012). There is also evidence that this action can in turn regulate GSK3β in mesenchymal stem cells (Case et al., 2011).

This glucose is then phosphorylated to glucose-6-phosphate (Glc-6-P) by the action of hexokinase (HK). Phosphorylation by HK serves to trap glucose in the cell, which along with the rate of efflux and metabolism maintains a gradient for facilitative transport. The neuronal HK1 isoforms also displays a low Km for glucose and can therefore work near maximally at intracellular glucose levels of 1mmol/L and can
also be inhibited by the presence of Glucose-6-phosphate (G-6-P) through a feedback mechanism (Wilson 2003). Flow of this glucose through the metabolic glycolytic and oxidative phosphorylation pathways is coupled to the generation of ATP. With the exception of these processes, the majority of cellular functions involve the consumption of ATP, which is hydrolysed to form adenosine diphosphate (ADP) and phosphate. Increases in ADP are in turn coupled to the activity of adenylate kinase, which catalyses a reversible conversion of ADP to form ATP and adenosine monophosphate (AMP). This places ATP, ADP and AMP levels at the centre of cellular nutrient production/use and makes the monitoring of these metabolites of particular importance to cells with high energy demand, such as neurons. A principal kinase in cellular fuel sensing is AMPK, discovered in 1988 and shown previously to act as a fuel gauge in a number of cell and tissue types (Sim and Hardie, 1988; for review see Hardie, 2007).

### 4.1.3 Role of AMPK in Glucose Uptake

AMPK comprises a heterotrimeric complex made up of a catalytic α- and regulatory β- and γ-subunits, which is rapidly activated (by up to 10 times) by AMP (Suter et al., 2006). However under the physiological conditions, it is unclear as to the role for this AMP-mediated allosteric modulation due to competition for the γ-subunit binding site by ATP and ADP (Oakhill et al., 2012). A second point activation mechanism for AMPK is the phosphorylation status of threonine 172 contained within the activation loop of the catalytic subunit (Suter et al., 2006). Under resting conditions, phosphorylation of the site is under the control of upstream kinases, the LKB1-STRAD-MO25 complex and Ca²⁺/calmodulin-activated protein kinases (particularly
CAMKKβ; Hawley et al., 2003; Shaw et al., 2004; Hawley et al., 2005). Binding of the γ-subunit by AMP then modulates this basal activation state, promoting phosphorylation and inhibiting dephosphorylation. The activity of AMPK in turn phosphorylates a number of downstream substrates including: acetyl-CoA carboxykinase (ACC) and Akt substrate of 160kDa (AS160, also known as TBC1D4).

In muscle, AMPK-mediated GLUT4 translocation is believed to augment glucose uptake in response to contraction (See Fig. 4.1). Like insulin-stimulated GLUT4 translocation discussed above, this mechanism also involves the fusion of GLUT4 storage vesicles to the plasma membrane, mediated by a RAB family of G-proteins (Sakamoto and Holman 2008). Under resting conditions, these RABs are maintained in their inactive state via association with RAB-GAPs such as; AS160 (alternatively, TBC1 domain family member 1 TBC1D1) and TBC1D4. AS160 has been shown previously to dissociate from these vesicles following phosphorylation by PKB in muscle and adipocytes in response to insulin (Geraghty et al., 2007, Treebak et al., 2009). Finally, AMPK was observed to phosphorylate AS160 in contracting muscles (Chen et al 2008; Pehmoller et al 2009).

Figure 4.1 Schematic diagram showing the convergent roles of insulin and muscle contraction on GLUT4 translocation (http://www.phoenixpeptide.com). The “?” is now thought to be AS160 (Chen et al., 2008; Pehmoller et al., 2009).
4.1.4 Insulin Signalling, Glucose Uptake and BACE1

Recently, studies have linked type 2 diabetes with an increased risk of AD, with people who develop AD also shown to be more prone to impaired glucose metabolism, hyperinsulinemia and insulin resistance (Janson et al., 2004; Strachan et al., 2011). Previous work from our lab has demonstrated that BACE1 may play a fundamental role in glucose uptake with BACE1 knockout mice displaying enhanced glucose disposal and insulin sensitivity (Meakin et al., 2012). These data suggested that BACE1 might therefore play a central role in AD and diabetes. More recently, through the use of rodent muscle myotube cell lines, enhanced glucose uptake and GLUT4 translocation were observed following BACE1 inhibition (Hamilton et al., 2014). It was also noted that these effects occurred independently of, but additively to, the action of insulin by stimulating GLUT4 translocation. Finally, it was seen that these changes were driven by a PI3K-dependent mechanism, with both being effectively attenuated by pre-exposure to the PI3K inhibitor, wortmanin. Mechanistically, it was hypothesized that these changes were likely due to the relative cleavage of the BACE1 substrate, APP. Indeed, it was subsequently shown that the action of the non-amyloidogenic cleavage pathway might play a key role in the observed effects. This was shown by a reduction in glucose uptake following $\alpha$-secretase inhibition, that increasing sAPP$\alpha$ levels through overexpression of APP stimulated glucose uptake and finally that direct application of sAPP$\alpha$ increased basal and insulin-stimulated glucose uptake and PI3K signalling (effects not replicated by the application of sAPP$\beta$). These findings implicate BACE1 as a novel regulator of glucose uptake into muscle and in the whole animal.
In an attempt to further these observations and see if APP processing may also have a role in central glucose uptake and insulin signalling, the human, neuronal SH-SY5Y cell line was examined. APP processing was manipulated in a number of ways, with stable overexpression of BACE1 (SH-SY5Y_{B1}), a BACE1 mutant devoid of secretase activity (SH-SY5Y_{mB1}), wild type APP (SH-SY5Y_{APPwt}) and APP harbouring the familial AD Swedish mutation (SH-SY5Y_{APPswe}) utilised. Cleavage of APP was also altered through the use of secretase inhibitors and media exchange experiments were performed to look at the effect of secreted factors from the various cell groups. It was hypothesised that, as had been observed previously, shifting APP metabolism down the amyloidogenic pathway would act to impair glucose uptake.
4.2 Results

4.2.1 2-Deoxyglucose Uptake Profile of the SH-SY5Y Cell Line

2-deoxyglucose (2-DG) is a non-metabolisable glucose analogue used commonly in ex vivo and cell-based assays to determine the rate of glucose uptake (Sylow et al., 2013; Tunduguru et al., 2014). Its structure is nearly identical to glucose with a switch of the 2-hydroxyl group to hydrogen, precluding its metabolism by glycolysis. 2-DG is taken up via GLUTs and undergoes phosphorylation by HK to form 2-DG-6-phosphate, retaining the 2-DG in the cell. Use of a tritiated (³H) 2-DG molecule means cells can be lysed upon completion of the assay and the uptake quantified via scintillation counting.

Initially, the 2-DG uptake profile for the SH-SY5Y cell line was conducted and showed that the rate of 2-DG uptake is linear at room temperature for up to 15 minutes (Fig. 4.2A). The 2-DG uptake assay works via the principle of bulk flow, meaning that the uptake of the labelled ³H-2-DG can be driven by increased concentrations of unlabelled 2-DG. This theory was displayed with the rate of ³H-2-DG uptake increasing and displaying Michaelis-Menton kinetics with respect to increasing concentrations of unlabelled 2-DG in the assay buffer (Fig. 4.2B). Finally it was observed that the uptake rate decreased following a 2-hour serum starvation (SS) period (Ctl; 3.20 ± 0.18 pmol/min/mg vs. SS; 2.15 ± 0.12 pmol/min/mg, p < 0.01, n = 4, Fig. 4.2C) and could be significantly induced by either the re-addition of serum (SS+S) or supraphysiological insulin (SS+I; 100 nM) (SS+S; 3.26 ± 0.18
Figure 4.2: $^3$H-2-Deoxyglucose (2-DG) uptake profile in SH-SY5Y cells.

(A) Linear increase in $^3$H-2-DG uptake with increasing incubation time up to 15 minutes. (B) $^3$H-2DG uptake rate displays Michaelis-Menten kinetics with respect to increasing concentrations of unlabelled 2-DG. (C) $^3$H-2DG is significantly reduced following a 2-hour serum starve (SS; $p < 0.01$, $n = 4$) and subsequently increased by either the re-addition of serum (SS+S; $p < 0.001$, $n = 4$) or 100 nM insulin (SS+I; $p < 0.01$, $n = 4$) 30 minutes prior to uptake measurement.
pmol/min/mg, p < 0.001 and SS+I; 3.24 ± 0.09 pmol/min/mg, p < 0.01, n = 4, Fig. 4.2C).

4.2.2 BACE1 Overexpression Significantly Reduces Basal and Maximal 2-DG Uptake

With the knowledge that glucose uptake and use are decreased in the AD brain and that BACE1 protein and activity levels have previously been shown to be increased, this state was mimicked via the stable overexpression of BACE1 in SH-SY5Y cells (Fig. 4.3A). BACE1 overexpression (of around 4 fold; EV; 1.00 ± 0.09 vs. BACE1; 3.97 ± 0.31, p < 0.001, n = 6-7, Fig. 4.3B) resulted in the expected shift in APP cleavage fragments released into the culture media, with a significant decrease in sAPPα (EV; 1.00 ± 0.03 vs. BACE1; 0.19 ± 0.02, p < 0.001, n = 4, Fig. 4.3D) and a significant increase in sAPPβ (EV; 1.00 ± 0.1 vs. BACE1; 4.41 ± 0.32, p < 0.01, Fig. 4.3E). These changes were accompanied by no significant change in the full-length APP protein levels in the cell (Fig. 4.3C). Following BACE1 overexpression, the rate of 3H-2-DG uptake was again assessed. BACE1 overexpressing cells (SH-SY5Y_{B1}) displayed a significantly reduced basal glucose uptake relative to the empty vector (SH-SY5Y_{EV}) control cells (EV; 5.83 ± 0.35 pmol/min/mg vs. BACE1; 3.83 ± 0.20 pmol/min/mg, p < 0.001, n = 8, Fig. 4.4A). Using increasing concentrations of unlabelled 2-DG in the assay buffer, it was also observed that SH-SY5Y_{B1} cells showed a significantly reduced maximal 3H-2-DG uptake rate (Fig. 4.4B).
Figure 4.3: Stable BACE1 overexpression significantly alters APP processing in SH-SY5Y cells.

(A) Representative Western blots showing stable overexpression of BACE1 and the resultant changes in APP and sAPPα, and β secreted into the culture media. BACE1 overexpression resulted in increased BACE1 protein levels (B, p < 0.001, n = 6-7), unchanged APP expression (C, n = 4) and a subsequent shift in APP processing with a significant reduction in sAPPα (D, p < 0.001, n = 4) and increase in sAPPβ (E, p < 0.01, n = 4) abundance revealed by densitometric analysis.
Figure 4.4: BACE1 overexpression significantly impairs basal and maximal $^3$H-2-DG uptake in SH-SY5Y cells.

(A) $^3$H-2-DG uptake rate showing a significant reduction in basal 2-DG glucose uptake following BACE1 overexpression ($p < 0.001$, $n = 8$). (B) Vmax curve showing significant reduction in maximal 2-DG uptake following BACE1 overexpression ($p < 0.01$ and $p < 0.05$, $n = 6 - 12$).
4.2.3 Reduced Glucose Uptake Following BACE1 Overexpression is Dependent Upon the Secretase Activity of BACE1

These results were further investigated by looking at whether the altered glucose uptake was reliant upon the secretase activity of BACE1. This was done in two ways, first, through the use of BACE1 inhibitor (Bi, Merck-3) that has been shown previously to decrease BACE1 activity in vitro and in vivo (Meakin et al., 2012; Hamilton et al., 2014). Following an overnight (20-hour) incubation with this BACE1 inhibitor, SH-SY5YB1 cells displayed a significant increase in 2-DG uptake rate (BACE1; 1.89 ± 0.04 pmol/min/mg vs. BACE1 + 1 µM Bi; 2.72 ± 0.23 pmol/min/mg, p < 0.05, and BACE1 + 10 µM Bi; 2.92 ± 0.26 pmol/min/mg, p < 0.01, n = 5, Fig. 4.5) with these rates no longer significantly different to SH-SY5YEV cells (EV; 3.2 ± 0.18 pmol/min/mg, n = 5, Fig. 4.5). A second method used to investigate the effect of BACE1 secretase activity removal was through the overexpression of a secretase-dead BACE1 mutant (mBACE1), which has been shown previously to be devoid of activity towards APP (Mowrer and Wolfe 2008). SH-SY5Y cells were then transfected with either an EV control or BACE1 containing cDNA vector as before or with one containing the mBACE1. Overexpression of both BACE1 constructs resulted in a significant increase in BACE1 protein level (EV; 1.00 ± 0.09 vs. BACE1; 3.97 ± 0.31 vs. mBACE1; 3.37 ± 0.31 Fig. 4.6B). Again, a significant increase in the relative abundance of sAPPβ was observed following BACE1 overexpression, with this shift in APP metabolism not observed in the mBACE1 overexpressing cells (SH-SY5YmB1; EV; 1.00 ± 0.05 vs. BACE1; 3.3 ± 0.34 vs. mBACE1; 0.62 ± 0.02, n = 6-10, Fig. 4.6D) with no changes observed in APP expression (Fig. 4.6C). Following these transfections, as previously, a significant
Figure 4.5: BACE1-mediated impairment in $^3$H-2-DG uptake can be attenuated by BACE1 secretase activity inhibition in SH-SY5Y cells.

Significant reduction in $^3$H-2-DG uptake rate following BACE1 overexpression ($p < 0.01$, $n = 5$) gradually attenuated by overnight treatment with a BACE1 inhibitor (1 µM; $p < 0.05$, $n = 5$ and 10 µM; $p < 0.01$ vs. BACE1, $n = 5$).
Figure 4.6: Stable overexpression of BACE1 and mBACE1 significantly alters APP processing in SH-SY5Y cells.
(A) Representative Western blots showing the stable overexpression of wild type and a secretase dead mutant (mBACE1) form of BACE1 and the resultant changes in APP and the initial BACE1-dependent cleavage product, sAPPβ. Analysis of the cell lysates and culture media revealed significant increases in BACE1 protein levels in both transfection groups (B). This resulted in a significant increase in sAPPβ in the BACE1 group (D; p < 0.001, n = 6-10) and a significant decrease in the mBACE1 group (D; p < 0.001, n = 6), with no observed change in total APP levels (n = 3) relative to EV cells (C).
reduction in $^3$H-2-DG uptake rate was observed in the SH-SY5Y$_{B1}$ but not in the SH-SY5Y$_{mB1}$ cells (EV; 8.12 ± 0.62 pmol/min/mg vs. BACE1; 5.43 ± 0.36 pmol/min/mg vs. mBACE1; 7.90 ± 0.57 pmol/min/mg, EV to BACE1 and BACE1 to mBACE1 p < 0.01, n = 5, Fig. 4.7).

This BACE1-dependent effect on glucose uptake was finally investigated using SH-SY5Y cells stably overexpressing either a wild type APP (APPwt) construct or one harbouring the familial AD Swedish (K670N/M671L) double mutation (APPswe). This mutation acts to enhance BACE1 binding to APP, thus improving it as a substrate. Overexpression of either APP construct (SH-SY5Y$_{APPwt}$ and SH-SY5Y$_{APPswe}$) resulted in a striking increase in the expression of full length APP (Fig. 4.8A), however, importantly, for the purposes of comparison, the degree of APP overexpression was even across transfection groups (Fig. 4.8B). Importantly, only the relevant sAPPβ was picked using antibodies specifically targeting either the wild type or mutated form and also the relative cleavage of APP in the expected manner by the Swedish double mutation (Fig. 4.8A). Following APP overexpression, changes in the rate of $^3$H-2-DG uptake were observed, with SH-SY5Y$_{APPwt}$ showing a significant increase and SH-SY5Y$_{APPswe}$ cells a trend toward a decrease in 2-DG uptake relative to SH-SY5Y$_{EV}$ cells (EV; 3.31 ± 0.43 pmol/min/mg vs. APP$_{wt}$; 5.52 ± 0.86 pmol/min/mg, p < 0.05, and APPswe; 1.65 ± 0.13 pmol/min/mg, n = 8, Fig. 4.9). Importantly when the groups are compared on the background of both having APP overexpressed, a highly significant decrease is observed between the SH-SY5Y$_{APPwt}$
Figure 4.7: BACE1-mediated impairment in $^3$H-2-DG uptake dependent upon BACE1 secretase activity in SH-SY5Y cells. Significant reduction in $^3$H-2-DG uptake rate following BACE1 overexpression ($p < 0.01$, $n = 5$) absent following overexpression of a BACE1 mutant devoid of secretase activity (mBACE1; $n = 5$).
Figure 4.8: Overexpression of wild type and Swedish mutation harbouring APP resulted in increased, but altered APP cleavage product generation in SH-SY5Y cells. (A) Representative Western blots showing the overexpression of APPwt and APPswe in SH-SY5Y cells and the resultant changes in sAPPα and sAPPβ release into the culture media. (B) Densitometric analysis showing the relative degree of APP overexpression achieved in the APPwt cells when compared to the APPswe cells.
Figure 4.9: Overexpression of wild type APP (APPwt) and APP harbouring the Swedish mutation (APPswe) significantly alters 2-DG uptake in SH-SY5Y cells. APPwt cells show a significant increase in "H-2-DG uptake rate compared to EV (p < 0.05, n = 8), while APPswe cells display a trend towards a decrease compared to EV cells and a significant reduction when compared to APPwt cells (p < 0.001, n = 8).
and the SH-SY5Y_{APP^{sw}} groups, suggesting a specific effect of the altered BACE1 activity observed between these groups (p < 0.001, n = 8, Fig. 4.9).

### 4.2.4 Inhibition of APP Secretase Proteins Significantly Alters 2-DG uptake

Having observed this BACE1-dependent shift in glucose uptake, manipulation of APP cleavage was next investigated for a potential role in altering 2-DG uptake. This was done using a range of inhibitors for different enzymes involved in APP cleavage, including: the Merck-3 BACE1 inhibitor mentioned earlier, TAPI-1 which is a general metalloprotease inhibitor shown to inhibit the α-secretase family of enzymes and DAPT, a γ-secretase inhibitor. An overnight (20-hour) treatment of naive SH-SY5Y cells (SH-SY5Y_{wt}) yielded significant alterations to the basal $^3$H-2-DG uptake rate with BACE1 inhibition (at 0.1 µM) eliciting a significant increase in 2-DG uptake (Ctl; 1.00 ± 0.05 vs. 0.1 µM Bi; 1.23 ± 0.05, p < 0.05, n = 8, Fig. 4.10A). Decreased 2-DG uptake was observed across a titration of α-secretase inhibitor, TAPI-1 (Ctl; 1.00 ± 0.07 vs. 0.1 µM TAPI-1; 0.89 ± 0.1, 1 µM TAPI1; 0.75 ± 0.08, p < 0.05 and 10 µM TAPI-1; 0.65 ± 0.05, p < 0.001, n = 10, Fig. 4.10B). Finally, no significant changes were observed following γ-secretase inhibition by DAPT (n = 4, Fig. 4.10C).

### 4.2.5 Altered 2-DG uptake involves an extracellular factor

In an attempt to better understand how manipulation of APP cleavage impinges upon glucose uptake, media exchange experiments were performed. For these, cell monolayers of the requisite cell type were plated into 12-well cultures plates and left
Figure 4.10: Inhibition of APP-cleaving enzymes impacts 2-DG uptake in SH-SY5Y cells.

(A) $^3$H-2-DG uptake rate following an overnight treatment with the BACEi Merck 3, with a significant increase in 2-DG uptake observed in the 0.1μM group (p < 0.05, n = 8). (B) $^3$H-2-DG uptake rate following an overnight treatment with the metalloprotease inhibitor TAPI-1, with a significant reduction observed following treatment with 1 and 10 μM (1 μM; p < 0.05 and 10 μM, p < 0.001, n = 10). (C) No significant changes observed in $^3$H-2-DG uptake rate following overnight treatment with the γ-secretase inhibitor DAPT (n = 4).
for 24 hours. 3 hours prior to $^3$H-2-DG uptake measurement, cells were incubated with conditioned cell culture media from separate flasks of cells. This allowed for treatment of 4 groups in each of the 2 experiments: SH-SY5Y$_{EV}$ and SH-SY5Y$_{B1}$ cells treated with either SH-SY5Y$_{EV}$ or SH-SY5Y$_{B1}$ conditioned media (giving EV/EV, EV/BACE1, BACE1/EV and BACE1/BACE1) and for the second experiment, SH-SY5Y$_{EV}$ and SH-SY5Y$_{APPwt}$ cells treated with SH-SY5Y$_{EV}$ or SH-SY5Y$_{APPwt}$ media (giving the following groups: EV/EV, EV/APPwt, APPwt/EV and APPwt/APPwt).

In the first experiment, as expected, SH-SY5Y$_{B1}$ cells display a significantly reduced 2-DG uptake rate when treated with SH-SY5Y$_{B1}$ conditioned media (BACE1/BACE1) relative to SH-SY5Y$_{EV}$ cells treated with SH-SY5Y$_{EV}$ media (EV/EV; EV/EV; 6.69 ± 0.22 pmol/min/mg vs. BACE1/BACE1; 4.15 ± 0.15 pmol/min/mg, p < 0.001, n = 4, Fig. 4.11A). There was however, also a significant effect of exchanging media across cell types, with SH-SY5Y$_{EV}$ cells treated with SH-SY5Y$_{B1}$ media (EV/BACE1) showing a significantly reduced 2-DG uptake rate relative to the EV/EV group (EV/EV; 6.69 ± 0.22 pmol/min/mg vs. EV/BACE1; 5.06 ± 0.27, p < 0.001, n = 4, Fig. 4.11A). Finally, there was a stimulatory effect elicited by the SH-SY5Y$_{EV}$ media, causing a significant increase in the 2-DG uptake rate of the SH-SY5Y$_{B1}$ cells relative to the SH-SY5Y$_{B1}$ media (BACE1/BACE1) treated group (BACE1/BACE1; 4.15 ± 0.15 pmol/min/mg vs. BACE1/EV; 5.75 ± 0.19 pmol/min/mg, p < 0.001, n = 4, Fig. 4.11A).

The same experimental protocol was then used to compare the EV and APPwt overexpressing cells. The control cells (those treated with their corresponding media) showed the same increase as previously shown (Fig. 4.9) in that SH-SY5Y$_{APPwt}$ cells treated with SH-SY5Y$_{APPwt}$ conditioned media (APPwt/APPwt) displayed
Figure 4.11: BACE1-mediated reduction in 2-DG uptake in SH-SY5Y cells involves an extracellular factor.

$^{3}$H-2-DG uptake rate measured following a 3 hour exposure to conditioned media across cell types. EV vs. BACE1 (A) and EV vs. APPwt (B) overexpressing cells treated with the corresponding media show a significant reduction and increase in $^{3}$H-2-DG uptake rate respectively (EV/EV vs. BACE1/EV/BACE1, p < 0.001 and EV/EV vs. APPwt/EV/APPwt, p < 0.01, n = 4). Significant differences were also observed following exchange of media across cell types (EV/BACE1, EV cells treated with BACE1 media and BACE1/EV, BACE1 cells treated with EV media; p < 0.001, n = 4 and EV/APPwt; EV cells treated with APPwt media and APPwt/EV; APPwt cells treated with EV media, p < 0.05, n = 4).
significantly enhanced 2-DG uptake relative to the SH-SY5Y\textsubscript{EV} cell and media (EV/EV) group (EV/EV; 4.19 ± 0.39 pmol/min/mg vs. APPwt/APPwt; 7.58 ± 0.5 pmol/min/mg, p < 0.01, n = 4, Fig. 4.11B). This uptake rate was also significantly higher than the SH-SY5Y\textsubscript{APP\textsubscript{wt}} cells treated with SH-SY5Y\textsubscript{EV} media (APP/EV) group (APPwt/APPwt; 7.58 ± 0.50 pmol/min/mg vs. APPwt/EV; 5.12 ± 0.47 pmol/min/mg, p < 0.05, n = 4, Fig.4.11B). Finally, there was a stimulatory effect observed when SH-SY5Y\textsubscript{APP\textsubscript{wt}} media was applied to SH-SY5Y\textsubscript{EV} cells (EV/EV; 4.19 ± 0.39 pmol/min/mg vs. EV/APPwt; 6.61 ± 0.53 pmol/min/mg, p < 0.05, n = 4, Fig. 4.11B). Taken together, these data suggests that the increased and decreased \textsuperscript{3}H-2-DG uptake rate observed following APPwt and BACE1 overexpression respectively is mediated by an extracellular factor present in conditioned media from each cell type.

Work by Mark Mattson has shown a stimulatory effect of sAPP\textsubscript{α} on glucose and glutamate transport in synaptosomes (Mattson \textit{et al.}, 1999). Following this experimental design, we too observed a significant increase in the rate of 2-DG uptake following incubation of SH-SY5Y\textsubscript{wt} cells with sAPP\textsubscript{α} (Ctl; 7.45 ± 0.85 pmol/min/mg vs. 5 nM sAPP\textsubscript{α}; 10.39 ± 0.84 pmol/min/mg, p < 0.05, n = 4, Fig. 4.12A). Hence modulation sAPP\textsubscript{α} i.e. depletion via overexpression of either BACE1 or APP\textsubscript{swe} (when compared to SH-SY5Y\textsubscript{APP\textsubscript{wt}} cells – Fig. 4.3A, D and 4.8A, C) and increased sAPP\textsubscript{α}, following overexpression of APP\textsubscript{wt} (Fig. 4.8A, C), appears to be a major driver of glucose uptake in SH-SY5Y cells.

Having previously observed PI3K-dependent changes in GLUT4 translocation and 2-DG uptake into muscle (Hamilton \textit{et al.}, 2014), the effect of sAPP\textsubscript{α} on this pathway was then examined. Following incubation with sAPP\textsubscript{α}, a significant increase in
Figure 4.12: sAPPα treatment significantly increases glucose uptake and protein kinase B (PKB) phosphorylation in SH-SY5Y cells.

(A) Wild type SH-SY5Y cells showed a significant increase in 3H-2-DG uptake rate following a 3 hour treatment with sAPPα (p < 0.05, n = 4). (B) Representative western blot of total and phosphorylated (threonine 308) PKB. (C) Densitometric analysis showing a significant increase in phosphorylation at threonine 308 of PKB following treatment with sAPPα (p < 0.05, n = 4)
phosphorylation of threonine 308 on PKB was observed (Ctl; 1.00 ± 0.05 vs. 3 nM sAPPα; 1.47 ± 0.05, p < 0.05, n = 4, Fig. 4.12B, C) and a trend towards an increase observed at a higher (5 nM) sAPPα concentration (Fig. 4.12B, C).

4.2.6 Manipulation of APP Metabolism Blunts Insulin-Stimulated 2-DG Uptake

Having previously observed that manipulation of BACE1 activity can significantly alter glucose uptake separately from and in addition to insulin via a PI3K-dependent effect on GLUT4 translocation in muscle cells, this signalling pathway was investigated as a potential driver of the changes observed here. With this in mind, initially, investigations into the BACE1-dependent effects on insulin-stimulated 2-DG uptake were performed. During these experiments, SH-SY5Y cells were serum starved for 2 hours and treated with a supraphysiological concentration of insulin (100nM) in an effort to obtain a reproducible response and is the same dose as has been used previously (Benomar et al., 2006). In the control, SH-SY5YEV cells, this stimulation resulted in a significant increase in 2-DG uptake rate (Ctl; 1 vs. 100nM Insulin; 1.46 ± 0.1, p < 0.001, n = 13, Fig. 4.13) while the transfected cell lines all displayed blunted insulin stimulated increases in 2-DG uptake (BACE1; 1.34 ± 0.09, p < 0.05, APPwt; 1.27 ± 0.02, p < 0.001 and APPswe; 1.35 ± 0.12, n = 4, Fig. 4.13).

In an attempt to more fully understand these effects, the PI3K-dependent phosphorylation of PKB, a key mediator of insulin-stimulated GLUT4 translocation was investigated. To do this, a 1-hour insulin time course was conducted following a 2-hour serum starvation with 10nM insulin in SH-SY5YEV and SH-SY5YB1 cells. As anticipated, a significant increase in phosphorylation of PKB at serine 473 was
Figure 4.13: BACE1 and APP(wt and Swe) overexpressing cells display blunted insulin-stimulated 2-DG uptake in SH-SY5Y cells. Significant increase in $^3$H-2-DG uptake rate in EV, BACE1 and APPwt overexpressing cells observed following a 2-hour serum starvation period and 30-minute stimulation with 100nM insulin (EV and APPwt, $p < 0.001$ and BACE1, $p < 0.05$, n = 4-13).
observed in both cell types, which reached significance after 5 minutes (EV; 8.12 ± 1.33 and BACE1; 9.18 ± 1.33, p < 0.01, n = 6, Fig. 4.14) and a peak after 15 minutes (EV; 11.3 ± 2.08 and BACE1; 10.08 ± 1.44, p < 0.01, n = 6, Fig. 4.14). There was however a significant difference in the phosphorylation response at threonine 308 with the SH-SY5Y<sub>EV</sub> cells displaying a significant, peak response vs. SH-SY5Y<sub>EV</sub> control cells after 1 minute (EV; 1.81 ± 0.18, p < 0.05, n = 4, Fig. 4.15), and remaining significantly elevated until 30 minutes after insulin stimulation. This response was almost entirely abolished in SH-SY5Y<sub>B1</sub> cells, with no significant changes in phosphorylation elicited by insulin treatment (Fig. 4.15).

A concurrent signalling pathway stimulated by insulin receptor activation is the mitogen-activated protein kinases (MAPKs). Signalling through the pathway occurs in response factors that mediate changes in cellular metabolism. Following BACE1 overexpression, there were significant increases in the phosphorylation of P44-42 (also known at ERK1 and 2) MAPK (EV; 1.00 ± 0.01 vs; BACE1; 1.37 ± 0.12, p < 0.05, n = 5, Fig. 4.16B) and the stress activated protein kinases (SAPK) 54 (EV; 1.00 ± 0.01 vs. BACE1; 1.28 ± 0.01, p < 0.001, n = 6, Fig. 4.16C) and 46 (EV; 1.00 ± 0.1 vs. BACE1; 1.23 ± 0.04, p < 0.01, n = 6, Fig. 4.16D).

**4.2.7 BACE1 Overexpression Blunts AMPK Protein Expression and Activity in SH-SY5Y Cells**

A second potential driver of altered GLUT4 translocation is AMPK, a kinase thought to play a central role in the sensing and modulation of cellular ATP levels. Therefore, its expression and activity was looked at the phosphorylation status of AMPK (at
Figure 4.14: BACE1 overexpressing cells retain their insulin-stimulated phosphorylation of serine 473 of PKB.
(A) Representative blot showing total and phosphorylated (serine 473) PKB levels in response to a 1-hour insulin (10nM) time course. (B) Densitometric analysis showing the significant increase in serine 473 phosphorylation over the 1-hour insulin timecourse relative to control, with no significant difference seen in the SH-SY5Y cells relative to the EV (p < 0.01, 0.001, n = 6).
Figure 4.15: BACE1 overexpressing cells display diminished insulin-stimulated phosphorylation of threonine 308 of PKB.

(A) Representative blot showing total and phosphorylated (threonine 308) PKB levels in response to a 1-hour insulin (10nM) time course. (B) Densitometric analysis showing the significant increase in threonine 308 phosphorylation over the 1-hour insulin time course relative to control (p < 0.05, n = 3-4) in EV cells with this effect absent in SH-SY5Y<sub>B1</sub> cells.
Figure 4.16: Stable BACE1 overexpression enhances MAPK signalling in SH-SY5Y cells. 

(A) Representative Western blot showing the changes in signal from the phospho-specific and total antibodies for p44-42 MAPK (ERK1 and 2) and SAPK54 and 46. Densitometric analysis displayed an increase in the phosphorylation of p44-42 (B, p < 0.05, n = 5), SAPK54 (C, p < 0.001, n = 6) and SAPK46 (D, p < 0.01, n = 6) in SH-SY5Y \textsubscript{B1} cells.
threonine 172) and saw that this was significantly reduced in SH-SY5Y_{B1} cells (EV; 1.00 ± 0.09 vs. BACE1; 0.61 ± 0.11, p < 0.05, n = 5-6, Fig. 4.17A, B) and a significant reduction in total AMPK protein level (EV; 1.00 ± 0.05 vs. BACE1; 0.63 ± 0.06, p < 0.01, n = 6, Fig. 4.17A, C) was also evident. Furthermore, there were also reduced total (EV; 1 ± 0.12 vs. BACE1; 0.71 ± 0.04, p < 0.05, n = 3-4, Fig. 4.18D, F) and phosphorylated levels (EV; 1 (EV; 1 ± 0.26 vs. BACE1; 0.42 ± 0.07, p < 0.01, n = 4, Fig. D, E) of the downstream target of AMPK, ACC.

Taken together, the above data implicate BACE1-mediated dysfunction in a number of cellular pathways that control energy balance and particularly insulin signalling as potential drivers of impaired homeostasis of GLUT protein expression. This in turn has the deleterious effect on basal and insulin-simulated glucose uptake into neuronal cells and may underlie some of the key, early physiological changes in AD progression.
BACE1 overexpression significantly blunts AMPK signalling in SH-SY5Y cells.

(A) Representative western blot showing phosphorylated (at threonine 172) and total AMPK levels in SH-SY5Y_EV and SH-SY5Y_BACE cells. (B) Densitometric analysis showing the significant reduction in both the proportion of phosphorylated AMPK (p < 0.05, n = 5-6) and in total protein level (C, p < 0.01, n = 6). (D) Representative blot showing phosphorylated (at serine 79) and total acetyl-CoA carboxylase (ACC) protein levels in SH-SY5Y_EV and SH-SY5Y_BACE cells. (E) Densitometric analysis showing a significant decrease in the proportion of ACC phosphorylated at serine 79 (p < 0.01, n = 4) and in total ACC levels (F, p < 0.05, n = 3-4).
4.3 Discussion

4.3.1 Manipulation of APP Processing Significantly Alters Glucose Uptake in SH-SY5Y Cells

Taken together, the present data suggest that the relative actions of the amyloidogenic and non-amyloidogenic arms of APP processing fundamentally act to regulate the basal glucose uptake rate of the human, neuroblastoma SH-SY5Y cell line. Here, pushing APP metabolism down the amyloidogenic pathway, via BACE1 overexpression (Fig. 4.3 and 4.4) or inhibition of the α-secretase enzyme family (through treatment with TAPI-1, Fig. 4.10B) resulted in significantly impaired 2-DG uptake. The effect of increasing BACE1 activity was also evident when SH-SY5Y cells overexpressing APPswe were compared to APPwt overexpressing cells (Fig. 4.8 and 4.9). In addition, when compared to SH-SY5Y cells bearing increased levels of BACE1, overexpression of BACE1 devoid of secretase activity yielded a significant increase in 2-DG uptake (Fig. 4.6 and 4.7), suggesting a vital role for the secretase activity of BACE1 in the changes observed.

These findings are in agreement with previous studies that have shown impaired glucose uptake following treatment with Aβ. One such study is the work by Keller and colleagues who demonstrated impaired glutamate and glucose transport into synaptosomes in culture as a result of Aβ-induced oxidative stress (Keller et al., 1997). Subsequent work also observed that glucose uptake into cultured primary hippocampal neurons was significantly altered by treatment with either Aβ25-35 or 1-42 (Prapong et al., 2002). A fascinating piece of data from this study was that the
inhibitory effect of Aβ came despite increased GLUT3 transcription and translocation to the membrane. It would therefore be intriguing to further investigate if alterations in BACE1 expression and/or activity may also alter GLUT3 transcription, translocation and/or function directly or whether changes are mediated via increases in Aβ.

4.3.2 BACE1-mediated Regulation of CREB Signalling

If such changes do occur via Aβ, one mechanism may be the activation of the calpain family of calcium-activated intracellular cysteine proteases. Increased calpain activation has previously been noted in the AD brain (Saito et al., 1993, Trinchese et al., 2008). Allied to this, reduced expression of the endogenous calpain inhibitor, calpastatin have also been noted in the prefrontal cortex in AD cases (Nixon et al., 2003; Rao et al., 2008). This aberrant activity of calpain may in turn contribute to the lowered GLUT3 levels observed in AD via cleavage of its transcription factor cyclic AMP (cAMP) response element (CRE)-binding protein CREB (Rajakumar et al., 1998). Recent work by Jin et al demonstrated that expression of full length CREB was required for GLUT3 transcription in neurons and that calpain I-induced truncation of CREB resulted in diminished GLUT3 transcription and expression (Jin et al 2013). These findings complement previous work that demonstrated that calpastatin-mediated inhibition of calpain I resulted in enhanced pCREB expression and improved learning and memory task performance in the APP/PS1 AD mouse model (Liang et al., 2010). This stimulatory effect on learning and memory was also observed in addition to enhanced synaptic marker expression (PSD95 and
synaptophisin) following treatment of the 3xTg AD mouse model with a calpain inhibitor (A-705253; Medeiros et al., 2013).

A recent study also suggests that calpain 1 could potentially mediate the diminished glucose uptake associated with AD and by extension, BACE1 overexpression (Jin et al., 2013). In this work, HEK 293 cells and human brain tissue from control and AD cases were used to demonstrate that under normal conditions, CREB transcription factor activity regulates neuronal expression of GLUT3. In turn, it was shown that levels of truncated CREB were increased in the AD brain and subsequently that this form is less able to bind the GLUT3 promoter and stimulate transcription (Jin et al., 2013). This work further demonstrated that GLUT3 mRNA and protein levels are diminished in AD. Additionally, the group showed that this aberrant proteolysis of CREB occurred via the action of calpain 1 (Jin et al., 2013). A second point of regulation of CREB activity is its phosphorylation at serine 133, and subsequent activation, by protein kinase A. Aβ has previously been reported to decrease this cAMP/PKA/CREB signalling (Tong et al., 2011). The work of Jin and colleagues demonstrated a key role for this event in GLUT3 expression, with S133A point mutation resulting in attenuated promoter activity (Jin et al., 2013). This correlates well with the finding that PKA activity is diminished as a result of calpain activation in the AD brain (Liang et al., 2007).

These calpain-induced changes in the transcription factor activity of CREB may represent a significant driver in AD pathogenesis over and above the altered glucose uptake. Indeed, CREB has been shown to exert regulatory control over a vast array of AD-associated genes with roles in CNS development, neuroprotection and synaptic
plasticity (Satoh et al., 2009). Furthermore, it has been postulated that it is via the CREB-signalling pathway that Aβ acts to impairs LTP and bring about synapse loss (Saura et al., 2011). Further evidence for this central role in hippocampal functioning comes from two studies showing that either postnatal removal of CREB or overexpression of a dominant mutant (harbouring a S133A mutation) result in neurodegeneration in the CA1 region (with additional loss in the DG) and the cortex respectively (Mantamdiotis et al., 2002; Ao et al., 2006). Interestingly, recent data has suggested a role for BACE1 in modulating camp/PKA/CREB signalling independently of Aβ (Chen et al., 2012). In this work, overexpression of BACE1 in MEF cells from APP/APLP2 knockout mice resulted in reduced CREB phosphorylation (Chen et al., 2012).

Taken together, the data presented herein and those of previous studies would suggest that calpain overactivation and resultant impairment of CREB signalling might mediate the altered glucose uptake observed upon BACE1 overexpression. This idea of enhanced BACE1 protein level and/or activity and dysfunctional CREB signalling may also link back to potential changes in eIF2α, resulting from cellular stress (3.3.5-8). In terms of AD progression, this BACE1-dependent change may then lead to downstream changes in hippocampal synaptic plasticity and neurodegeneration.

**4.3.3 Promotion of sAPPα Production and Glucose Uptake**

Conversely, it has been shown here that under conditions of enhanced non-amyloidogenic processing (i.e. stimulation of sAPPα production/levels), 2-DG uptake is enhanced. This has been demonstrated via inhibition of BACE1 (Fig. 4.10A) and
also noted following APPwt overexpression (Fig. 4.9). Furthermore, direct stimulation of naïve SH-SY5Y cells with sAPPα resulted in a significant increase in 2-DG uptake (Fig. 4.12A). This finding is supported by work by Mark Mattson and colleagues that showed a stimulatory effect of sAPPα on glucose uptake into synaptosomes (Mattson et al., 1999). These data are also in agreement with our previous work in both muscle cell lines and the whole animal (Meakin et al., 2012; Hamilton et al., 2014). Indeed, in C2C12 myotubes, BACE1 inhibition resulted in a PI3K-dependent stimulation of glucose uptake, which occurred in addition to, but separately from the action of insulin. In agreement with this mechanism, here, a significant increase in the phosphorylation of threonine 308 was observed in response to sAPPα (Fig. 4.12B, C). This stimulation may, as in the case of insulin stimulated glucose uptake, lead to the mobilization of GLUT4-containing vesicles to the plasma membrane to augment basal, neuronal glucose uptake via GLUT3. It would therefore be interesting to see if BACE1 inhibition and/or sAPPα are able to stimulate, through a PI3K dependent mechanism, GLUT4 translocation in neuronal cells, as was previously observed in muscle (Hamilton et al., 2014). Some evidence for diminished GLUT4 translocation may be provided by the finding that insulin stimulated 2-DG uptake was blunted in the cells where BACE1 (SH-SY5Y_B1) or APP (SH-SY5Y_APPwt and APPswe) had been overexpressed all displayed a blunted response to supraphysiological insulin stimulation with regards 2-DG uptake (Fig. 4.13). Having seen a trend towards significance in the difference between these responses it would be interesting to observe how the cells responded when treated with a more physiological insulin dose. A previous study by Benomar and colleagues also observed a significant, PI3K-dependent increase in glucose uptake in SH-SY5Y cells owing to enhanced GLUT4 translocation to the membrane in response to insulin (100
nM) and leptin (15 nM) (Benomar et al., 2006). A second beneficial alteration to the current study protocol may be a longer period of serum starvation prior to insulin-stimulated 2-DG uptake measurements being taken. This paper utilised a 16-hour serum starve and obtained a larger fold change in glucose uptake (around 2-fold) and this may help to delineate the changes observed between groups of the current study.

4.3.4 Insulin Resistance as a Driver of AD Pathogenesis

Allied to these potential changes in glucose uptake, a number of alterations in cell signalling indicative of insulin resistance were observed upon enhancement of amyloidogenic processing (via BACE1 overexpression). These included a loss of the phosphorylation of the threonine 308 site on PKB in response to insulin (Fig. 4.15). It is this event that mediates a conformational change in PKB allowing full activation of the kinase. This finding might also be of importance as it was observed that treatment of naïve SH-SY5Y cells with sAPPα resulted in a significant increase in phosphorylation at this site. It should however be noted that the degree of phosphorylation at the secondary serine 473 site of PKB was retained in response to insulin in SH-SY5Y_{B1} cells (Fig. 4.14). It would therefore be of interest to look at the functional PKB kinase activity to more fully understand if this reduced threonine phosphorylation is of physiological relevance. Allied to these changes at the level of PKB, changes in MAPK signalling were also observed (Fig. 4.16). Phosphorylation of both P44-42 (ERK1-2) and SAPKs (JNK) were significantly increased in BACE1 overexpressing cells (Fig. 4.16). This enhanced JNK signalling is of particular interest, as this was previously noted to potentially be a driver of enhanced BACE1, and APP, protein expression in the hippocampus of HFD fed animals (Figs. 3.15 and
Previous work has shown that the BACE1/APP pathway can become altered under treatment with both palmitate and ceramide (Hamilton et al., 2014). There is also evidence that implicates increased fat supply with skeletal muscle insulin resistance in the context of obesity. This change is proposed to come as the result of increased levels of intracellular lipid intermediates (such as diacyl-glycerol (DAG) and ceramide), which act to impair PI3K-PKB signalling (for review see Eckardt et al 2011).

These data are suggestive of a potentially vicious cycle elicited by an initial stress event (such as HFD) that acts to increase BACE1 expression/activity through enhanced JNK pathway activity, which in turn enhances JNK and ERK1-2 signalling independent of the causative stressor. The present data also show that this aberrant BACE1 expression may then act to significantly reduce cellular glucose uptake.

Modulation of insulin signalling has received a great deal of attention with regards a role in AD pathogenesis and the link between T2DM and AD. Indeed, impaired insulin signalling is seen as a fundamental link between these conditions (Zhao et al., 2008a; Bomfim et al., 2012). Furthermore a longitudinal study has shown that neurodegeneration is exacerbated in the presence of impaired insulin signalling in the brain (Matsuzaki et al., 2010). In a model proposed by Bosco and colleagues, this loss of insulin signalling, particularly that through PI3K/PKB may result in decreased GLUT expression and a resultant reduction in intracellular ATP levels. Evidence for such a model has been provided by a number of recent studies. One such work is that of Moloney et al. showed the presence of decreased insulin and insulin-like growth factor (IGF1) receptor expression is diminished in AD neurons. Interestingly,
increased levels of IGF1 receptors were increased in and around Aβ plaques, which may be a protective mechanism to conserve insulin signalling and promote survival (Moloney et al., 2010). A second study found a similar trend of reduced IGF1/2 receptor expression in AD and showed that this reduction correlated with enhanced levels of GSK3β activity and the presence of increased APP mRNA (Steen et al., 2005). There also appears to be the possibility that the presence of central insulin resistance may underlie AD progression, with a study by Chua and colleagues showing that impaired insulin signalling occurs prior to Aβ accumulation in the APP/PS1 AD mouse model (Chua et al., 2012). When added to the current findings, the enhanced BACE1 activity of the APPswe mutation would appear to be a driving force behind this decreased insulin signalling. In attempt to combine previous findings with regards the connection between T2DM and AD, Liu and colleagues compared the insulin-PI3K-PKB function in the frontal cortices of autopsied brains from AD, T2DM and T2DM-AD individuals (Liu et al., 2011). They showed that decreased insulin signalling was present in both T2DM and AD cases but that the greatest reduction was observed in the T2DM-AD brains (Liu et al., 2011).

Taken together, the present data and the findings of previous works strongly implicate BACE1 as a key modulator of neuronal glucose uptake and insulin signalling. Indeed, recent work from our lab has shown that reduction or removal of BACE1-mediated APP processing can alter whole body glucose homeostasis and insulin sensitivity, through modulation of GLUT4 translocation in peripheral tissues (notably muscle). The data herein takes these findings a step further and demonstrate a reciprocal effect of shifting APP cleavage towards Aβ production, with impaired glucose uptake observed. Aberrant BACE1 expression/activity also resulted in a shift
in cellular signalling pathways (enhanced MAPK and diminished PI3K) indicative of insulin resistance. Having observed fundamental changes in glucose uptake as a result of manipulation of APP processing, it was postulated that these alterations might also mediate a change in cellular substrate use.

Figure 4.18: Hypothetical model showing the potential points of interaction of sAPPα and Aβ with insulin signalling. The present data suggest that sAPPα may act through IR activation of PKB phosphorylation (at threonine 308) or directly to stimulate GLUT4 translocation to the membrane. In the case of BACE1 overexpression, reduced pathway activity (via reduced sAPPα production and/or Aβ-induced alterations) may impair GLUT4 translocation. Alternatively, increased Aβ production may, through calpain-mediated CREB truncation, reduced GLUT3 expression.
Chapter 5

Effect of BACE1 Overexpression on Neuronal Substrate Use in SH-SY5Y Cells
5.1 Introduction

5.1.1 Central Metabolism

It has long been recognised that glucose is the predominant substrate utilised by the adult brain under physiological conditions. Indeed, while only providing around 2% to body weight, the brain consumes around 20 and 25% of the total body oxygen consumption and glucose respectively (Mckenna et al., 2006). It has also been demonstrated that the respiratory quotient of the brain is almost exactly 1, indicating almost universal carbohydrate metabolism (for review see Sokoloff 1999). Alternative substrates, such as glycogen and amino acids can play a role in central metabolism, however due to limited supply and storage capacity, this is thought to be small under physiological conditions (Henderson et al., 2009). There is also potential for lactate and ketone bodies to supplement brain energy supply via dietary-induced ketonemia, or during periods of prolonged exercise, starvation. This is facilitated by the upregulation of the monocarboxylate transporter expression at the blood-brain-barrier (BBB; Cunnane et al., 2011; Lutas and Yellen 2013). The potential role for ketones in AD-mediated metabolic decline has also been demonstrated via the reversal of early bioenergetic changes seen in 3xTg AD mouse model by 2-DG-induced ketonemia (Yao et al., 2011). The glucose metabolism of the brain is subsequently coupled to the generation of the intracellular currency of energy, ATP, as well as neurotransmitter production.
5.1.2 Neuronal Glucose Metabolism

The vast majority of energy consumed by the brain goes towards neuronal functions, encompassing action potential and subsequent post-synaptic potential generation as well as the maintenance of ion gradients (Ivannikov et al., 2010; Howarth et al., 2012). Of this, synaptic events are the predominant point for neuronal energy consumption, with the release of a single synaptic vesicle calculated at close to $2 \times 10^5$ ATP molecules (Harris et al., 2003). There are three key junction points in neuronal metabolism: G-6-P, pyruvate and acetyl CoA. As mentioned previously, glucose is taken up into neurons via a combination of GLUT3 and GLUT4–mediated transport before being phosphorylated by HK. The resultant Glc-6-P can then undergo two fates, acting either as a substrate for glucose 6-phosphate dehydrogenase, the rate-limiting enzyme of the pentose phosphate pathway (PPP) or being converted to its isof orm fructose 6-phosphate. In neurons, the PPP contributes to the production of nucleotides as well as playing a role in oxidative stress defence by maintaining the glutathione redox status through the generation NADPH (Vaughn et al., 2008; Herrero-Mendez et al., 2009). The post mitotic nature of most neurons, would suggest little need for nucleotides, however this pathway was recently shown to consume a meaningful fraction of neuronal metabolic flux (Rodriguez-Rodriguez et al., 2013).

Fructose 6-phosphate on the other hand serves as a substrate for the key glycolytic enzyme, phosphofructokinase (PFK). This conversion of fructose 6-phosphate to fructose marks the first reaction of glycolysis and due to the requirement for ATP, effectively commits substrate to this pathway. Taken together, the steps of glycolysis
act to convert glucose to pyruvate and generate a small amount of ATP and involve 10 catalysed reactions (see Figure 5.1).

The resultant pyruvate is then subject to oxidative decarboxylation, catalysed by the PDH complex, effectively linking anaerobic (glycolysis) to oxidative metabolism in the mitochondria. It is at this level that lactate enters metabolism following its uptake to the neuron (via MCT2) and subsequent conversion to pyruvate by the action of lactate dehydrogenase. PDH is a multimeric complex, encompassing around 30 E1, 60 E2 and 6 E3 subunits and lies at the heart of carbohydrate metabolism, playing a key role in cellular fuel partitioning. The E1 subunit (pyruvate dehydrogenase) is comprised of a tertramer made up of 2α and 2β subunits (41 and 36kDa respectively) while the E2 subunit represents the 74-kDa dihydrolipoyl transacetylase. Finally the E3 subunit is the 55kDa dihydrolipoyl dehydrogenase, which also forms part of the alpha-ketoglutarate dehydrogenase complex (Patel and Harris 1995). The activity of the PDHC relies upon a number of cofactors and substrates including Ca²⁺, Mg²⁺, ATP:ADP as well as the complexes phosphorylation status (Huang et al., 1998). The phosphorylation and subsequent inactivation of the PDHC is controlled by a family of 4 pyruvate dehydrogenase kinases (PDK1-4) of which PDK2, 3 and 4 are expressed in the brain with PDK2 showing the highest expression in the rat brain, particularly in the hippocampus (Nakai et al., 2000; Xing et al., 2012). These kinases can themselves be regulated by a number of factors and are activated by increased ratios of NADH:NAD⁺, ATP:ADP and acetyl CoA:CoA while inhibition has also been observed in response to increased NAD⁺ and ADP as well as the drug dichloroacetate (DCA; Bowker-Kinley et al., 1998; Baker et al., 2000; Wilson et al., 2003). PDK2 and 4 expression has also been shown to be increased in conditions of starvation and
diabetes, through the influence of fatty acids, glucocorticoids and insulin (Huang et al., 2002).

Figure 5.1: Schematic diagram of the steps involved in the glycolytic pathway (www.conceptdraw.com)

Following the PDHC-catalysed conversion of pyruvate to acetyl-CoA, NADH and CO₂, the acetyl CoA serves as the primary substrate for the first committed stage of oxidative metabolism, the citric acid (TCA) cycle (see Figure 5.2). The turning of which encompasses a series of enzymatically controlled reactions resulting in the degradation of pyruvate coupled to the generation of 1 molecules of guanosine-5'-triphosphate (GTP) and reducing equivalents (3 molecules of NADH and 1 FADH₂).
These reduced, high-energy molecules go on to serve as a substrate for oxidative phosphorylation via the actions of complexes I-IV of the electron transport chain (ETC; see Figure 5.2). The hydrogen of NADH and FADH$_2$ is transferred to oxygen to produce water by the action of the four complexes in the ETC: NADH dehydrogenase succinate ubiquinone oxidoreductase, ubiquinol cytochrome c oxidoreductase and cytochrome c oxidoreductase. The single electron of the hydrogen has a large amount of transfer energy and the movement of this through the ETC complexes is coupled to the extrusion of protons across the inner mitochondrial membrane. The dissipation of the resultant proton gradient is coupled to the generation of ATP via the action of F$_1$F$_0$ ATP synthase, functioning as an ATP hydrolyser running in reverse (see Figure 5.2).

### 5.1.3 NAD$^+$ and SIRT1 as Regulators of Ageing and AD

The sirtuins (SIRT1-7) are an evolutionarily conserved family of histone deacetylases (HDAC) that utilise NAD$^+$ as an essential cofactor (Imai et al., 2000; Landry et al., 2000). Their cellular localisation varies across the family with SIRT1 (associated with euchromatin), 6 (associate with heterochromatin) and 7 found in the nucleus while SIRT2 is cytosolic and SIRT3, 4 and 5 are found in the mitochondria (Michishita et al., 2005; Ford et al., 2006; Tanno et al., 2007).

SIRT proteins have a number of transcription factor substrates and are best characterised as mediators of the anti-ageing effects attributed to calorie restriction (CR; Bonda et al., 2011). SIRT activation results in transcriptional programs involved in cell apoptosis, cell differentiation and oxidative metabolism, mitochondrial efficiency and oxidative stress resistance (for review see Haigis and Sinclair 2010; Chang and Guarente 2013).
This interrelationship between SIRT and NAD$^+$ was recently highlighted as a potential mediator of ageing itself in the NAD$^+$ World Hypothesis (Imai 2009). This theory proposes that NAD$^+$ acts as a major controller of tissue metabolism and exerts its effect via SIRT1, through transcriptional regulation of metabolic change. In
particular, the brain is highlighted as a “critical tissue” in the World Hypothesis due to its regulatory control of other tissues (Revollo et al., 2007).

The process of ageing reflects the progressive decline of physiological functions and an increasing risk of degenerative disease state such as: CVD, stroke, Parkinson’s, Huntington’s and Alzheimer’s disease. Maintenance of NAD$^+$ biosynthesis is believed to play a role in healthy ageing through its control the anti-ageing effects of CR. As a result of these potential connections, interest has grown in trying to understand the effect of alterations in NAD$^+$ and SIRT1 in normal and pathogenic brain function. The predominant SIRT in the neurons was observed to be SIRT1, which shows a nuclear import/export sequence and has been observed in the cytosolic fraction of rat brain tissue (Tanno et al., 2007; Ramadori et al., 2008). SIRT1 expression has been shown to decline in models of ageing and neurodegeneration and has been suggested to play a role in neuronal stress response and in the prevention of axonal loss (Araki et al., 2004; Alvira et al., 2007). SIRT1 has also been postulated to impact dendritic maturation and development, with removal of its deacetylase activity being associated with impaired dendritic development in the hippocampus and SIRT knockout brains displaying reduced dendritic branching that can be reversed with the SIRT activator, resveratrol (Michan et al., 2010; Braidy et al., 2012). These data have prompted groups to begin to look at the effect of manipulating SIRT1 on markers of AD.

Recent studies have shown that increased activity of the SIRT1 pathway protects against amyloid pathology in AD animal models (Qin et al., 2006; Albani et al., 2009) and that brain (hippocampal) SIRT1 and PGC-1 expression are reduced in AD
animal models and AD patients (Marques et al., 2012; Theendakara et al., 2013). Furthermore, impaired SIRT1 activity exacerbates AD pathology and behavioural deficits, whereas increased brain SIRT1 suppresses this phenotype in AD mice. Importantly, concerted activity of the SIRT1 pathway and retinoic acid receptor-β impinge directly on APP processing through increased activation of the α-secretase ADAM10 (Lee et al., 2014) and down-regulation of BACE1 transcription and protein levels (Wang et al., 2013), which in combination reduce Aβ generation. In addition SIRT1 plays an important role in learning and memory through synaptic plasticity (Gao et al., 2010; Michan et al., 2010). Thus the SIRT1 pathway links cellular metabolism and stress with regulation of BACE1. However, whether the converse relationship exists, whereby BACE1 activity influences SIRT1-PGC-1 signalling has not been investigated.

Previous work from our lab has shown that manipulation of APP cleavage can impact substrate use and metabolic flexibility in the rodent myotube C2C12 cell line. Therefore the aim of this study was to see if shifting APP processing towards Aβ production could alter substrate metabolism in the human neuronal SH-SY5Y cell line and whether enhanced BACE1 activity could alter the NAD+/SIRT1 systems.
5.2 Results

5.2.1 BACE1 Overexpression Impairs Glucose Use in SH-SY5Y Cells

As glucose is a primary and fundamentally required neuronal substrate glucose metabolism was investigated following BACE1 overexpression in SH-SY5Y cells. SH-SY5Y\textsubscript{B1} cells displayed a significant reduction in the rate at which $^{14}$C-labelled glucose was oxidised to $^{14}$C-labelled CO\textsubscript{2} (EV; $11.12 \pm 1.59$ pmol/min/mg vs. BACE1; $8.01 \pm 0.98$ pmol/min/mg, $p < 0.01$, $n = 7$, Fig. 5.3A). This was not observed as a result of impaired uptake and incorporation of the labelled glucose into the cell, as a trend towards an increase in the rate of glucose incorporation in the SH-SY5Y\textsubscript{B1} cells was observed (EV; $285.6 \pm 66.52$ pmol/min/mg vs. BACE; $366.1 \pm 88.2$ pmol/min/mg, $n = 9$, Fig. 5.3B). Taken together these data reflect a robust change in the efficiency with which SH-SY5Y\textsubscript{B1} cells can utilise the glucose they take up as illustrated by the significant reduction in the $^{14}$C-glucose oxidation to incorporation ratio (EV; $1 \pm 0.18$ vs. BACE; $0.62 \pm 0.09$, $p < 0.001$, $n=8$, Fig. 5.3C).

To see if this change was directly attributable to the secretase activity of BACE1, a BACE1 mutant devoid of secretase activity (mBACE1) was overexpressed as in the 2-DG uptake investigations. Following mBACE1 overexpression there was a complete reversal of the impaired $^{14}$C-glucose oxidation (EV; $17.74 \pm 0.48$ pmol/min/mg vs. BACE; $14.05 \pm 0.28$ pmol/min/mg vs. mBACE1; $17.87 \pm 0.44$ pmol/min/mg, $p < 0.001$, $n = 5$, Fig. 5.4A), glucose incorporation (EV; $228 \pm 10.55$ pmol/min/mg vs. BACE; $252.3 \pm 18.06$ pmol/min/mg vs. mBACE1; $204.4 \pm 10.80$ pmol/min/mg, $n = 5$, Fig. 5.4B) and oxidation to incorporation ratio (EV; $1 \pm 0.04$ vs.
Figure 5.3: BACE1 overexpression significantly impairs glucose oxidation in SH-SY5Y cells.
(A) Significant reduction in $^{14}$C-glucose oxidation rate following BACE1 overexpression ($p < 0.01$, n=7). (B) No significant change in $^{14}$C-glucose incorporation rate observed in SH-SY5Y$_{B1}$ cells (n=9). (C) Significant reduction in the ratio of $^{14}$C-glucose oxidation to incorporation following BACE1 overexpression ($p < 0.01$, n=7).
Figure 5.4: Absence of BACE1-mediated glucose oxidation impairment following overexpression of BACE1 mutant devoid of secretase activity in SH-SY5Y cells.

(A) Significant reduction in $^{14}$C-glucose oxidation rate following BACE1 overexpression not observed following overexpression of BACE1 mutant devoid of secretase activity (mBACE1; p < 0.001, n=5). (B) No significant changes observed in $^{14}$C-glucose incorporation rate following overexpression of BACE1 or mBACE1 (n=5). (C) Significant reduction in the ratio of $^{14}$C-glucose oxidation to incorporation following BACE1 overexpression not observed in the mBACE1 cells (EV to BACE1 p < 0.01, BACE1 to mBACE1 p < 0.001, n=5).
BACE; 0.73 ± 0.055 vs. mBACE1; 1.13 ± 0.06, EV to BACE1 p < 0.01 and BACE1 to mBACE1 p < 0.001, n = 5, Fig. 5.4C). It can therefore be concluded that the reduction in glucose oxidation following BACE1 overexpression is dependent upon its secretase activity.

5.2.2 BACE1 Overexpression Alters Respiration Towards Aerobic Glycolysis in SH-SY5Y Cells

In an attempt to examine the impaired glucose use more closely, a Seahorse Extracellular Flux Analyser was utilised. This instrument measures cellular respiration in real time. Actively respiring mitochondria consume oxygen and therefore oxygen consumption rate (OCR) can be taken as a measure of the flux of substrate through the oxidative phosphorylation pathway while extracellular acidification (ECAR), a measure of glycolysis, occurs via the release of lactic acid from the cell, following its conversion from glucose through the glycolytic pathway. Overexpression of BACE1 resulted in a major shift in the respiration profile in the cells, with a significant reduction in OCR (EV; 8.23 ± 0.27 pmolO₂/min/µg vs. BACE1; 5.86 ± 0.42 pmolO₂/min/µg, p < 0.001, n = 11-12, Fig. 5.5A) and a significant increase in ECAR (EV; 0.8 ± 0.06 mpH/min/µg vs. BACE1; 1.59 ± 0.12 mpH/min/µg, p < 0.001, n = 7-11, Fig. 5.5B) observed in response to 2.5 mM glucose as the substrate. Taken together these data reflect a highly significant change in the balance of glycolysis and oxidative phosphorylation as given by the OCR to ECAR ratio (EV; 1 ± 0.08 vs. BACE1; 0.36 ± 0.04, p < 0.001, n = 7-11, Fig. 5.5C). These changes are indicative of a reduced flux of substrate through the oxidative phosphorylation pathway and a compensatory increase in glycolysis and suggest an
Figure 5.5: BACE1 overexpression significantly alters cellular respiration in the presence of 2.5mM glucose in SH-SY5Y cells.

(A) Significant reduction in oxygen consumption rate (OCR) following BACE1 overexpression (p < 0.001, n=11-12). (B) Significant increase in extracellular acidification rate (ECAR) in SH-SY5Y cells (p < 0.001, n=7-11). (C) Significant reduction in OCR:ECAR ratio following BACE1 overexpression (p < 0.001, n=7-11).
increased reliance upon glycolysis for ATP generation when BACE1 levels are increased.

5.2.3 BACE1 Overexpression Significantly Increases all Glycolytic Measures in SH-SY5Y Cells

To further investigate the altered glycolytic rate observed in SH-SY5Y<sub>B1</sub> cells, a Glycolysis Stress Test protocol was utilised. Prior to beginning this experiment, cells were washed with warm (37°C) PBS and switched into assay media containing 2.5mM pyruvate and 4mM L-glutamine to deplete the cells of glucose until the ECAR rate decreased to a steady state level termed non-glycolytic acidification. The assay was then started by the addition of 2.5mM glucose to stimulate basal glycolysis. Maximal glycolysis can be induced via the inhibition of F<sub>1</sub>F<sub>0</sub> ATP synthase by the addition of 1µM oligomycin, making the cells wholly reliant upon glycolysis for ATP generation. The assay was ended by the addition of a competing dose of 2-DG to inhibit glucose uptake and reduce ECAR back to baseline (Fig. 5.6A). Following BACE1 overexpression, stimulation of basal glycolysis resulted in a significantly greater change in ECAR (EV; 6.06 ± 0.4 mpH/min vs. BACE1; 9.67 ± 0.52 mpH/min, p < 0.001, n = 18-21, Fig. 5.6B) upon glucose addition. The maximal rate of glycolysis achieved by the cells was also significantly increased following BACE1 overexpression (EV; 15.45 ± 0.84 mpH/min vs. BACE1; 19.3 ± 0.94 mpH/min, p < 0.01, n = 18-21, Fig. 5.6C). Finally the difference between these two rates represents the glycolytic reserve and this was also significantly increased in BACE1 overexpressing cells (EV; 7.82 ± 1.16 mpH/min vs. BACE1; 11.19 ± 1.28 mpH/min, p < 0.05, n = 6, Fig. 5.6D).
Figure 6.6: BACE1 overexpression significantly enhances the rate of glycolysis in SH-SY5Y cells.

(A) Glycolysis stress test profile of EV and BACE1 cells showing the additions of 2.5mM glucose, 1mM oligomycin (oligo) and 25mM 2DG. (B) Significantly increased ΔECAR from baseline in BACE1 overexpressing cells following addition of glucose to stimulate basal glycolysis (p < 0.001, n = 18-21). (C) Significant change in ECAR from baseline in BACE1 overexpressing cells following glucose and oligomycin additions (p < 0.01, n = 18-21). (D) Significant increase in the glycolytic reserve following BACE1 overexpression, given by the difference between basal and maximal ECAR rates (p < 0.05, n = 6).
This altered metabolism has previously been suggested to occur in areas of the brain containing increased Aβ deposition and correlated with areas of the brain that are most susceptible to cell death in AD (Vaishnavi et al., 2010; Vlassenko et al., 2010). The highest rate of aerobic glycolysis was observed in the default network, an area known for its high neuronal activity and as a site of major amyloid deposition (Gusnard et al., 2001, Buckner et al., 2005). In an attempt to connect some of these experimental observations, increased energy demand (as occurs during neuronal activation) was mimicked by the application of the mitochondrial uncoupler 2, 4-dinitrophenol (DNP). These compounds work by punching holes in the mitochondrial membrane causing the dissipation of the ETC-driven proton gradient and this movement is not coupled to ATP generation as the protons can bypass ATP synthase. In an effort to maintain ATP levels, the cell must then increase substrate flux through the mitochondria (via increased glucose uptake, glycolysis and oxidative metabolism). To test the cellular response to increased energy demand, SH-SY5Y<sub>EV</sub> and SH-SY5Y<sub>B1</sub> cells were exposed to increasing dose of DNP prior to measurement of <sup>3</sup>H-2-DG uptake measurement or cell death detection.

In response to a 30-minute treatment with low dose DNP (0.01μM), both SH-SY5Y<sub>EV</sub> and <sub>B1</sub> cells displayed significantly increased rates of 2-DG uptake (increased by EV; 49.7 ± 6.0 %, p < 0.001 vs. BACE1; 52.3 ± 10.4 %, p < 0.05, n = 3, Fig. 5.7A). The percentage increase in 2-DG uptake rate remained significantly higher in the SH-SY5Y<sub>EV</sub> cells up to and including the highest DNP dose (1μM; increased by 43.8 ± 4.0 %, p < 0.001, n = 3, Fig. 5.7A). Whereas, the percentage 2-DG uptake rate increase of the SH-SY5Y<sub>B1</sub> cells significantly declined with increasing DNP, with no significant increase observed following 0.5 and 1μM treatment (Fig. 5.7A). This
Figure 5.7: BACE1 overexpression leaves SH-SY5Y cells more susceptible to cell death during increased energy.

(A) SH-SY5Y_EV cells can maintain significantly increased glucose uptake rates under challenge with the mitochondrial uncoupler dinitrophenol while SH-SY5Y_BACE1 cells display an increased uptake initially but this soon reduces and this decline in ³H-2-DG uptake correlates with the increased cytoplasmic appearance of fragmented DNA mono- and oligonucleosomes, a marker of apoptotic cell death (B).
declining 2-DG uptake was reasoned to be as a result of cell death and indeed, a significant increase in the cytoplasmic enrichment levels of fragmented DNA mono- and oligo-nucleotides (a marker of apoptotic cell death) following treatment with 0.5 and 1μM DNP (0.5μM; 2.84 ± 0.18, p < 0.01 and 10μM; 5.09 ± 0.09, p < 0.001, n = 4, Fig. 5.7B) relative to SH-SY5Y EV cells. Taken together these data suggest that chronic elevation of BACE1 protein levels results in a shift towards aerobic glycolysis to maintain intracellular ATP levels, however this changes appear to leave cells more susceptible to death in response to increased energy demand.

5.2.4 BACE1 Overexpression Impairs Lactate use in SH-SY5Y Cells

It has been proposed that lactate can also act as a substrate for neurons at rest, during activity and in recovery from stress/injury. In an effort to assess the effect of increased BACE1 activity on lactate use, a 14C-glucose oxidation assay was performed on EV and SH-SY5Y B1 cells while titrating in cold, unlabelled lactate. This experimental design has been utilised by Luis Sokoloff’s group to show that primary neurons will preferentially use lactate over glucose and that the extent to which increasing lactate infusion can repress the rate of labelled glucose oxidation mirrors the rate of lactate oxidation (Itoh et al., 2003). SH-SY5Y B1 cells displayed a significantly reduced ability to utilise lactate was observed at physiological levels (0.5 and 2mM) as indicated by a significantly lower percentage repression of 14C-glucose oxidation (0.5mM – EV; repressed to 53.29 ± 8.2 % vs. BACE1; 77.41 ± 9.2 %, 2 mM – EV; repressed to 36.1 ± 6.7 % vs. BACE1; 48.5 ± 6.9 %, p < 0.05, n = 5-6, 5.8A). This impaired lactate use also presented as a significantly lower OCR when
Figure 5.8: BACE1 overexpression significantly impairs cellular lactate use in SH-SY5Y cells

(A) Percentage $^{14}$C-glucose oxidation rate with increasing infusion of cold-unlabelled lactate (0.5, 2 and 4mM) showing significant reduction in lactate use following BACE1 overexpression at 0.5 and 2mM lactate ($p < 0.05$, $n = 5-6$). (B) Seahorse experiments showing significant reduction in OCR with 2mM lactate as a substrate but not 4mM following BACE1 overexpression ($p < 0.05$, $n = 6-11$).
cells were provided 2 mM lactate as a substrate (EV; 6.76 ± 0.48 pmolO₂/min/µg vs. BACE1; 4.35 ± 0.51 pmolO₂/min/µg, p < 0.05, n = 5, Fig. 5.8B). These significant reductions were not however observed at higher lactate concentrations (4 mM) with a non-significant difference observed in both percentage repression of glucose oxidation (4 mM – EV; repressed to 33.8 ± 9.6 % vs. BACE1; 42.2 ± 7.5 %, n = 5-6 Fig. 5.8A) and OCR (EV; 7.87 ± 0.93 pmolO₂/min/µg vs. BACE1; 7.02 ± 0.62 pmolO₂/min/µg, n = 6, Fig. 5.8B).

5.2.5 BACE1-mediated Impairment in Oxidative Phosphorylation due to Reduced Substrate Delivery to the Mitochondria and not Impaired Functioning

It was hypothesised that the reduced oxidative metabolism was as a result of either impaired substrate delivery to the mitochondria or reduced functioning of the mitochondria themselves. To assess mitochondrial health, a Mito Stress Test was utilised to look at mitochondrial efficiency. Due to the limited uncoupling rate achievable in undifferentiated SH-SY5Y cells with only glucose as a substrate, the typical stress test profile (Appendix I Fig. 1.4) was separated and the altered OCRs observed previously have been normalised to 100% for each cell type. In the first experiment, F₁F₀-ATP synthase was inhibited by the addition of oligomycin (1µM) and the reduction in OCR represents the proportion of cellular oxygen consumption going towards ATP generation. The rest of the electron transport chain ETC was then inhibited by the addition of the complex I and III inhibitors rotenone and antimycin-A (each 2µM). The percentage difference between the OCR following this addition and that following the oligomycin addition reflects the amount of oxygen needed to maintain the mitochondrial leak. Following BACE1 overexpression there was no
significant difference in the ATP-linked OCR (EV; 63 ± 3.7 % vs. BACE1; 54.5 ± 5.2 %, n = 8, Fig. 5.9B), the leak OCR (EV; 13.7 ± 1.9 % vs. BACE1; 17.2 ± 3.8 %, n = 8, Fig. 5.9C) or the non-mitochondrial oxygen consumption rate (EV; 20.6 ± 6.8 % vs. BACE1: 25.8 ± 9.2 %, n = 8, Fig. 5.9D). The second part of the split Mito Stress Test protocol looked at cellular reserve capacity. This is the inherent ability of cells to increase their respiration when substrate oxidation is uncoupled from ATP generation. To look at this, a carefully titrated dose of the mitochondrial uncoupler FCCP is added and the increase in OCR monitored. SH-SY5Y_B1 cells displayed no significant change in the percentage increase in maximal OCR achieved (EV; 155.4 ± 8.1% vs. BACE1; 161 ± 14.2 %, n = 9-10, Fig. 5.10B). With no significant changes observed following any of these additions, it can be concluded that mitochondrial function is largely maintained in SH-SY5Y_B1 cells. This result left impaired substrate delivery as a potential driver of the reduced substrate oxidation observed in BACE1 overexpressing cells. As a result of its position at the heart of carbohydrate metabolism and its key role in cellular fuel partitioning, PDH function was assessed. This was done through the use of an indirect reaction based activity assay and the Seahorse. SH-SY5Y_B1 cells showed a significant decrease in the rate of this PDH governed reaction (Fig. 5.11A) with a significant reduction observed following BACE1 overexpression (p < 0.01, n = 5, Fig. 5.11B). This depressed PDH activity also presented as a significantly reduced OCR when cells were provided pyruvate as the sole substrate (EV; 9.88 ± 1.40 pmolO₂/min/µg vs. BACE1; 5.49 ± 0.75 pmolO₂/min/µg, p < 0.05, n = 5, Fig. 5.11C). Taken together, these data suggest that the BACE1-mediated shift in metabolism is likely brought about as a result of impaired PDH activity and not through a reduction in mitochondrial efficiency.
Figure 5.9: BACE1 overexpression does not significantly alter ATP-linked, mitochondrial leak or non-mitochondrial oxygen consumption in SH-SY5Y cells.
(A) Modified Mito Stress Test profile showing the normalised baseline OCR readings for EV and BACE1 overexpressing cells and the percentage change in OCR following the additions of oligomycin (oligo; 1μM) and rotenone and antimycin A (Rot/Ant; 2μM). (B) No significant difference in the ATP-linked OCR following BACE1 overexpression (n = 8). (C) No significant difference in the OCR required to maintain the mitochondrial leak following BACE1 overexpression (n = 8). (D) No significant difference in the non-mitochondrial oxygen consumption following BACE1 overexpression (n = 8).
Figure 5.10: BACE1 overexpression does not significantly alter cellular reserve capacity for glucose metabolism in SH-SYSY cells.

(A) OCR profile showing the normalised baseline OCR readings for EV and BACE1 overexpressing cells and the maximal OCR attained following addition of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 0.2μM). (B) No significant difference in percentage change from baseline in response to FCCP addition following BACE1 overexpression (n = 9-10).
Figure 5.11: BACE1 overexpression significantly impairs pyruvate dehydrogenase function in SH-SY5Y cells.

(A) Schematic diagram showing the enzymatic conversion of pyruvate produced by glycolysis to acetyl CoA in the mitochondria controlled by PDH. (B) Significant reduction in PDH activity as measured by specific enzyme assay following BACE1 overexpression (p < 0.01, n=5). (C) Seahorse measurement showing significantly reduced OCR following BACE1 overexpression when 2.5mM pyruvate provided as a substrate (p < 0.05, n = 5).
These results also provided a molecular target for the reversal of the altered glucose metabolism phenotype.

5.2.6 Impaired Glucose Oxidation in SH-SY5Y_{B1} Cells can be Partially Reversed by Treatment with Dichloroacetate (DCA)

A key point of regulation for PDH is the phosphorylation status of its e1α subunit, controlled by the antagonistic actions of PDKs and PDPs. In an attempt to restore oxidative metabolism, cells were treated with the PDK inhibitor DCA. DCA is used clinically to restore the balance of glycolysis and oxidative metabolism in cases of metabolic acidosis and has been shown previously to enhance oxidative phosphorylation of glucose in the brain (Itoh et al., 2003). EV and SH-SY5Y_{B1} cells were treated overnight with increasing concentrations of DCA before a $^{14}$C-glucose oxidation assay was performed. In the absence of DCA treatment, a significant reduction in glucose oxidation was again observed in BACE1 overexpressing cells (EV; 5.05 ± 0.17 pmol/min/mg vs. BACE1; 3.55 ± 0.10 pmol/min/mg, p < 0.05, n = 5, Fig. 5.12). Following overnight treatment with 100μM DCA, a significant increase in glucose oxidation rate is observed in both cell types when compared their control states, however the BACE1 cells remain significantly impaired (EV; 6.64 ± 0.52 pmol/min/mg vs BACE1; 5.23 ± 0.17 pmol/min/mg, p < 0.05, n = 5, Fig. 5.12). Next, the phosphorylation status of the PDH e1α subunit was investigated to better understand the reduced enzyme activity and function (5.13A). In SH-SY5Y_{B1} cells, there was a significant increase in the ratio of phosphorylated to unphosphorylated PDH e1α subunit (EV 1 ± 0.06 vs. BACE1; 1.52 ± 0.14, p < 0.04, n = 4, Fig. 5.13B). Treatment with DCA resulted in a significant reduction in the phosphorylation of
Figure 5.12: BACE1-mediated impairment in glucose oxidation is partially reversed following dichloroacetate treatment in SH-SY5Y cells.

(A) $^{14}$C-glucose oxidation performed following an overnight treatment with the pyruvate dehydrogenase kinase inhibitor dichloroacetate (DCA). In control conditions, BACE1 overexpression again results in a significant reduction in glucose oxidation rate ($p < 0.05, n = 5$). DCA treatment ($100\mu M$) results in a significant increase in glucose oxidation rate from baseline in both cell types ($p < 0.05, n = 5$). However, the glucose oxidation rate in BACE1 overexpressing remains significantly lower than EV following this treatment ($p < 0.05, n = 5$).
Figure 5.13: BACE1 overexpressing SH-SY5Y cells display enhance phosphorylation of the e1α subunit of PDH under control and DCA-treated conditions.

(A) Representative western blot showing phosphorylated and total levels of the pyruvate dehydrogenase e1α subunit in SH-SY5Y cells. (B) Densitometric analysis showing a significant increase in the phosphorylation of the e1α subunit of PDH in BACE1 overexpressing cells under control conditions (p < 0.05, n = 4). The level of phosphorylation in both cell types decreases significantly (relative to control) following overnight treatment with DCA (p < 0.05, n = 4), with the level remaining significantly higher in the BACE1 overexpressing cells (p < 0.05, n = 4).
PDH in both EV (10µM DCA; reduced to 0.69 ± 0.05 relative to EV control, 100 µM; 0.61 ± 0.06 relative to EV control, p < 0.05, n = 4, Fig. 5.13B) and BACE1 cells (10µM; 0.96 ± 0.15 relative to EV Control, 100µM; 0.89 ± 0.07 relative to EV control, p < 0.05, n = 4, Fig. 5.13B). However, there remained significantly increased e1α phosphorylation in SH-SY5Y_B1 cells (EV; 0.61 ± 0.06 vs. BACE1 0.89 ± 0.07 relative to EV control, p < 0.05, n = 4, Fig. 5.13B).

5.2.7 BACE1 Overexpression Results in Impaired TCA Cycle Enzyme Function in SH-SY5Y Cells

It has been shown previously that TCA cycle enzyme function is impaired in AD brain tissue, with notable reductions in α-ketoglutarate (α-KGDH) dehydrogenase activity being observed (Gibson et al., 1998, Bubber et al., 2005). α-KGDH controls the second oxidative reaction of the TCA cycle, the decarboxylation-mediated conversion of α-ketoglutarate to succinyl CoA (Fig 5.14A). BACE1 overexpression resulted in a large reduction in α-KGDH activity, which was not observed in SH-SY5Y_mB1 cells (EV; 1 ± 0.14 vs. BACE1; 0.61 ± 0.06 vs. mBACE1; 0.98 ± 0.13, p < 0.01, n = 6, Fig. 5.14B). These data indicate a requirement for BACE1 activity to produce the inhibition of α-KGDH.

Thus, increased BACE1 activity appears to induce a large inhibition of PDH and α-KGDH activity, these enzymes control decarboxylation reactions in the mitochondrial bioenergetic pathway. Consequently, the activity of the final enzyme controlling such a reaction in the TCA cycle, isocitrate dehydrogenase (IDH) was examined for sensitivity to BACE1 activity. The IDHs catalyse the oxidative decarboxylation of
Figure 5.14: BACE1 overexpression impairs α-ketoglutarate dehydrogenase activity in SH-SY5Y cells.

(A) Schematic diagram showing the α-ketoglutarate dehydrogenase-controlled conversion of α-ketoglutarate to succinyl CoA in the TCA cycle. (B) Significant reduction in α-ketoglutarate dehydrogenase activity following BACE1 overexpression (p < 0.01, n = 6) with this change absent in cells overexpressing the secretase dead mutant BACE1 (mBACE1; p < 0.01, n = 6).
isocitrate to $\alpha$-ketoglutarate while reducing NAD(P)$^+$ to NAD(P)H (Fig. 5.15A). IDH3 is a multisubunit enzyme found in the mitochondrial matrix, where it plays a key role in the TCA cycle and is modulated positively (ADP, calcium and citrate) and negatively (ATP, NADH, NADPH) by a number of intracellular factors (Gabriel et al., 1985). The NADP$^+$-dependent isoforms (IDH1 and 2) however share considerable homology, and are unrelated to IDH3. IDH1 displays a mainly cytoplasmic and peroxisomal distribution, while IDH2 localises to the mitochondria (Nekrutenko et al., 1998; Geisbrecht and Gould 1999; Yoshihara et al., 2001). NADPH generated by IDH1 has previously been shown to play a role in oxidative stress response, peroxisomal fat and cholesterol metabolism and pancreatic islet glucose sensing (Minard et al., 1999; Ronnebaum et al., 2006). IDH2 meanwhile has been proposed to provide a reverse pathway for the IDH3-driven isocitrate to $\alpha$-ketoglutarate conversion and has been shown previously to catalyse the conversion of $\alpha$-ketoglutarate and NADPH to isocitrate and NADP$^+$ (Comte et al., 2002). Ward and colleagues provided further evidence of this phenomenon by demonstrating reduced glutamine to citrate flux (requiring this reverse reaction) upon knockdown of IDH2 in glioma cells (Ward et al., 2010). These previous studies implicate IDH as a potential modulator of a number of cellular metabolic processes.

Through the use of differential supplementation in the reaction mixes during the IDH activity assay, the NAD$^+$-supplemented reaction showing the activity of the mitochondrial IDH3 isoform while the NADP$^+$-dependent activities of the cytoplasmic IDH1 and mitochondrial IDH2 isoforms can be observed. In SH-SY5Y$_{B1}$ cells, there was a significant reduction in NAD$^+$-dependent IDH activity (EV; 29.51 ± 2.54 nmole/min/ml vs. BACE1; 18.92 ± 2.40 nmole/min/ml, p < 0.05, n
Figure 5.15: BACE1 overexpression impairs isocitrate dehydrogenase activity in SH-SY5Y cells.

(A) Schematic diagram showing the isocitrate dehydrogenase-controlled conversion of isocitrate to α-ketoglutarate in the TCA cycle. (B) BACE1 overexpression results in significant reductions in the activity of the NAD⁺-dependent IDH isoform (IDH3; p < 0.05, n = 4), the NADP⁺ isoforms (IDH1 and 2; p < 0.001, n = 4) and in total IDH activity (p < 0.001, n = 4).
= 4, Fig. 5.15B), NADP⁺-dependent IDH activity (EV; 145.30 ± 6.23 nmole/min/ml vs. BACE1; 89.92 ± 2.11 nmole/min/ml, p < 0.001, n = 4, Fig. 5.15B) and total (NAD⁺ and NADP⁺-dependent activity; EV; 149.80 ± 2.57 nmole/min/ml vs. BACE1; 71.60 ± 2.00 nmole/min/ml, p < 0.001, n = 4, Fig. 5.15B).

Impaired TCA cycle function has previously been shown to result in a shift in the metabolism of glutamine, which is a key amino acid in neuronal function, as it contributes to both metabolic pathways as a contributor of carbon to the TCA cycle and neurotransmitter production (for review see DeBerardinis and Cheng 2010). During periods of hypoxia and impaired mitochondrial function, it has been shown that glutamine metabolism may also be switched from oxidative to reductive (Mullen et al., 2011; Wise et al., 2011). Glutamine is first converted to glutamate by the action of glutamine dehydrogenase and subsequently to α-ketoglutarate by glutamate dehydrogenase. Under normal conditions, this additional source of α-ketoglutarate can supplement ATP generation through oxidative metabolism, however it can also be metabolised in a reductive manner to form citrate to supplement cell proliferation (Wise et al., 2011). To look at the potential effect of increased BACE1 activity on glutamine metabolism, the Seahorse was again utilised. First OCR and ECAR were measured when EV and SH-SY5Y_B1 cells were provided either glucose alone or glucose and L-glutamine as substrate. As was observed previously, EV cells displayed a significantly increased OCR (EV; 8.42 ± 0.28 pmolO₂/min/µg vs. BACE1; 5.64 ± 0.39 pmolO₂/min/µg, p < 0.001, n = 10, Fig. 5.16A) and significantly increased ECAR (EV; 1.19 ± 0.13 mpH/min/µg vs. BACE1; 1.80 ± 0.10 mpH/min/µg, p < 0.01, n = 10, Fig. 5.16B) when provided 2.5mM glucose as a substrate and this trend was unaltered with 4mM L-glutamine present (OCR – EV;
Figure 5.16: BACE1 overexpression induces reductive glutamine metabolism in SH-SY5Y cells.

Significant reduction in OCR (A; p < 0.001, n = 4-10) and OCRE:ECAR ratio (C; p < 0.001, n = 4-10) and significant increase in ECAR (B; p < 0.01 and p < 0.05, n = 6-10) again observed in the presence of 2.5mM glucose alone with no significant change elicited by addition of 4mM L-glutamine. (D) SH-SY5Y cells provided 4mM glutamine as the sole substrate show a significant reduction in OCR (p < 0.05, n = 8).
9.06 ± 0.50 pmolO₂/min/µg vs. BACE1; 5.29 ± 0.55 pmolO₂/min/µg, p < 0.001, n = 4, Fig. 5.16A, ECAR; 0.99 ± 0.27 mpH/min/µg vs. BACE1 1.70 ± 0.17mpH/min/µg, p < 0.05, n = 6, Fig. 5.16B). Taken together, these changes reflect a large decrease in the OCR:ECAR ratio with and without L-glutamine present (Glucose – EV; 1.00 ± 0.12 vs. BACE1; 0.41 ± 0.04, p < 0.001, n = 10 and Glucose + L-Glutamine – EV; 1.00 ± 0.10 vs. BACE1; 0.42 ± 0.1, p < 0.001, n = 4, Fig. 5.16B). Finally there was significant reduction in the oxygen consumption when SH-SY5YEV and SH-SY5YB1 cells were provided 4mM L-glutamine as a substrate (EV; 7.07 ± 0.75 pmolO₂/min/µg vs. BACE1; 4.59 ± 0.55 pmolO₂/min/µg, p < 0.05, n = 8, Fig. 5.16D). These data suggest that L-glutamine does not play a significant role in basal metabolism in SH-SY5Y cells and that increased BACE1 activity may blunt its oxidative metabolism.

This effect whereby increased BACE1 protein expression inhibits enzyme activity was however not universal to all TCA cycle enzymes. As way of a control, fumarase activity was measured, as changes in its activity have not been implicated in AD. Both SH-SY5YB1 and SH-SY5YmB1 displayed significant reductions in fumarase activity (EV; 1.00 ± 0.02 vs. BACE1; 0.93 ± 0.01 vs. mBACE1; 0.91 ± 0.02, to BACE1 p < 0.001 and to mBACE1 p < 0.001, n = 7, Fig. 5.17A) and quantity (EV; 1.00 ± 0.02 vs. BACE1; 0.94 ± 0.01 vs. mBACE1; 0.91 ± 0.02, to BACE1 p < 0.01 and to mBACE1 p < 0.01, n = 7, Fig. 5.17B). Taken together these data reflected no change in fumarase specific activity (EV; 1 ± 0 vs. BACE1 1.00 ± 0.00 vs. mBACE1; 0.99 ± 0.00, n = 7, Fig. 5.17C). These data suggest that increased BACE1 activity has caused metabolic lesions at the level of specific enzymes and not simply a universal decrease in TCA cycle enzyme function or mitochondrial mass.
Figure 5.17: BACE1 overexpression results in no change to fumarase specific activity in SH-SY5Y cells.

(A) SH-SY5Y_{31} and SH-SY5Y_{m31-} cells display a significantly reduced fumarase activity (A, BACE1, p < 0.001 and mBACE1, p < 0.01 relative SH-SY5Y_{Ev}, n = 7) as a result of having a significantly decreased fumarase quantity (B, BACE1 and mBACE1, p < 0.05 relative to SH-SY5Y_{Ev}, n = 7) reflecting no change in fumarase specific activity (C, n = 7).
5.2.8 Impaired Glucose Oxidation Following BACE1 Overexpression can be Effectively Attenuated by α-Lipoic Acid Supplementation in SH-SY5Y Cells

α-lipoic acid is a naturally occurring cofactor (in the form of lipoyllysine) for a number of mitochondrial proteins (notably PDH and α-KGDH) and plays a major role in regulating the redox status of the cell. Taken as either a dietary supplement or a therapeutic agent, lipoic acid plays a key role in antioxidant defence, metal chelation transcriptional regulation, and metabolic cell signalling (for review see Shay et al., 2009).

To investigate the effect of α-lipoic acid supplementation, EV and SH-SY5Y<sub>B1</sub> cell monolayers were plated into 6-well culture plates and the media was supplemented with increasing concentrations of α-lipoic acid for 48 hours prior to the glucose oxidation rate being measured. In the absence of α-lipoic acid supplementation, again there was a significant reduction in glucose oxidation observed in the SH-SY5Y<sub>B1</sub> cells (EV; 11.88 ± 0.31 pmol/min/mg vs. BACE1; 9.06 ± 0.19 pmol/min/mg, p < 0.001, n = 6, Fig. 5.18). Supplementation with α-lipoic acid resulted in a trend toward an increase in the glucose oxidation rate of the EV cells (25µM; 13.99 ± 0.99 pmol/min/mg, 50µM; 13.79 ± 0.39 pmol/min/mg, n = 6, Fig. 5.18) while there was a significant increase in the glucose oxidation rate of the SH-SY5Y<sub>B1</sub> cells (25µM; 11.6 ± 0.58 pmol/min/mg, 50µM; 12.61 ± 0.38 pmol/min/mg, p < 0.05 and p < 0.001 respectively, n = 6, Fig. 5.18) with the 50 µM rate no longer significantly different to that of the corresponding EV rate. Taken together these data suggest that the impaired glucose oxidation observed in SH-SY5Y<sub>B1</sub> cells can be more effectively reversed with lipoic acid supplementation than DCA treatment. This is likely due to
Figure 5.18: BACE1-mediated impairment in glucose oxidation fully reversed by α-lipoic acid supplementation in SH-SY5Y cells.

(A) 14C-glucose oxidation assay performed following 48 hour α-lipoic acid treatment. In the control condition, BACE1 overexpression results in a significant reduction in glucose oxidation rate ($p < 0.001$, $n = 6$). Supplementation with α-lipoic acid results in a significant increase in glucose oxidation in the BACE1 overexpressing cells (25μM; $p < 0.05$, $n = 6$ and 50μM; $p < 0.001$, $n = 6$), with the rate following 50μM α-lipoic acid no longer significantly lower than corresponding EV cells.
the fact that it acts as a cofactor for both PDH and α-KGDH and alleviates the inhibited respiration at two points rather than just PDH (as is the case for DCA).

5.2.9 BACE1-mediated Impairment in Oxidative Glucose Use can be Effectively Attenuated by Ketone Body Supplementation

Ketone bodies are natural by-products of fatty acid β-oxidation in the liver and were first discovered in the urine of diabetic patients and subsequently thought to be the result of incomplete fat oxidation. However further research found that they were in fact a meaningful metabolite for extra-hepatic tissues and their use by the brain was first described by Cahill and colleagues in 1966 and demonstrated by Owen et al in 1967. Using brain catheterisation in obese patients undergoing sustained starvation it was shown that ketone bodies could replace glucose as a fuel for the brain (Owen et al., 1967). Following uptake by hepatocytes, acyl-CoA synthetases convert fatty acids into CoA esters. In the case of medium and short chain fatty acids, these acyl-CoAs can then readily cross the mitochondrial membrane for β-oxidation to produce acetyl-CoA and acetoacetate. Ketogenesis will occur when the energy requirements of the liver are met. It involves the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA via the action of thiolases first described by Lynen and colleagues. This can then be condensed further with acetyl-CoA to form hydroxymethylglutaryl-CoA (HMG-CoA) synthase and this can then be dissociated to acetoacetate and acetyl-CoA by HMG-CoA lyase. The final ketogenic reaction is the NADH-dependent reduction of acetoacetate to form the body’s most abundant ketone body, β-hydroxybutyrate (β-HB; for review see Van Itallie and Nufert 2003).
To look at the effectiveness of ketone body supplementation, increasing concentrations of β-HB were added during the course of a $^{14}$C-glucose oxidation assay. In the absence of ketone body supplementation, a significantly reduced glucose oxidation rate was again observed in SH-SY5Y B1 cells (EV; 1 vs. BACE1; $0.74 \pm 0.03$, $p < 0.001$, $n = 4-6$, Fig. 5.19). Increasing doses of β-HB effectively attenuated this deficit, with 1 and 10µM concentrations returning the glucose oxidation rate to EV levels (1µM; $0.91 \pm 0.05$, 10µM; $1.09 \pm 0.04$ relative to control, $n = 4-6$, Fig. 5.19).

Work by Danial and colleagues has shown that knocking out of the anti-apoptotic Bcl2 family protein BAD, resulted in a significant switch in the substrate preference of primary neurons, with an enhanced capacity to utilise ketones being observed in the BAD knockout neurons (Gimenz-Cassina et al., 2012). Having observed a positive effect of ketone body supplementation on oxidative metabolism in SH-SY5Y B1 cells, we then looked at BAD protein levels (Fig. 5.20A). A significant reduction in BAD protein levels was noted in the SH-SY5Y B1 cells (EV $1.00 \pm 0.10$ vs. BACE1; $0.39 \pm 0.05$, $p < 0.01$, $n = 4$, Fig. 5.20B). Taken together, these data suggest that ketone bodies are able to preferentially increase glucose oxidation in SH-SY5Y B1 cells and that this enhanced use of alternate substrates to glucose may be driven by a reduction in BAD protein levels.
Figure 5.19: BACE1-mediated impairment in glucose oxidation effectively attenuated by ketone body supplementation

(A) $^{14}$C-glucose oxidation assay performed in the presence of β-hydroxybutyrate. In the control condition, BACE1 overexpression results in a significant percentage reduction in glucose oxidation rate ($p < 0.001$, $n = 4-6$). Supplementation with β-hydroxybutyrate gradually reverses this, with the 1 and 10µM groups no longer significantly different to corresponding EV groups ($n = 4-6$).
Figure 5.20: Enhanced ketone body use following BACE1 overexpression due to reduced BAD protein levels

(A) Representative western blot showing the total level of the pro-apoptotic Bcl-2 family protein, BAD, in EV and BACE1 overexpressing cells. (B) Densitometric analysis showing a significant reduction in BAD protein levels following BACE1 overexpression (p < 0.01, n = 4).
5.2.10 BACE1 Overexpression Significantly Alters the Balance of Oxidised and Reduced Nicotinamide Dinucleotide and Alters SIRT1-PGC1 Signalling in SH-SY5Y Cells

A key coenzyme involved in a number of reactions of cellular substrate metabolism is NAD, which is an essential regulator of the redox status of the cell. The oxidised form of NAD (NAD$^+$) is reduced to NADH regularly as part of the early metabolic pathways (glycolysis and the TCA cycle) and this reaction is reversed by the action of enzymes comprising the ETC during oxidative metabolism. Having observed a shift away from oxidative metabolism of substrate following BACE1 overexpression and with a compensatory increase in glycolytic, it was hypothesised that there would be resultant shift in the NAD$^+$/NADH balance. This was looked at using a specific NAD$^+$ to NADH ratio assay and following BACE1 overexpression a significant, secretase activity dependent, reduction in NAD$^+$ was observed (EV; 25.17 ± 2.18 pmole/well vs. BACE1; 9.14 ± 0.98 pmole/well vs. mBACE1; 23.92 ± 1.63 pmole/well, $p < 0.001$, $n = 7$, Fig. 5.21A). Allied to this there was no significant change observed in NADH (EV; 3.12 ± 0.22 pmole/well vs. BACE1; 2.54 ± 0.3 pmole/well vs. mBACE1; 3.34 ± 0.34 pmole/well, $n = 7$, Fig. 5.21B) combining to reveal a significant reduction in the NAD$^+$ to NADH ratio in the BACE1 overexpressing cells (EV; 8.35 ± 0.97 vs. BACE1; 4.04 ± 0.78 vs. mBACE1; 7.79 ± 1.14, $p < 0.05$, $n = 7$, Fig. 5.21C).

In an effort to better understand the potential mechanism underlying these BACE1-mediated changes in oxidative metabolism, focus shifted to the SIRT pathway, best characterised for its role in the anti-ageing effects of calorie restriction. The
Figure 5.21: BACE1 overexpression significantly alters nicotinamide adenine dinucleotide (NAD) redox status in SH-SYSY cells. (A) Significant reduction in the oxidised NAD form, NAD\(^+\) following BACE1 overexpression (p < 0.001, n = 7). (B) No significant change in the level of the reduced form of NAD, NADH following BACE1 overexpression (n = 7). (C) Resultant significant change in the ratio of NAD\(^+\)/NADH ratio following BACE1 overexpression (p < 0.05, n = 7).
activation of this pathway is known to activate transcriptional programs that increase oxidative metabolism, enhance mitochondrial efficiency and increase oxidative stress resistance via the deacetylation of its substrate PGC1α (for review see Haigis and Sinclair 2003). When EV and SH-SY5Y_{B1} cells were compared, a significant reduction in the protein level of both SIRT1 (EV; 1.00 ± 0.06 vs. BACE1; 0.74 ± 0.09, p < 0.05, n = 6, Fig. 5.22A) and PGC1 (EV; 1.00 ± 0.06 vs. BACE1; 0.86 ± 0.04, p < 0.01, n = 6-8, Fig. 5.22B) was observed. A second point of regulation of PGC1 is its phosphorylation by AMPK (Jager et al., 2007), and as previously mentioned (Fig. 4.17) AMPK activation and signalling was significantly blunted in SH-SY5Y_{B1} cells.
Figure 5.22: BACE1 overexpression significantly alters SIRT1-PGC1 protein levels in SH-SY5Y cells.

(A) Representative western blot showing total SIRT1 protein levels in EV and BACE1 overexpressing cells. (B) Densitometric analysis showing a significant reduction in SIRT1 protein levels following BACE1 overexpression (p < 0.05, n = 8). (C) Representative western blot showing total PGC1 protein levels in EV and BACE1 overexpressing cells. (D) Densitometric analysis showing a significant reduction in PGC1 protein levels following BACE1 overexpression (p < 0.01, n = 8-8).
5.3 Discussion

5.3.1 Hypometabolism in AD

As previously described, decreased central metabolism has been as an invariant pathophysiological event occurring in AD progression, and it has been hypothesised that this may occur years, and even decades prior to symptom presentation (Section 1.4.6). Despite these observations of reduced glucose metabolism being predictive of later cognitive decline and AD symptom presentation, relatively little about the cellular mechanisms underlying these changes is known. Here, it was observed that BACE1 overexpression significantly impairs full oxidation of glucose and that this is not simply as a result of reduced incorporation of glucose into the cell. This significantly reduced glucose oxidation was directly attributable to the secretase activity of BACE1 having not been observed following the overexpression of a secretase dead mutant form of BACE1 (Fig. 5.3 and 5.4).

The impairment in glucose oxidation rate appears to be driven by a change in which the cell handles glucose, with the data from Seahorse Extracellular Flux Analyser reflecting a shift in respiration favouring enhanced glycolysis and reduced oxidative phosphorylation in normoxic conditions, so called aerobic glycolysis (Fig. 5.5). Previous studies have suggested that this metabolic adaptation confers later susceptibility to cell death of neurons in the default network (Vaishnavi et al., 2009). In an attempt to model this high energy demand (as would occur in an active neuronal network) was mimicked by treatment with the mitochondrial uncoupler, DNP. Following this treatment, chronic elevation of BACE1 protein expression left cells
unable to maintain enhanced 2-DG uptake under increasing energy demand (Fig. 5.7A). The ensuing impairment in 2-DG uptake also correlated with the increased appearance of apoptotic cell death markers (5.7B).

This increased proportion of glucose used by the glycolytic pathway may in turn diminish the activity of the other glucose-consuming pathway in neurons, the PPP. This may ultimately underlie the enhanced cell death marker production under energy challenge. Recently, it has been shown that glucose use, via the PPP, acts to inhibit apoptosis in both cancer cells as well as neurons via a mechanism of cytochrome c activation (Vaughn et al., 2008). It may therefore be beneficial to see whether BACE1 overexpression does alter the substrate flux through the PPP and this could be done through the use of differentially $^{14}$C-labelled glucose molecules and PPP inhibitors as previously described in neurons (Rodriguez-Rodriguez et al., 2013). With these observations in mind, it would be interesting to move the present findings into an electrically active model and to see whether increased BACE1 results in impaired neuronal activity.

5.3.2 Altered Mitochondrial Function Following BACE1 Overexpression

It was hypothesised that this altered cellular respiration came as the result of either impaired mitochondrial functioning or as an adaptation to reduced substrate flow to the mitochondria. Through the use of the Seahorse, the function of the ETC as a whole was examined by the additions of carefully titrated doses of mitochondrial inhibitors (oligomycin, FCCP, rotenone and antimycin-A) and these data suggest that chronic elevation of BACE1 does not impair ETC function. This is a little surprising
as previous studies suggest that Aβ can directly impact on ETC function. Work by Anne Eckert’s group has demonstrated a significant Aβ-dependent reduction in the activity of complex IV in mitochondria isolated from APP<sub>wt</sub> overexpressing SH-SY5Y cells and neurons from the 3 x Tg AD mouse model (Rhein et al., 2009a; 2009b). This direct effect on complex IV was also previously reported by Casley et al who demonstrated a significant reduction in complex IV activity (around 50%) following treatment of non-synaptic neuronal mitochondria with Aβ<sub>25-35</sub> (Casley et al., 2002b).

It could be that the Mito Stress Test protocol does not have the resolution to observe these changes at the single complex level and therefore, repeating the assessment of mitochondrial function in a mitochondrial preparation may prove beneficial. It is also possible that these changes in the activity of a single complex are not significant enough to alter whole cell respiration and so are not observed in the whole cell setting. There is also the issue of how these experiments were performed, with the isolated mitochondria work being conducted with organelles in buffered suspension solution while the current studies are conducted on adherent cells in culture. This set up would allow the native cellular mechanisms to determine substrate delivery and impart any compensatory mechanisms in response to ETC manipulation that would not be present in the isolated mitochondrial preparation. With this in mind, work by Yao et al demonstrated that the cellular response of primary hippocampal neurons obtained from 3 x Tg AD animal model displayed an unaltered response to oligomycin and rotenone/antimycin A additions as is the case in our cells. This group did however observe a small but significant decrease in the maximal respiration in these neurons but this may be an effect elicited by the cooperative impairment of mitochondria by Aβ and tau pathologies in this model (Yao et al., 2009).
5.3.3 BACE1-mediated Regulation of Substrate Flux

This left reduced substrate delivery to the mitochondria as the driver for the altered respiration observed in SH-SY5Y_{B1} cells. Indeed, significant reductions in both the activity of PDH and also oxidation when cells were provided pyruvate as a substrate were observed. This impairment at the level of PDH, a key enzyme in cellular fuel partitioning, appears to be a core adaptation in AD aetiology and has been noted in a number of settings and cellular models. One of the first such descriptions came in the form of a reduced activity of the PDHC in the AD brain (Sheu et al., 1985). Work by Yao et al also demonstrated a reduced protein level of the E1α subunit of the PDHC in the brains of 3 x Tg AD animals (Yao et al., 2009). Casley and colleagues, who demonstrated a significant effect of Aβ25-35 on PDHC activity in isolated mitochondria, postulated a causative role for Aβ in the change. This work also showed that the effect was not dependent on the presence of biological membranes as the same results were obtained in permeabilised mitochondria and argue that Aβ directly impairs PDH activity (Casley et al., 2002b). There may however also be a role for the oxidative stress conditions that is directly induced by Aβ. Work by a number of groups have demonstrated that treatment of primary neuronal cultures with Aβ results in an increase in oxidative stress markers such as, protein carbonyls and lipid peroxidation products (Harris et al., 1995; Mark et al., 1997). This state of oxidative stress may also directly impact PDHC activity as demonstrated in a study by Humphries and Szweda. This work showed that treatment of cardiac mitochondria with HNE resulted in a significant reduction in PDHC activity. This impairment was shown to occur as a result of a reduction in the amount of lipoic acid (essential PDH cofactor) that was covalently bound to the E2 subunit of the PDHC with increasing
concentrations of HNE (Humphries and Szweda 1998). Subsequent evidence for this impairment in PDH activity under oxidative stress conditions come from a study by Sims and colleagues who showed that isolated brain mitochondria showed a significant impairment in pyruvate metabolism in response to hydrogen peroxide (Sims et al., 2000). This oxidative stress-induced reduction in lipoic acid binding to the PDHC may be reversed by supplementation, hence the observed reversal of the impaired glucose oxidation shown here (Fig. 5.18).

A key point of regulation for PDHC activity is the ATP-dependent phosphorylation status of the α-subunit, PDH. Serine phosphorylation can occur at 3 potential sites with phosphorylation at site 1 causing inactivation of the complex while sites 2 and 3 can be phosphorylated with minimal loss of enzymatic activity (Jha et al., 2012). Phosphorylation at sites 1 and 2 can be achieved by any of the PDK isoforms, with site 3 phosphorylation specifically performed by PDK1 (Bowker-Kinley, 1998). This phosphorylation-mediated control of PDH may also play a key role in vivo with the active component of total PDH only thought to be around 30% in the rat brain at rest (Ksiezak et al., 1982).

Following BACE1 overexpression, a significant increase in the phosphorylation of the E1α subunit of PDH was observed and this was partially reversible by PDK inhibition following treatment with DCA (Fig. 5.12 and 5.13). The fact that E1α phosphorylation remains significantly higher in the BACE1 overexpressing cells in the presence of PDK inhibition suggests that there may be a secondary factor contributing to phosphorylation of PDH. Work by Hoshi and colleagues propose a role for the potentially AD-associated kinase glycogen synthase kinase 3β (GSK-3β;
alternatively tau protein kinase 1 (TPK1)) in the regulation of PDH. This study demonstrated that GSK-3β could phosphorylate the α-subunit of PDH in an ATP-dependent manner in mitochondria isolated from pig heart. This work also demonstrated that GSK-3β was localised to the mitochondria in hippocampal neurons and that its activity could be induced in primary neuronal cultures by treatment with Aβ25-35 (Hoshi et al., 1996). This potential connection was observed in work by Takashima and colleagues who showed that introduction of antisense oligonucleotides reduced GSK-3β levels and resulted in a significant increase in PDH activity in primary neurons (Takashima et al., 1993). There may also be the potential for altered balance between the PDHC inhibiting PDKs and the activating pyruvate dehydrogenase phosphatases (PDP1 and 2; Roche et al., 2001). In this regard, it has previously been shown in the HEPG2 liver cells line that insulin can produce a coordinated increase and decrease in PDP and PDK gene promoter activity respectively (Wang et al., 2009). With this in mind, it may be useful to see if the markers of reduced insulin signalling (decreased PI3K/PKB and increased MAPK signalling; Fig. ??) has the same effect on the balance of PDK/PDP actions. Reduced signalling through PI3K and PKB may also underlie enhanced GSK3β activity and subsequent phosphorylation of PDHC. Previous studies have shown that blunted PI3K signalling (through PKB) result in reduced phosphorylation (and subsequent activation of GSK3β) in both cell lines and the APP/PS1 AD mouse model (Arboleda et al., 2010; Jimenez et al., 2011). Finally, evidence from cancer cell lines have suggested that tyrosine phosphorylation of PDK1 (at sites 143, 243 and 244) leads to increased ATP and PDH binding leading to PDHC inactivation and a Warburg effect driven shift towards aerobic glycolysis (as observed with increased ECAR and decreased OCR following BACE1 overexpression). Taken together, the present and
previous data suggest a system whereby increased BACE1 and/or aberrant Aβ production can result in either direct or indirect regulation of PDH activity and protein level, fundamentally altering mitochondrial function. It may therefore be beneficial to look at the potential regulation of the other PDK isoforms to see if these are regulated in the same way.

Further investigation of mitochondrial enzyme function focussed on the TCA cycle, with significant reductions observed in the activities of α-KGDH and both NAD⁺- and NADH-dependent IDH reactions. Gibson and colleagues first described reduced α-KGDH activity in cases of AD in 1988 as part of their work into the effect of thiamine deficiency on mitochondrial enzyme function (Gibson et al., 1988). The group hypothesised that impaired functioning of PDH and α-KGDH linked to altered calcium homeostasis and oxidative metabolism and may underlie neuronal degeneration. It has also been reasoned that altered oxidative metabolism and TCA cycle function could result in a loss of cholinergic signalling as well as impaired glutamate uptake/metabolism resulting in uncontrolled release and resultant excitotoxic injury in the brain (Hirsch and Gibson 1984). Subsequent studies have demonstrated that, like PDH, α-KGDH can be fundamentally impaired in an Aβ-dependent manner and following exposure to HNE (Humphries and Szweda 1998; Casley et al., 2002b). A similar mechanism whereby oxidative stress-induced depletion of lipoic acid drives impairments in PDH and α-KGDH may also underlie the currently observed reduction in oxidative metabolism as it is effectively attenuated following supplementation with α-lipoic acid (Fig. 5.18). This HNE-induced impairment in α-KGDH was also shown to fundamentally impair NADH-linked respiration (Humphries et al., 1998) as its activity plays a key, rate determining, role
in respiration. Inhibition of α-KGDH, in addition to the direct effect on respiration has also been shown to result in the release of cyochrome c from the mitochondria and this was associated with the induction of pro-apoptotic pathways and collapse of the mitochondrial membrane potential (Huang et al., 2003). Chronic depletion of α-KGDH activity through heterozygotic deletion of DLST (the gene that encodes the E2k subunit of α-KGDH) has been shown to alter brain metabolism and also results in accelerated learning and memory deficits and a more aggressive Aβ pathology when these animals are crossed with AD transgenic animals (Tg19959; Dumont et al., 2009).

Recently, a paper by Bubber and colleagues demonstrated a down regulation in PDH, α-KGDH and IDH activity in AD cases, with the decline in PDH most closely correlating with clinical stage cognitive decline (Bubber et al., 2005). In further agreement with the present data this group also observed that the decline was not a universal event with increases in the activities of succinate and malate dehydrogenase observed and unchanged activities of the remaining TCA cycle enzymes reported. As reported here, following BACE1 overexpression, no change in fumarase specific activity was observed. This concerted upregulation of the second half of the TCA in the face of progressive decline in the initial reactions has been reported during chronic hypoxia (Weinberg et al., 2000). In yeast exposed to hypoxic conditions, a novel anaerobic mitochondrial pathway was uncovered whereby oxaloacetate is converted to succinate via the reversal of malate dehydrogenase which is then utilised by complex I of the ETC (McCammon et al., 2003). As this screen was carried out in yeast, it remains unclear if the same adaptation can occur in mammals and indeed neurons. It has also been hypothesised that under conditions of cerebral ischemia and
hypoglycemia succinate can be formed as an end product of anaerobic glycolysis and via the action of malate dehydrogenase and the reversal of the succinate dehydrogenase reaction (Hoyer and Krier 1986). Taken together, these data suggest that under conditions of both chronic elevation of BACE1 and AD, alternate metabolic pathways may be initiated in an attempt to alleviate impaired metabolic flux.

5.3.4 Potential Benefits of Ketone Body and Alpha-Lipoic Acid Supplementation in AD

The current studies also highlighted the potential for ketone supplementation as an effective mediator to alleviate the BACE1-driven impairment in glucose oxidation. Ketone body supplementation was first shown to provide an extremely efficient aid to metabolism in the perfused, working heart model whereby addition of ketone bodies (β-HB/acetoacetate) to the heart perfusate resulted in a large increase in TCA cycle intermediate levels, an increase akin to application of insulin (Kashiwaya et al., 1994; 1997; Sato et al., 1995). Ketone supplementation also resulted in a significant increase in the force generated by the heart and a corresponding decrease in oxygen consumption, suggesting enhanced metabolic efficiency. It was also stated during these studies by Robert Veech that “ketones bypassed the block in glucose transport caused by lack of insulin…(and) also bypassed the block in PDH by providing an alternative source of acetyl CoA”. More recently, this idea that ketone bodies have the ability to increase metabolic efficiency was also observed in the mouse brain whereby a one-month ketogenic diet altered mitochondrial components at the DNA and protein level (Selfridge et al., 2014). Ketone bodies have also proved effective at
the cellular level where it has been shown that β-HB can protect primary neuronal cultures from Aβ-induced toxicity and that transgenic mice carrying the “London” APP mutation displayed decreased Aβ levels (Van der Auwera et al., 2005). A concerted effect of reducing oxidative damage and increasing maximal, complex I-driven respiration of parietal lobe mitochondria in an aged dog model was also accompanied by a reduction in APP levels following ketosis induction (Studzinzki et al., 2008). Finally, an early bioenergetic failure in 3xTg AD model can be reversed by 2-DG-induced ketonemia (Yao et al., 2009). In addition to these effects on mitochondrial function and respiration, the present data also show that ketone body supplementation can also specifically increase the rate at which glucose is oxidised to CO₂ in SH-SY5Y_B1 cells (Fig. 5.20). This effect presumably comes as a result of a reversal of the observed impairment in PDH activity, however the precise mechanism by which this occurs cannot be proved at this time. One potential mechanism may be that the ketone body-derived oxaloacetate drives the TCA cycle and thereby relieves inhibition of PDH allowing increased substrate flux. Some positive effects on cognition have also been described following ketogenesis brought about by oral administration of medium chain triglycerides (MCTs) in cases of mild and moderate AD. One example study saw an increase in the ADAS-Cog score in APOE4 negative patients while impairment was observed in APOE4 carriers (Reger et al 2004).

Allied to the observed beneficial impact of ketone body supplementation, there was an effective attenuation of the BACE1-mediated impairment in glucose oxidation (Fig. 5.19). This finding mirrors the beneficial effects that have been reported previously in reversing diminished cerebral metabolism in models of both ageing (senescence accelerated prone mouse strain (SAMP) 8 and aged rats) and AD mice
(Farr et al 2003; Jiang et al 2013; Sancheti et al 2013). The findings of these studies demonstrated that lipoic acid supplementation effectively reversed declining glucose uptake by the brain over the course of ageing in the models. The present data add to this that the full oxidation of glucose shows a trend towards an increase in control cells, but has a stronger effect of reversing the BACE1-induced reduction. These studies also show the positive effects of lipoic acid in redressing insulin-signalling changes (reduced PI3K/PKB and increased MAPK pathway activities) over the course of ageing (Jiang et al., 2013; Sancheti et al., 2013). These findings mirror those observed earlier, whereby BACE1 overexpression induced similar changes (Fig. 4.15 and 4.16). Therefore, this restoration of effective insulin signalling may also play a role in the enhanced glucose oxidation in BACE1 overexpressing cells. Evidence from cell models also showed that lipoic acid and not its reduced form (didydro-lipoic acid; DHLA) treatment conferred resistance to Aβ and H2O2-induced cells death in primary cortical neurons (Zhnag et al 2001; Lovell et al 2003).

There is also growing evidence to support a potential role for lipoic acid supplementation as part of an AD treatment regimen. Hager and colleagues made an initial finding; whereby lipoic acid treatment was able to stabilise the MMSE scores in 9 probable AD cases (Hager et al., 2001). A more recent study also showed that declining cognitive performance could be averted via combined treatment with lipoic acid and omega-3 fatty acids. Taken together, the present data and those of previous studies represent a growing body of work that shows the potential for therapeutic benefit of neutraceutical interventions in AD. However, there remains a great deal more to be done; with larger cohort studies that encompasses double blind, placebo methodologies required.
5.3.5 BACE1-induced Changes in NAD\(^+\) and SIRT1

The present data showed a significant, BACE1 secretase activity-dependent, reduction in NAD\(^+\) levels and a resultant decrease in the NAD\(^+\):NADH ratio. The ratio determined in the current work (NAD\(^+\):NADH ~ 8) compares favourably with previous work showing that SH-SY5Y cells display a whole cell NADH:NAD\(^+\) ratio of around 0.1 (Gimenez-Xavier et al., 2006). Initially it was thought that these changes came as a result of the shift to the NAD\(^+\) consuming glycolytic pathway and the diminished activity of the NADH generating oxidative phosphorylation pathway in the mitochondria. However, for this to be true there should have been a reciprocal increase in NADH which was not observed here. This would suggest an alteration in the balance of NAD\(^+\) production and degradation/consumption. Two potential mediators of increased NAD\(^+\) consumption with links to ageing and AD are the zinc-binding nuclear enzyme poly (ADP-ribose) polymerase (PARP-1) and the type II membrane protein cluster of differentiation 38 (CD38). Recently, it has been shown that the level and activity of these two factors increase with age and play a fundamental role in the age-related decline in NAD\(^+\) levels (Braidy et al., 2013). CD38 is a multifunctional enzyme that utilises NAD\(^+\) to generate the second messenger, cyclic adenosine diphosphate-ribose (cADPR) and it was shown in this study that both its NADase and ADP ribosyl cyclase activities increase markedly in the rat brain during ageing. A significant (~ 5 x) increase in NAD\(^+\) levels was also observed in neurons from CD38 knock out animals and this was accompanied by a significant increase in the activity of the NAD\(^+\)-dependent deacteylase activity of SIRT1. Production of a single cADPR is coupled to the hydrolysis of 100 NAD\(^+\) molecules so aberrant CD38 activity could be a reason for the significant reduction
observed here (de Toledo et al., 2000). It has also been noted recently that CD38 levels are increased in the peripheral blood mononuclear cells in AD cases (Kassner et al., 2008).

Recently, interest has increased as to the potential role of PARP-1 in a number of central nervous system disorders, including: ischemia, brain injury, glutamate toxicity, Parkinson’s and amyloid lateral sclerosis (ALS; Eliasson et al., 1997; Mandir et al., 1999; 2000; Chung et al., 2004). PARP-1 overactivation has also been suggested to play a role in AD progression and an increased level of polyribosylated proteins in the AD brain has previously been observed (Love et al., 1999). Subsequent studies have also shown that Aβ can induce activation of PARP1 in astrocytes and induce neuronal death in co-cultures and that PARP-1 inhibition and PARP-1 knockout mice display enhanced protection to Aβ toxicity (Abeti et al., 2011; Kaupinnen et al., 2011). The work by Kaupinnen and colleagues also demonstrated a role for PARP-1 in Aβ-induced microglial activation through its activation of NF-κB (Kaupinnen et al., 2011). A direct role for PARP-1 in Aβ-mediated neuronal damage has also been shown in SH-SY5Y cells (Martire et al., 2013). Taken together, these data would strongly implicate PARP1 and CD38 as potential mediators of the significantly reduced NAD⁺ level observed following BACE1 overexpression.

The reduction in NAD⁺ is also likely to significantly impair the NAD⁺-dependent deacetylase activity of the SIRT family of proteins. Presently, BACE1 overexpression resulted in a significant reduction in the protein levels of both SIRT1 and one of its key targets PGC1. In work by Braidy and colleagues showed that in
addition to reduced NAD\(^+\) during ageing, there was an accompanying decrease in SIRT1 activity, which became significant across the brain regions between 12 and 24 months of age (Braidy et al., 2013). Reduced expression of SIRT1 has also been noted in neurodegenerative models and following exposure to neurotoxins (Pallas et al., 2008).

5.3.6 BACE1-dependent Alterations to the PGC1\(\alpha\)-SIRT1-AMPK Energy Sensing Network

Inactivation of SIRT1 directly impacts mitochondrial function through PGC-1 (Nemoto et al., 2005; Rodgers et al., 2005). PGC1\(\alpha\) plays a role in mitochondrial biogenesis through its action as a transcriptional coactivator of the peroxisome proliferator-activate receptor \(\gamma\) (PPAR\(\gamma\); Fernandez-Marcos and Auwrex 2011). Recently, both PGC1\(\alpha\) and PGC1\(\beta\) were shown to play a key role in the regulation of neuronal mitochondrial density (Wareski et al., 2009). As well as this role in biogenesis, PGC1\(\alpha\) has also been postulated to play a role in the composition of the mitochondria themselves, with mitochondria from muscle expressing increased PGC1\(\gamma\) showing enhanced levels of ETC complexes and ATP synthase (Wenz et al., 2009; Austin et al., 2011). Another major consequence of impaired SIRT1-PGC-1 signalling is altered mitochondrial quality control. SIRT1 up-regulation induces the mitochondrial unfolded protein response (UPR\(^{mt}\)), while inactivation of the UPR\(^{mt}\) prevented the longevity effects of the SIRT1 ortholog, SIR2.1, in worms (Mouchiroud et al., 2013). It is therefore conceivable that BACE1-induced changes in NAD\(^+\) could result, via impaired SIRT1 activity, in reduced clearance of malfunctioning mitochondria. Such an impairment of mitochondrial function and quality control may
play a role in neurodegenerative disease (e.g. AD) development as these display chronic oxidative damage to nuclear DNA (Rass et al., 2007; Wang et al., 2013). This damage may stem from the increased oxidative stress associated with defective mitochondria not cleared via mitophagy.

In addition to its regulation through deacetylation by SIRT1, PGC1α can also be regulated by phosphorylation by AMPK. AMPK is a cellular fuel sensor that is activated under low energy conditions and increases processes dedicated to ATP catabolism while reducing ATP anabolism (for review see Hardie 2007). Phosphorylation of PGC1α at threonine 177 and serine 538 are required for induction of the PGC1α promoter and it has previously been shown in muscle cells that activation of AMPK can increase NAD+ levels and that phosphorylation of PGC1α actually facilitates SIRT1-dependent deacetylation (Canto et al., 2009). The resultant aberrant PGC1α activity, brought about by impaired activation by both SIRT1 and AMPK may then induce significant changes to neuronal function. Previous studies have shown a significant reduction in both the activation of AMPK (phosphorylation of threonine 172) and PGC1α mRNA levels and hypothesise a role for these changes in an early metabolic change in the APPswe/PS1dE9 AD mouse model (Pedros et al., 2014). The current data suggest a direct role for the enhanced BACE1 activity elicited by the APPswe mutation in these observed changes. As well as potential changes in its regulation, BACE1 overexpression also resulted in a significant reduction in PGC1α protein level. This was recently also shown to occur in the AD brain and that reduction in PGC1α protein correlated with dementia severity (Qin et al., 2009). The current data showed that AMPK activity, as indicated by its
phosphorylation at threonine 172 and phosphorylation of its downstream target ACC, is significantly impaired in SH-SY5Y_{B1} cells.

Taken together, these data strongly implicate BACE1 as a central enzyme in cellular nutrient detection; given its potential inhibition of the key metabolic PGC1 transcriptional pathway via reduced SIRT1 protein expression and cofactor (NAD^+) levels as well as AMPK signalling. This comes in addition to its effect of reducing overall oxidative metabolism, with a compensatory shift to aerobic glycolysis and increased reliance on alternate fuel sources (extracellular lactate and ketone bodies). In the current cell model, these adaptations allow for continued functioning under resting conditions. However, when energy demand is increased, cells with chronically enhanced BACE1 are unable to further adapt and ultimately die. The data presented herein propose BACE1 as a potential mediator of major metabolic change in neuronal cells and further investigation is required to better understand these and identify novel targets for intervention in AD and other disorders.
Chapter 6

Final Conclusions
6.1 Role of BACE1 in Sporadic AD and Other Disorders

Currently, AD represents a major unmet medical and socioeconomic need Worldwide. In light of the ageing population demographic across the world, it is thought that the number of dementia cases (umbrella terms encompassing AD and other causes) will quadruple in the next 50 years (Agosta et al., 2013). At present, there are very few effective treatments available to halt or reverse symptom presentation, with only palliative care available. A core tenent of AD research over the last 20 years has been the amyloid cascade hypothesis. This theory is based on the observation of familial mutations that all, in one way or another act to enhance the production, and subsequent aggregation, of Aβ; beginning a series of events that culminate in substantial neuronal death (Hardy and Higgins, 1992). This led to a great deal of interest in viable targets to preclude Aβ overproduction and subsequent aggregation. One such area of interest has been the development of BACE1 inhibitors (for review see Menting and Claasen, 2014). BACE1 was initially characterised as the rate-controlling enzyme in Aβ production, with removal resulting in a complete attenuation of plaque formation in the brain (Luo et al., 2001; Roberds et al., 2001). Allied to this, increased BACE1 levels (consistently protein and activity and occasionally mRNA) have been identified in autopsied brains from AD cases (Holsinger et al., 2002; Fukomoto et al., 2002; Tyler et al., 2002; Stockley et al. 2006).

It should however be noted that recent results from potential AD treatments where the goal has been Aβ removal (either γ-secretase inhibition or targeted antibody treatment) have failed to alleviate symptoms, and in some cases have exacerbated
cognitive decline. Furthermore, BACE1 knockout animals were initially described as phenotypically normal, however subsequent studies demonstrated cognitive deficits in learning and memory tasks (Ohno et al., 2004; 2006). These data suggest a potentially detrimental effect of removing endogenous BACE1 activity completely. Indeed, recent in vitro screens have revealed a wide array of potential BACE1 substrates (Hemming et al., 2009). For this reason, a better fundamental understanding of the physiological roles for BACE1 may allow for novel targets and better understanding of the pathogenesis of sporadic AD as well as identification of novel targets for intervention in AD and other disorders.

6.2 Regulation of BACE1 and APP by Cellular Stress

The present data suggests BACE1 protein expression and activity are fundamentally regulated by a number of cellular stressors (including mitochondrial inhibition, oxidative stress, fatty acid excess and protein synthesis inhibition). These findings compare favourably with the current literature and also implicate the eIF2α-ATF4 driven integrated stress response as a mediator of this aberrant BACE1 protein expression and activity (and APP expression). This places BACE1 amongst a select number of proteins (around 3-4% of those transcribed) that are upregulated by eIF2α activity, with a more general downregulation of protein synthesis resulting from its activation (for review see Barid and Wek, 2014). Previous reports have shown the involvement of three upstream kinases (GCN2, PKR and PERK) in translating an array of stress signals (nutritional changes, viral infection and oxidative stress) to enhanced BACE1 and subsequent impairments in learning and memory (for review see Ohno 2014). In the case of the present cell model, it would be interesting to
further investigate the relative involvement of these kinases in the observed enhancement of BACE1-mediated APP processing with a view to moving the findings into a model whereby electrophysiological responses could be observed.

6.3 BACE1 as a Regulator of Glucose Uptake

Previous work from our lab has shown that BACE1 may play a central role in whole body and muscle glucose metabolism. Whole body knockout of BACE1 resulted in resistance to diet induced obesity and these animals displayed significantly enhanced glucose disposal and insulin sensitivity (Meakin et al 2012). Further work has revealed that these changes may, at least in part, be mediated via enhanced muscle glucose uptake, as a result of increased GLUT4 translocation following BACE1 inhibition (Hamilton et al., 2014). The data presented herein showed both the same basic outcomes, with BACE1 inhibition or increased sAPPα production stimulating 2-DG uptake with a reciprocal relationship whereby enhancing BACE1 activity (via protein overexpression) impaired 2-DG uptake. It would therefore be interesting to further investigate the relative contribution of the APP-derived extracellular factors (as suggested by media exchange experiments) on glucose uptake in neurons. Allied to this, Aβ-induced, calpain 1 dependent, decreases in GLUT3 expression have previously been reported (Jin et al., 2013) and so is a potential mechanism to explain the reduced 2-DG uptake following BACE1 overexpression. There may also be a role for diminished GLUT4 translocation, as evidenced by the finding that intrahippocampal application of Aβ42 induced impaired GLUT4 translocation (Pearson-Levy and McNay 2012). This attenuated transport was in addition to blunted PKB signalling and this mirrors the findings in C2C12 muscle cells that
enhanced GLUT4 translocation, following BACE1 inhibition was dependent upon PI3K signalling (Hamilton et al., 2014). GLUT4 translocation may also occur via an AMPK-AS160 pathway process as has been observed in response to contraction in muscle (Chen et al., 2008; Pehmoller et al., 2009) and it would be interesting to determine whether a similar mechanism exists in neurons. Therefore, an investigation of the relative GLUT expression in the plasma membrane under steady state and under insulin-stimulated conditions may help to delineate the mechanisms by which BACE1 overexpression impedes glucose uptake. A better working understanding of these BACE1-dependent mechanisms in the brain may in turn lead to novel targets for reversal of reduced glucose uptake, an early and potentially causative factor in AD pathogenesis.

6.4 BACE1-dependent Regulation of Neuronal Substrate Use

In addition to altering SH-SY5Y neuroblastoma 2-DG uptake, stable overexpression of BACE1 also brought about a number of changes in cellular substrate use. The focus here was placed on diminished glucose and lactate use, with both being significantly reduced following BACE1 overexpression. This impaired glucose oxidation was accompanied by a shift in metabolic profile of the cells, with a shift towards aerobic glycolysis observed. This is a novel finding and strongly implicates aberrant BACE1 activity as a potential driver of some the earliest changes observed in the brains of people who later develop AD (Vaishnavi et al., 2009; Vlassenko et al., 2009). This impaired glucose use also appeared to be mediated via inhibition of key enzymes that determine oxidative phosphorylation pathway activity: pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase. These observations are in
broad agreement with studies looking at the activity of these enzymes in autopsied brain from AD cases (Bubber et al., 2005). Taken together these decreases in glucose uptake and use are suggestive of specific, BACE1-dependent lesions that act to impair oxidative metabolism of neuronal substrates.

6.5 BACE1-mediated Change in SIRT1 signalling

In an attempt to find a mechanism that may be driving the BACE1-induced changes in metabolism, attention was turned to the SIRT1 pathway. Best characterised for its role in the longevity promoting effects of calorie restriction, more recent studies have shown a role for activated SIRT1 signalling (via PGC1α) in APP processing; through enhanced ADAM10 activity (Lee et al., 2014) and decreased BACE1 transcription (Wang et al., 2013). Herein, the data indicates a possible reciprocity, whereby shifting APP metabolism towards Aβ resulted in significantly reduced protein expression of both SIRT1 and PGC1α, blunted the activity of AMPK (a second modulator of PGC1α) as well as significantly decreased levels of the essential SIRT1 co-factor NAD⁺. This observed decrease in NAD⁺ levels was not coupled to an increase in intracellular NADH levels, as is the case in steady state physiology. These data suggest that BACE1 overexpression, or resultant downstream changes have led to activation of NAD⁺ consuming processes. Two potential mediators of such a change are PARP1 and CD38; both of which become activated during ageing and in may play a role in a number of brain disorders. It would therefore be interesting if it were via the activation of either of these proteins that the observed BACE1-dependent reduction in NAD⁺ occurs.
These data are indicative of a significant decline in SIRT1-PGC1α signalling. To further investigate these changes, the functional regulation of PGC1α, notably the level of SIRT1-mediates acetylation and AMPK-driven phosphorylation (at threonine 177 and serine 538) would be investigated in addition to looking at the downstream effects of the diminished PGC1α activity. One such event may be impaired initiation of the SIRT1-PGC1α driven mitochondrial unfolded protein response (UPR\textsuperscript{mt}; Mouchiroud et al., 2013). It is therefore conceivable that BACE1 driven changes in SIRT1 signalling (resulting from decrease NAD\textsuperscript{+} levels) may hamper clearance of malfunctioning mitochondria. This reduced mitochondrial quality control may in turn lead to enhanced ROS production and further metabolic decline, two precipitating factors in AD progression. Through the use of confocal microscopy, colocalisation of stained mitochondria (using Mitotracker) with markers for autophagic machinery (MAP1LC3; Kawajiri et al., 2010) or the lysosome (LAMP1; Pankiv et al., 2007) may reveal altered efficiency of mitophagy following chronic BACE1 elevation.

### 6.6 Potential for Reversal of BACE1-dependent Metabolic Changes

Upon observation of specific impairments to oxidative metabolism following BACE1 overexpression; effective attenuation of these changes was achieved through supplementation with either ketone bodies (notably β-hydroxybutyrate) or alpha-lipoic acid. These findings provide a clear, cell model that mirrors the in vivo findings of restoration of reduced cerebral oxidative metabolism by alpha-lipoic acid in both ageing and AD models in response to ALA (Farr et al., 2007; Jiang et al., 2013; Sancheti et al., 2013) and ketones (Yao et al., 2009). Additionally, these nutraceutical approaches have shown initial promise in small, clinical studies
showing beneficial effects of ketogenesis stimulation by medium chain triglycerides (Reger et al., 2004) and lipoic acid (Hager et al., 2001) as well as a recent study showing that combined omega-3 fatty acid and lipoic acid treatment slowed cognitive decline in a small AD cohort (Shinto et al., 2014). Taken together, these data are suggestive of the potential for intervention with ALA and or ketogenesis-stimulating agents in AD treatment and this may fundamentally be due to a reversal of the impaired oxidative metabolism induced by increased BACE1 expression/activity. There is however a need for larger, multi-centre studies to look at these effects in a double blind, placebo trial setting.

An additional avenue of enquiry is the potential for reversal of the altered oxidative metabolism through combined SIRT1 activation and supplementation with NAD\(^+\) precursors. Previous studies have shown that activation of SIRT1 signalling is partially effective in reversing neurodegeneration (for review see Donmez et al 2012). This partial effectiveness may be due to the fact that activating SIRT1 (via resveratrol) whilst BACE1 protein levels are elevated (and hence NAD\(^+\) levels are reduced) deacetylation cannot occur due to lack of cofactor. It may therefore be more beneficial to combine treatment with NAD\(^+\) precursors (nicotinamde riboside (NR) and nicotinamide mononucelotide (NMN)) along with lipoic acid to restore oxidative metabolism. This combination therapy would seek to reverse the two major metabolic lesions brought about by BACE1 overexpression, which mirror those seen in AD.
6.7 Physiological Relevance

From a physiological viewpoint, BACE1 may represent a fundamental site of integration for a number of cellular stress events and subsequently acting to suppress metabolism. During times of stress (impaired mitochondrial functioning, substrate delivery or oxidative stress) BACE1 is up-regulated, which in turn inhibits metabolism at a general (glucose uptake) and specific enzyme (PDH and α-KGDH) level, yielding diminished production of precursors for acetylcholine and glutamate in neurons. This reduced neurotransmitter formation in concert with reduced bioenergy availability would act to reduce synaptic events (the major ATP consuming process in neurons), hence conserving cellular resources during the period of stress. However, during prolonged stress and subsequent chronic elevation of BACE1 protein and/or activity these physiological processes become deranged and aberrant overactivation of certain proteins and pathways (e.g. calpain 1, PARP1 and CD38) leads to further metabolic change that cannot be fully recovered upon stress stimulus removal (i.e. downregulation of GLUT3 expression, and NAD⁺ consumption). This spiral leads to further changes in cellular physiology (i.e. reduced mitochondrial quality control and impaired calcium homeostasis) that exacerbate the problem and result in neuronal death.

The data presented herein, along with recent findings, suggest that attenuation of prolonged, aberrant BACE1 activity and not complete removal may be of potential therapeutic benefit in the management of AD. This could in turn be combined with nutraceutical interventions aimed at mitigating the downstream metabolic decline
induced during these periods (and AD pathogenesis) and may represent the best long-term treatment options for halting the progress towards AD.
Appendix I

Seahorse Characterisation of SH-SY5Y Cells
Figure A1.1: Measurement of oxygen consumption rate (OCR) by the Seahorse XF24
(A) The probe of the XF-24 in the “wait” position, allowing oxygenation of the media prior to OCR measurement. (B) XF-24 oxygen sensing probe during measurement of OCR, with a “closed” volume of media (7µl) allowing for the change in oxygen tension in the media to be plotted over time. (C, D, E) Decreasing oxygen tension (grey and black circles) plotted against time, from which the rate of oxygen consumption is calculated. Also visible is the effect of the mix, wait periods in returning O₂ levels after each measurement. (C, D) Representative plots showing the changes in declining O₂ tensions for untreated (C) and drug treated cells in response to oligomycin, FCCP and rotenone and antimycin A (C, D; A, B, C) with these traces combined in (E).
Figure A1.2: Cell titration assay of SH-SY5Y cells
(A, B) Representative control traces showing that relative oxygen consumption and extracellular acidification rates correlate with increasing cell number.
Figure A1.2: Modulation of OCR by the addition of various mitochondria modulating drugs.

(A) Initial FCCP titration showing sequential additions of the mitochondrial uncoupler (each 0.2µM), showing a maximal increase in OCR in response to 0.2µM, with no beneficial effect of increasing FCCP, with most likely cell death occurring upon 0.6µM. (B) Robust inhibition of OCR in response to addition of the electron transport chain inhibitors rotenone and antimycin A (Rot+Ant) at either 1 (solid line) or 2µM (broken line). (C) Promotion of aerobic glycolysis-like state (decreased OCR (solid line) and increased ECAR (broken line)) in response to ATP synthase inhibition by oligomycin (1µM).
Figure A1.4: Shortcomings of standard Mito Stress Test Protocol in the present cell model.

(A) Prototypical representation of the Seahorse Mito Stress Test experimental paradigm and the data surmised: basal respiration (1), ATP-linked respiration (2), mitochondrial leak (3), maximal respiration (4), reserve capacity (5) and non-mitochondrial oxygen consumption (6) following additions of oligomycin, FCCP and rotenone + antimycin A. (B) Example trace showing SH-SY5Y neuroblastoma cell performance during the Mito Stress Test (solid line) in comparison to those cells where control media is injected (broken line). The undifferentiated state and use of only glucose as a substrate leads to little uncoupling capacity in response to FCCP and therefore maximal respiration and reserve capacity are not measurable in this setup.
Chapter 8

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