Metabolic reprogramming and the role of the BCAT protein – implications for type 2 diabetes and Alzheimer's disease

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Abstract

Introduction: Oxidative stress and impaired homeostasis are key features of type 2 diabetes mellitus, which is comorbid with Alzheimer's disease. Dysregulation of the branched-chain aminotransferase protein (BCAT), which is overexpressed in AD, is suggested to be involved in metabolic reprogramming, thus impacting on oxidative stress and protein misfolding and aggregation. However, the precise changes in cellular health resulting from BCAT dysregulation remain unclear.

Methods and Results: Using SH-SY5Y neuronal models and a combination of metabolomics, biochemical and molecular techniques, we showed that changes in the expression of BCATc affect metabolite load and impair enzymes of glycolysis therefore acting as a glycolytic regulator, with changes in its redox status shifting its binding abilities to enzymes of the TCA cycle and oxidative phosphorylation. In addition, BCATc dysregulation led to impairment of proteins of the antioxidant system and an increase in ROS generation, disruption of the autophagy pathway and increased expression of toxic protein aggregates such as amyloid β and hyperphosphorylated tau.

Conclusion: These results demonstrate that BCATc dysregulation leads to metabolic reprogramming such as observed in type 2 diabetes mellitus and the redox environment of the cell has a key role in these outcomes. Although further studies are required to evaluate whether BCATc overexpression occurs as a result of chronic levels of branched-chain amino acids or otherwise, this study showed that its expression levels are key in the regulation of energy pathways. This in turn impacts on oxidative stress and clearance mechanisms such as autophagy, ultimately contributing to protein aggregation and cellular death as observed in AD.

Publications

The following list of publications includes work produced during this project.

Harris, M.*, El Hindy, M.*, **Usmari Moraes, M.***, Hudd, F., Shafei, M., Dong, M., Hezwani, M., Clark, P., House, M., Forshaw, T., Kehoe, P., Conway, M.E. (2020) BCAT-induced autophagy regulates Aβ load through an interdependence of redox state and PKC phosphorylation-implications in Alzheimer's disease. *Free Radical Biology and Medicine* [online]. 152 (January), pp. 755–766.

*Represents papers with joint first authorships

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Abbreviations

ArAA	Aromatic Amino Acids
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AGE	Advanced glycation end products
Akt	Protein Kinase B
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
AMPK	5' adenosine monophosphate-activated protein kinase
ANS	Autonomic nervous system
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APS	Ammonium persulphate
AR	Aldose reductase
ATP	Adenosine triphosphate
Aβ	Amyloid-beta
BACE1	β-site APP cleavage enzyme
BBB	Blood-brain barrier
BCAA	Branched-chain amino acids
BCAT	Branched-chain aminotransferase
BCAT1	BCAT gene 1 cytosolic
BCAT2	BCAT gene 2 mitochondrial
BCATc	Branched-chain aminotransferase (cytosolic isoform)
BCATm	Branched-chain aminotransferase (mitochondrial isoform)
BCKA	Branched-chain α-keto acid
BCKD	Branched-chain α-keto acid dehydrogenase
BCKDHA	Branched-chain α-keto acid dehydrogenase, E1α subunit
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
cdk5	Cyclin-dependent protein kinase 5
CNS	Central nervous system
CSF	Cerebral Spinal Fluid
DAPI	4',6-Diamidino-2-phenylindole
DCFD-A	2′,7′-Dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMEM	Dulbeco Modified Eagle Media

DN	Diabetic nephropathy
DTT	Dithiothreitol
EAAT	Excitatory amino acid transporters
EBSS	Earle's balanced salt solution
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EOAD	Early-onset AD
ER	Endoplasmic reticulum
ERK	Extracellular Signal-regulated Kinase
F6P	Fructose-6-phosphate
FBS	Foetal Bovine Serum
FCCP	4-(trifluoromethoxy)phenylhydrazone
G6P	Glucose-6-phosphate
GABA	Gamma-aminobutyric Acid
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography mass spectroscopy
GDH	Glutamate dehydrogenase
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
Grx	Glutaredoxin
GSH	Glutathione (reduced)
GSIS	Glucose stimulated insulin secretion
GSK3β	Glycogen synthase kinase 3 β
GSSH	Glutathione oxidised
GSV	GLUT4-containing vesicles
GTP	Guanosine-5'-phosphate
HbA1c	Haemoglobin A1c
H_2O_2	Hydrogen peroxide
HK	Hexokinase
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IDH	Isocitrate dehydrogenase
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein

iGluR	Ligand-gated ionotropic glutamate receptor
ILβ	Interleukin β
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Insulin resistance
IRS-1/2	Insulin receptor substrates 1/2
JNK	c-Jun N-terminal kinases
LAT1	Large Neutral Amino Acid Transporter
LC3	Microtubule-associated light chain 3 phosphatidylethanolamine
LC-MS/MS	Liquid Chromatography Tandem Mass Spectroscopy
LOAD	Late-onset AD
LTD	Long-term depression
LTP	Long-term potentiation
mAChR	Muscarinic ACh receptor
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of Rapamycin complex 1
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
nAChR	Nicotinic ACh receptor
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NDMA	N–methyl-D-aspartate
NMDAR	N-methyl-d-aspartate Receptors
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OCR	Oxygen consumption rate
PBS	Phosphate buffered Saline
PDH	Pyruvate dehydrogenase
PFK-1	Phosphofructokinase
PHF-tau	Hyperphosphorylated tau
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PNS	Parasympathetic nervous system
PP1A/PP2A	Protein phosphatase 1A and 2A
PPI	Polyphosphoinositides

PSEN1/2	Presenilin-1/2
RAGE	AGE receptor
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
S6K1	Ribosomal protein S6 kinase beta-1
SAM	S-adenosyl methionine
SDS	Sodium Dodecyl Sulphate
siRNA	Small interfering RNA
SLC1	Solute-carrier 1
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
T2DM	Type 2 diabetes mellitus
TBST	Tris-buffered Saline Tween
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
Tris-HCL	Tris(hydroxymethyl)aminomethane hydrochloride
Trx	Thioredoxin
TSC1/2	Tuberous sclerosis complex 1/2
WB	Western blotting
α-KG	α-ketoglutarate

Chapter 1

Introduction & Aims

1.1. Introduction

"[...] when the heart is affected it reacts on the brain; and the state of the brain again reacts through the pneumo-gastric nerve on the heart, so that under any excitement there will be much mutual action and reaction between these, the two most important organs of the body" (Darwin, 1872).

There is increasing evidence to support the relationship between the pathophysiology of type 2 diabetes mellitus (T2DM) and the development of Alzheimer's disease (AD) (Biessels *et al.,* 2006; Barbagallo and Dominguez, 2014; Li *et al.,* 2015; Beeri and Bedlin, 2020); T2DM has not only been associated with 60% higher risk of AD development, but also with an increase in mortality rate in individuals with dementia (Cholerton *et al.,* 2016). With a growing number of studies showing the effects of high concentrations of plasma glucose, branched-chain amino acids (BCAAs) and fatty acids in the development of insulin resistance and subsequently T2DM (Yoon, 2016; Bloomgarden, 2018; White *et al.,* 2021), it is equally important to investigate such underlying metabolic irregularities in the context of AD pathogenesis.

This project aimed to address some of the unanswered questions in the context of metabolic imbalances and redox regulation that span the pathologies of both diseases, with particular focus on the first protein of BCAA catabolism, the branched-chain aminotransferase (BCAT), which has been previously shown to be significantly increased in AD brains relative to age-matched controls (Hull *et al.,* 2015). Moreover, concentrations of BCAT-associated metabolites BCAAs and glutamate correlate with measures of cognitive assessments and can be used as markers to predict the development of AD (Hudd *et al.,* 2019). Given the role of leucine as a nutrient signal and its linked control of insulin secretion, it is vital to investigate whether the end point measures of AD pathology are linked to metabolic reprogramming by BCAA

metabolites, impacting on hyperglycaemia and altered cellular redox conditions (Conway *et al.,* 2020).

1.2. The pathogenesis of Alzheimer's disease

Alzheimer's disease is one of the most prevalent types of neurodegenerative dementias worldwide. It accounts for approximately 60-70% of the estimated 50 million dementia cases across the globe, a figure expected to rise above 150 million by 2050 (Kumar and Ekavali, 2015; Van Cauwenberghe *et al.,* 2015; World Health Organization*,* 2017; Marshe *et al.,* 2018). The disease was first described by physician Alois Alzheimer in 1906 following the death of his 50-year-old patient Auguste D, who presented with key AD features such as comprehension deficit, memory impairment, erratic sleeping patterns, paranoia, aggressiveness, and confusion (Maurer *et al.,* 1997; Hippius and Neundörfer, 2003). *Post-mortem* histopathological observations reported severe loss in cerebral mass and volume relative to healthy tissue, abnormal extracellular plaque formation and accumulation of intracellular tangles (Figure 1). AD is characterised by functional effects such as progressive cognitive decline and distinct short-term memory impairment (Figure 2), which have been linked with cholinergic and glutamatergic neuronal loss involved in spatial, semantic, and episodic memory in the hippocampal region of the brain (Perl, 2010). This loss is associated with extracellular amyloid β (Aβ) fibrils and intracellular neurofibrillary tangles (NFTs) from hyperphosphorylated microtubule-associated protein tau (MAP-tau), respectively (Perl, 2010).

Currently, AD is categorised as early onset familial AD (<65 years of age, EOAD) and late-onset sporadic AD (>65 years of age, LOAD), with EOAD accounting for approximately 1 to 6% of cases (Liu *et al.,* 2019). In contrast to LOAD, EOAD is

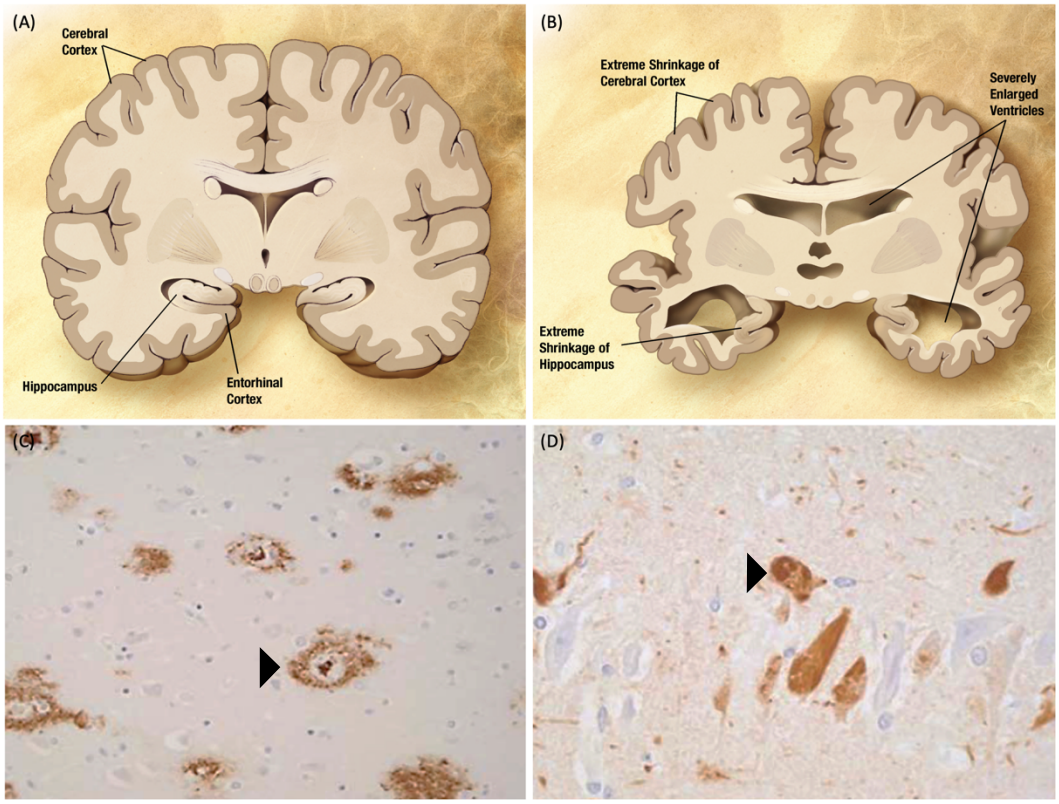

Figure 1. Morphological and histological changes in the brains of AD patients. Under normal conditions (A), brain volume and mass remain unaltered with well-structured and defined morphological characteristics. Under pathological conditions such as AD (B), loss of cerebral mass, shrinkage of hippocampal region and cortex, and enlarged ventricles are prominent. Moreover, accumulation of extracellular Aβ plaques (C) and intracellular hyperphosphorylated tau (D) are also observed in patients with AD. A and B. Wikimedia Commons; C and D adapted from Taipa *et al*. (2016).

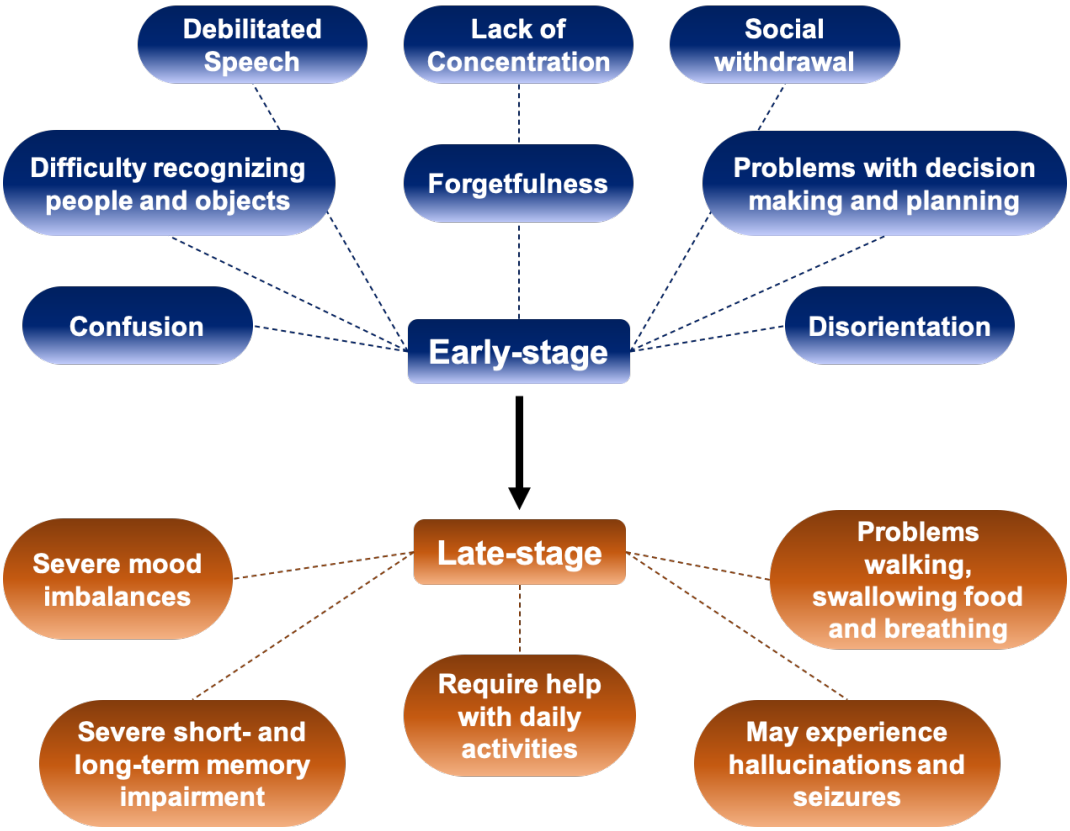

Figure 2. Early- and late-stage symptoms of AD. Early symptoms of AD dementia are often associated with normal ageing. Early AD symptoms closely resemble mild cognitive impairment (MCI), which similarly to AD presents with forgetfulness, disorientation, and confusion. In late stages, most activities are severely compromised, and neurodegeneration progresses more rapidly, also affecting general muscle control.

highly heritable, presenting with atypical neuropsychological symptoms and disease progression. Both subsets share similar clinical outcomes, and many EOAD cases are also observed in families with LOAD, including the morphological (loss in cerebral volume and overall tissue shrinkage), histological (protein aggregation), and symptomatic (progressive cognitive decline, disorientation) changes (Chiaravalloti *et al.,* 2016). However, more than 100 years after the pathophysiology of AD was first described, the underlying mechanisms which are dysregulated and trigger the disease remain largely unknown.

1.2.1. Diagnostic tools for Alzheimer's dementia

Diagnosis of AD relies on clinical manifestation of symptoms (cognitive decline) and neuroimaging assessment (primarily brain atrophy), with accuracy estimated at approximately 85%. Neuropathological studies provide *post-mortem* confirmation by identifying extracellular Aβ tangles and intracellular NFTs aggregation, relative to age-matched controls without dementia (Bird, 2008). Differential diagnosis relies on assessment scales such as the ADAS Cog (Alzheimer's Disease Assessment Scale Cognitive Subscale) as well as imaging technology such as computed tomography (CT) and magnetic resonance imaging (MRI) scans, used to discriminate between other types of dementia (i.e., frontotemporal and Lewy body dementia) or other causes of cognitive impairment such as tumours (McGleenon *et al.,* 1999).

1.2.2. Therapeutic targets in the management of AD

1.2.2.1. Neurotransmitter and receptor inhibitors

Current treatments for AD rely primarily on symptomatic relief, exploiting acetylcholinesterase (AChE) inhibitors (galantamine, rivastigmine and dopenezil) and N-methyl-d-aspartate (NMDA) receptors (NMDARs) antagonist (memantine), which selectively block extra synaptic NMDARs function; combination therapy with AChE

inhibitors and memantine have been shown efficient in individuals with moderate to severe symptoms (Table 1).

Table 1. Current drug therapies for the treatment of AD.

Drug	Commercial name	Target	Treatment Stage
Donepezil	Aricept	AChE inhibitor	All stages
Galantamine	Razadyne	AChE inhibitor	Mild to moderate
Rivastigmine	Exelon	AChE inhibitor	Mild to moderate
Memantine	Namenda	NMDAR antagonist	Mild
Aducanumab*	Aduhelm	Amyloid aggregates	Mild to severe
Lecanemab*	Leqembi	Amyloid aggregates	Early stages
Combination therapy (Dopenezil+ Memantine)	Namzaric	AChE inhibitor + NMDAR antagonist	Moderate to severe

**FDA-approval in the USA only. Adapted from Conway (2020).*

However, these therapies provide modest and transient improvement and do not directly address the underlying mechanisms leading to the development and progression of the disease (Usmari Moraes and Gaudet, 2018). For that reason, the focus has shifted to exploring the Aβ and tau processing pathways as potential targets for the treatment of AD. β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) and γ-secretase inhibitors have been prime candidates for new treatment avenues in the APP/Aβ pathway (Ohno *et al.,* 2004; Li *et al.,* 2007; Albright *et al.,* 2013; Doody *et al.,* 2013; Porrini *et al.,* 2015; Kennedy *et al.,* 2016), whereas kinases such as glycogen-synthase kinase-3β (GSK-3β) and phosphatases such as

phosphatase 2A (PP2A) are the most explored targets in tau hyperphosphorylation (Nakashima *et al.,* 2005; Mangialasche *et al.*, 2010).

1.2.2.2. Immunotherapeutic approaches

Immunotherapeutic therapies directly targeting Aβ fibrils and neurofibrillary tangles of hyperphosphorylated tau have also been widely explored. Trials for Aducanumab, a monoclonal antibody targeting Aβ oligomers and fibrils which had initially demonstrated promising results in mice models, were paused following preliminary futility analysis failure (less than 20% chance the trial would return a positive finding). Recently, however, Biogen, the company responsible for drug development, has suggested one of the trials provided significant results, although discrepancies remain between the two studies (Sevigny *et al.,* 2016; Schneider, 2019). Despite the contradicting results, the USA's Food and Drug Administration (FDA) approved the drug (marketed Aduhelm) under its accelerated approval pathway, as it predicts the clinical benefits outweigh the need for Phase III trials (Walsh *et al.,* 2021).

AADvac-1, an active immunisation targeting epitopes in tau protein to prevent aggregation and promote clearance, has been on trial since 2014, with new data showing that although the vaccine was shown to be safe and immunogenic, further studies are required to understand clinical efficacy and implications on AD biomarkers (Kontsekova *et al*., 2014; Novak *et al.,* 2017; Novak *et al.,* 2019; Novak *et al.,* 2021). Nonetheless, most studies have been conducted in AD mice models, with many clinical trials halted due to unforeseen toxicity in humans. Although AD is considered a disease of ageing, young animals have a distinct immune system in comparison with aging individuals and are commonly no more than a few months old when Aβ plaques are observed, thus challenging the transferability of such models (King, 2018). This highlights that further *in vitro* research into the biochemical pathways

which are dysregulated and contribute to the development of the disease are critical for more robust, efficacious treatment avenues.

1.2.3. Synergistic hypotheses in AD pathogenesis

There are numerous hypotheses concerning the development and progression of AD. Whilst these are particularly focused on aggregation of toxic peptides such as Aβ and hyperphosphorylated tau, and neurotransmitter imbalance, more recent studies have shown the association of metabolic reprogramming (i.e., T2DM) in disease progression (Liu *et al.,* 2019). This work has discussed the Aβ, tau and neurotransmitter hypotheses in detail, both individually and in synergy, and in the context of both AD and T2DM.

1.2.3.1. The amyloid β hypothesis and peptide processing

The amyloid hypothesis is based on Aβ aggregation as a trigger. It suggests that accumulation of Aβ and related intracellular deposition of hyperphosphorylated tau, likely as a result of failure in clearance processes (such as the autophagy mechanism), lead to memory loss and result in the observed cognitive decline over time (Chen *et al.,* 2017). Genetic and environmental factors, as well as traumatic brain injury, microbiota dysregulation and, most importantly, T2DM and oxidative stress, are all suggested to play a role in Aβ aggregation, however the exact mechanisms contributing to the initial aggregation remain elusive (Zhang *et al.,* 2018).

Aβ is produced via endoproteolytic cleavage of the amyloid precursor protein (APP), a large type-1 transmembrane protein located along the neuronal body (soma, dendrite, and axons) (Glenner and Wong, 1984). Synthesis of the shorter peptide, $A\beta_{40}$, via α-secretase accounts for approximately 90% of the total Aβ production and has been implicated in the development of synaptic plasticity, with synthesis shown

to increase formation of long-term potentiation (LTP), the primary mechanism studied as a model of learning and memory in the mammalian brain (Koudinov and Koudinova, 2005; Kelleher and Shen, 2017) (Figure 3, non-amyloidogenic). There are studies suggesting additional physiological roles for APP and Aβ in the brain, particularly in the regulation of LTP, glutamatergic and cholinergic responses in the hippocampus (Puzzo *et al.,* 2011), and neurite outgrowth and axonal guidance (Billnitzer *et al.,* 2013). Pearson and Peers (2006) also suggested that APP processing and subsequent Aβ production are closely related with regulation of synaptic activity, and likely protect against excessive release of glutamate (Pearson and Peers, 2006).

In contrast, APP cleavage by β-secretase (β-site APP cleaving enzyme 1, BACE1), enzyme which has been shown to be upregulated in the AD brain (2.7-fold) relative to age-matched controls, can produce the longer fragment $A\beta_{42}$, the primary biomarker in the development and progression of AD (Molinuevo *et al.,* 2018) (Figure 3, amyloidogenic). However, BACE1 upregulation and further APP cleavage may suggest an additional event in the development of AD prior to Aβ accumulation. Aβ has been shown to be generated as a result of autophagic turnover of APP-rich organelles during autophagy, key clearance mechanism in maintaining cellular health by removal of damaged organelles and protein aggregates (Glick *et al.,* 2010), with engulfing of Aβ peptides by synaptic-generated autophagosomes allowing for prompt clearance (Nixon, 2007). Under pathological conditions such as AD, where both maturation and transport are severely impaired, this process results in further accumulation of autophagic vacuoles (immature autophagosomes) and generation of Aβ fibrils in affected areas, which accelerate disease progression (Nixon, 2007).

Gu and Guo (2013) showed that although $A\beta_{40}$ and $A\beta_{42}$ can aggregate in an interlaced manner, $A\beta_{42}$ is more efficient in developing fibrils (Gu and Guo, 2013). This is likely as a result of conformational differences between the peptides, as $A\beta_{42}$

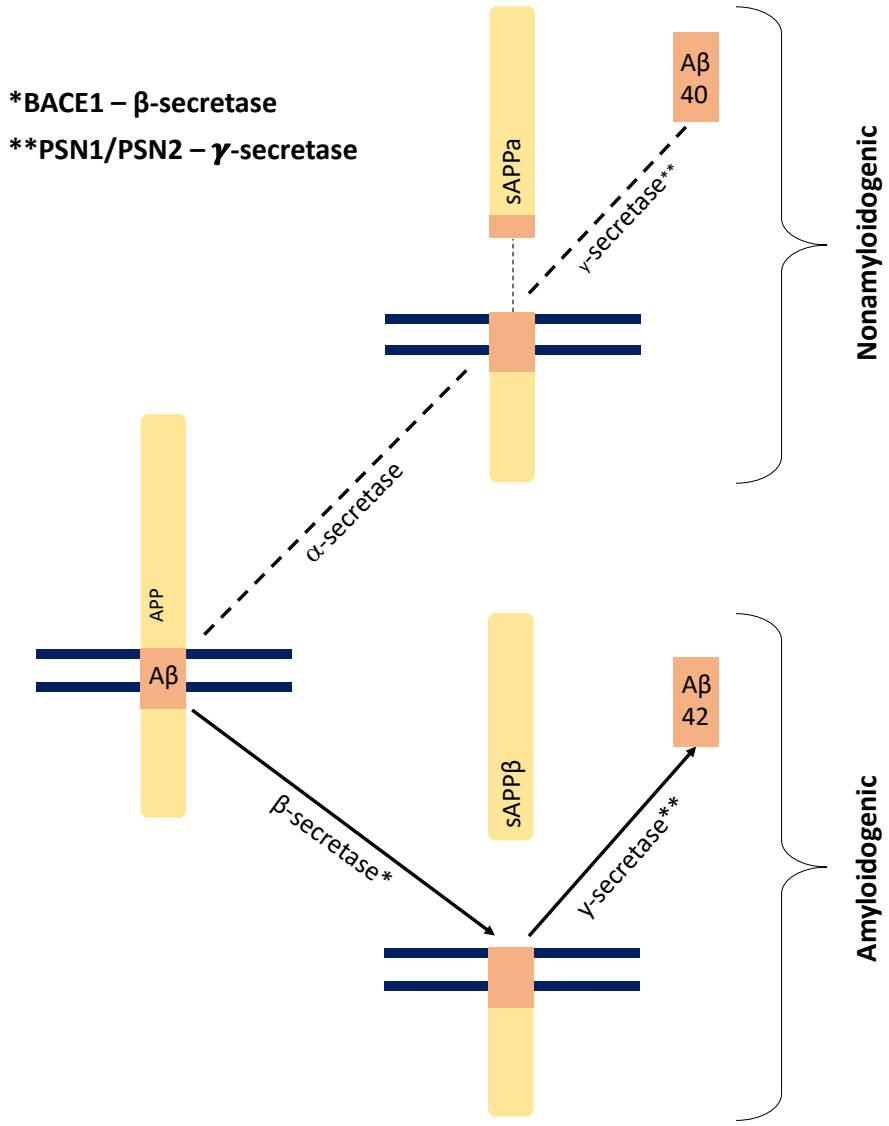

Figure 3. Nonamyloidogenic and amyloidogenic APP processing. Cleavage of APP by α- and γ-secretase (non-amyloidogenic pathway) produces a peptide which is released in the extracellular space and rapidly degraded or removed (sAPPα to $A\beta_{40}$, α-pathway) (Coimbra *et al*., 2018; Liu *et al*., 2019). Alternatively, APP can undergo processing via the β-pathway where it is initially cleaved by β-secretase, followed by subsequent cleavage by γ-secretase (Holsinger *et al*., 2002). This alternative cleavage process generates the more hydrophobic, insoluble 42-amino acid long Aβ peptide ($A\beta_{42}$), which accumulates and produces the distinct senile plaques observed in AD patients (sAPPβ to $A\beta_{42}$, amyloidogenic pathway) (Zhang *et al*., 2014; Chen *et al*., 2017).

presents two additional C-terminal residues which form a β-hairpin structure and confer the peptide less flexibility, thus rendering it more prone to aggregation (Chen *et al.,* 2017). Aβ clearance through enzymatic (Aβ-degrading enzymes) and non-enzymatic (transcytosis via vascular Aβ receptors) mechanisms are therefore fundamental for removal of such toxic aggregates, which is particularly important as impairment of processes exacerbating accumulation was shown to be increased with normal aging (Majdi *et al.,* 2020).

1.2.3.2. Aβ and its genetic associations

In both subsets of AD (EOAD and LOAD), polymorphisms in genes encoding for carrier proteins such as apolipoprotein E (ApoE) confer the highest genetic risk as shown by several genome-wide association studies, although the APP gene, presenilin-1 (PSEN1) and presenilin 2 (PSEN2) also show strong association particularly with EOAD (Table 2) (Selkoe, 1997; Vassar, 2003; Van Cauwenberghe, *et al.,* 2016; Marshe *et al.,* 2018).

Table 2. Genes associated with early onset AD.

Location	Gene	Function	Inheritance
Chr. 21q21	APP	Synapse formation and plasticity, cell surface receptor	Autosomal dominant
Chr. 19q13.32	ApoE	Lipid transport (cholesterol and triglycerides)	Semi-dominant
Chr. 14q24.2	PSEN1	Regulation of y-secretase	Autosomal dominant
Chr. 1q42.13	PSEN2	Regulation of y-secretase	Autosomal dominant

Adapted from Bekris et al. (2010).

Mutations in the genes encoding for APP or elements of the APP processing pathway such as PSEN1/2 directly increase $A\beta_{42}$ turnover and are associated with increased risk of EOAD. PSEN1 mutations lead to a shift in the location γ-secretase cleaves sAPPβ, which in turn favours the production of the longer Aβ fragment (Zhang *et al.,* 2014). This is of particular importance in cellular integrity, where Rolland *et al.* (2020) showed that Aβ aggregation significantly reduced synaptic transmission and long-term potentiation in glutamatergic neurons when APP was present. This aggregation can also lead to an increase in reactive oxygen species (ROS) and upregulation of BACE1 via the c-*Jun* N-terminal kinase (c-JNK) pathway, further increasing β-cleavage APP processing and $A\beta_{42}$ production in a feedback loop reflecting on the pathogenesis of AD (Tong *et al.,* 2005; Tamagno *et al.,* 2008; Rolland *et al.,* 2020).

Another strong example of such genetic predispositions is polymorphisms in the ApoE gene. It is composed of three polymorphic alleles, ε2, ε3 and ε4, with the ε3 allele most frequently observed in the population (Farrer *et al.,* 1997). In patients with AD, however, the frequency of the ε4 allele (ApoE-ε4) is approximately 40% higher, is associated with increased risk of developing the disease (20 to 90%) and significant decrease in the age of onset (>10 years) (Corder *et al.,* 1993). ApoE is a glycoprotein involved in lipid transport across tissues, playing a vital role in the metabolism of both endogenous and dietary cholesterol and triglycerides (Mahley and Rall, 2000; Marshe *et al.,* 2018), with ongoing discussions over impaired lipid metabolism and the potential link between the metabolic dysregulation observed in T2DM and the development and progression of AD (Kulas *et al.,* 2020). This is important as the protein mediates *in situ* synthesis of cholesterol in astrocytes and acts as a carrier protein between astrocytes and neurons in the brain, with recent studies showing that cholesterol synthesis and astrocytic transport of ApoE are strict regulators of Aβ

accumulation in neurons (Wang *et al.,* 2021). In fact, ApoE has been shown to co-localise with Aβ aggregates and neurofibrillary tangles of hyperphosphorylated tau protein in AD, and patients with the ApoE-ε4/4 genotype have been shown to present with more Aβ-derived senile plaques than those with ApoE-ε3/3. (Kok *et al.,* 2009; Bilousova *et al.,* 2019). In light of the protein's role in lipid metabolism, Aβ and tau development, it is crucial to understand its role in neuronal health and outcomes of its dysregulation, which will provide additional clues on the association between T2DM and AD.

1.2.3.2. Tau hyperphosphorylation and neurofibrillary tangles

Deposits of hyperphosphorylated tau protein are the main observable characteristics in the brains of individuals with secondary tauopathies, a family of neurodegenerative conditions including AD and chronic traumatic encephalopathy (CTE) and anti-IgLON5-related tauopathy (Wang *et al.,* 2013). Tau is localised in the axon of mature neurons, in the somatodendritic compartment and nucleus, as well as (in lower levels) in astrocytes and oligodendrocytes, where it plays a key role in microtubule dynamics and assembly (Mandelkow *et al.,* 1995; Naini and Soussi-Yanicostas, 2015). It originates from a single gene (MAPT) following alternative mRNA splicing and contains approximately 80 serine/threonine and 5 tyrosine phosphorylation sites (Stoothoff and Johnson, 2005; Wang *et al.,* 2013). *In vitro* studies demonstrated that tau phosphorylation at the Ser235, Thr231 and Ser262 sites inhibit its microtubule binding ability by approximately 10, 25 and 35%, respectively, and further phosphorylation of sites Ser396/422 and Thr231 contributes towards tau self-aggregation (Sengupta *et al.,* 1998; Alonso *et al.,* 2004). Although phosphorylated tau is commonly observed in healthy brains and believed vital for foetal development by contributing towards neuronal growth and plasticity, it has been

reported 2-3 times more phosphorylated under pathological conditions, with biological impact severity correlating with phosphorylation site (Xia *et al.,* 2021).

Post-translational modifications such as phosphorylation, aggregation, polyamination and nitration of the tau protein are features of pathological conditions such as AD and have been suggested to contribute to tau hyperphosphorylation and generation of NFTs, most likely due to destabilisation of microtubule interactions leading to instability and transport defect (Martin *et al.*, 2011; Naini and Soussi-Yanicostas, 2015) (Figure 4). NFTs are thought to accumulate starting from subcortical regions of the brain, trans entorhinal and entorhinal cortex, and subsequently develop in the hippocampal region and some areas of the neocortex largely correlating with disease progression (Miao *et al*., 2019). Evidence also suggests that Aβ and APP may be involved in triggering tau misfolding and hyperphosphorylation. Toxic Aβ fragments can induce ROS production and trigger a stress/inflammatory response via tau kinases such as c-JNK (Blurton-Jones and LaFerla, 2006; Barron *et al*., 2017; Du *et al*., 2022), upregulate GSK-3β and inhibit PP1/2A from dephosphorylating both APP, thus sustaining the phosphorylation cycle and increasing the accumulation and aggregation of plaques and NFTs (Oliveira *et al.,* 2015). In addition, increased β-secretase processing affects the axonal transport of APP leading to axonal and synaptic defects that contribute towards tau mis-localisation, which can accumulate and form distinct tau fibrils. This accumulation can in turn disrupt the axonal transport further, increasing tau fibrils and enhancing the damage via a continuous cycle (Zheng *et al*., 2002; Rusu *et al*., 2007; Wu *et al*., 2018). However, the abnormal mechanisms leading to the development of PHF-tau and NFTs are not completely understood and require further elucidation.

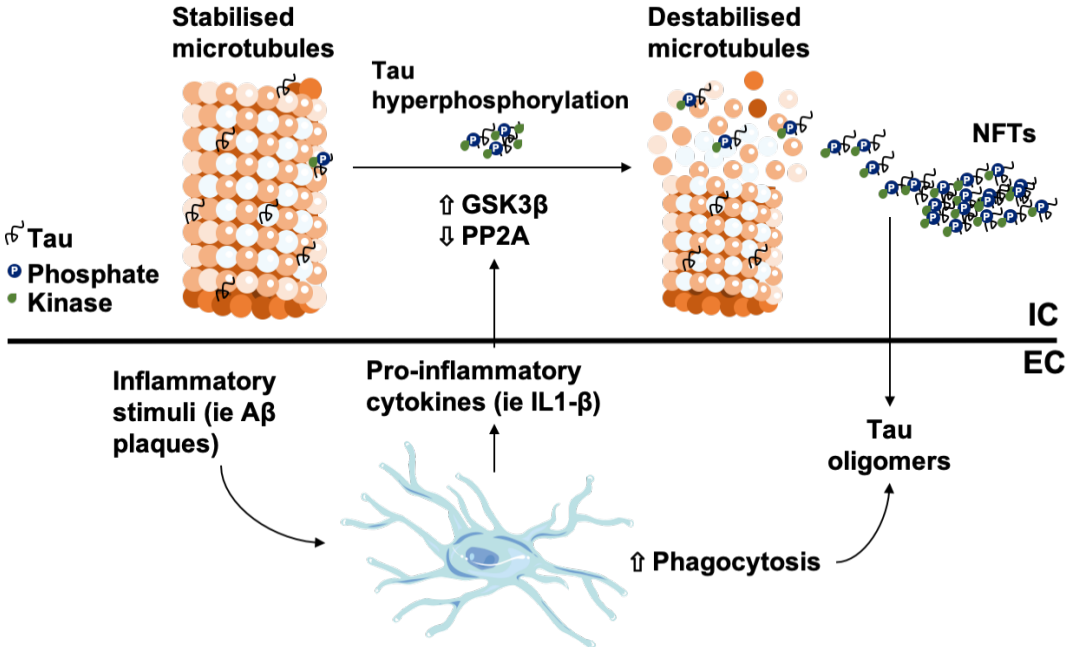

Figure 4. NFTs from tau hyperphosphorylation. Accumulation of PHF-tau occurs prior to the development of NTFs and impacts on the functionality of multiple proteins throughout affected areas. Following phosphorylation, p-tau detaches from microtubules in the cytoplasm, forming paired helical filaments which accumulate as NFTs and ultimately lead to cell death (Götz *et al.,* 2011). Upregulation of GSK-3β and downregulation of PP2A are key processes in tau hyperphosphorylation in AD brains (Gong and Iqbal, 2008). Extracellular inflammatory stimuli, particularly Aβ plaques, can induce pro-inflammatory cytokines production and directly impact on kinase activity. Adapted from Barron *et al.* (2017) and Echeverry *et al.* (2018).

1.2.3.3. Cholinergic hypothesis

The cholinergic hypothesis and its contribution to AD pathology have been explored for more than 35 years, and propose that the dysfunction in the synthesis and release of the neurotransmitter acetylcholine (ACh) in cholinergic neurons contributes to the development of AD. The cholinergic system originates in the basal forebrain, with the release of ACh signalling to areas such as the anterior cingulate, medial prefrontal, parietal and sensory cortices, and the hippocampus, thus playing a major role in cognitive behaviour, attention and memory retrieval and consolidation (Avery and Krichmar, 2017).

ACh is synthesised in the presynaptic terminal from Acetyl CoA and choline by choline acetyltransferase (ChAT, EC 2.3.1.6) and released in the synaptic cleft where it is rapidly hydrolysed and inactivated by acetylcholinesterase (AChE), the primary cholinesterase in the human brain cortex. ACh is synthesised in the presynaptic terminal from Acetyl CoA and choline by choline acetyltransferase (ChAT, EC 2.3.1.6) and released in the synaptic cleft where it is rapidly hydrolysed and inactivated by acetylcholinesterase (AChE), the primary cholinesterase in the human brain cortex. Hydrolysis produces acetate and choline which can re-enter neurons and reinitiate the ACh synthesis cycle. In health, ACh plays key roles in synaptic plasticity and transmission as well as neuronal excitability, enhancing LTP in multiple regions of the brain with particular focus in the stimulation of hippocampal muscarinic and nicotinic ACh receptors; thus, it acts as a neuromodulator by modifying internal and external inputs of neuronal networks throughout the brain and contributing to cognitive function (Hasselmo, 2006; Picciotto *et al.,* 2012; Yakel, 2014).

In AD, however, aggregation of Aβ oligomers for instance can lead to significant loss in cholinergic neurons particularly in the nucleus basalis of Meynert (~70%), with the remaining cells having a significant decrease in ChAT transcription and thus ACh synthesis and release (Ferreira-Vieira *et al.,* 2016). In contrast, tau

hyperphosphorylation, as well as the kinase most linked to its dysregulated pathway, GSK-3β, have been suggested to increase activity of AChE, with inhibition of the kinase showing the opposite effect (Cortéz-Goméz *et al.,* 2020). Considering Aβ is also involved in inducing GSK-3β overactivation via the inflammatory pathway, it can be assumed that it is the conjunction in pathway disruptions, rather than each individually, that is most likely responsible for the severe neuronal loss subsequently leading to AD.

Damage to the cholinergic system, as a result of Aβ and tau dysregulation or otherwise, evidently correlates with neurodegeneration and thus the cognitive impairment observed in AD, which led to most treatment strategies focusing on AChE inhibitors to prevent ACh turnover. However, such therapies provide little more than symptomatic relief, and further understanding of the AD pathology and the mechanistic dysregulation as a whole are required to allow for the development of disease modifying drugs (Francis *et at.*, 1999; Herholz *et al.,* 2008; Du *et al.,* 2018).

1.2.3.4. Exploring glutamate excitotoxicity and NMDA receptors hypothesis

Glutamate is the major excitatory neurotransmitter in the human brain, and glutamatergic neurotransmission via NMDARs is essential for neuronal survival as well as synaptic transmission and plasticity, with dysregulation associated with apoptosis and neurodegeneration (Padurariu *et al.,* 2012; Wang and Reddy, 2017). Glutamate mediates the majority of neurotransmission in the CNS alongside its ligand-gated ionotropic glutamate receptors (iGluRs), with extracellular levels closely regulated by release-and-uptake ratio (Wang and Reddy, 2017). In the hippocampus, released glutamate acts on NMDARs, as well as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, with excess glutamate in the synaptic junction removed by solute-carrier 1 (SLC1)-type excitatory amino acid

transporters (EAAT) in pre- and post-synaptic membranes (EAAT2 and 3, respectively) as well as astrocytes (EAAT2) (Vandenberg and Ryan, 2013). Considering its fundamental role as a neurotransmitter, minor shifts in glutamate levels in the synaptic junction (either from excessive production and release or decreased removal) can create an optimal environment for over-stimulation of its receptors, with subsequent excitotoxicity and neuronal damage.

NMDARs have been extensively linked with synaptic dysfunction in AD, normally as a result of extra-synaptic overactivation leading to superoxide production by neuronal NADPH oxidase-2 (NOX2), damage to mitochondrial membrane potential, oxidative stress, and cellular death (Zhang *et al.,* 2016). Increasing evidence associates Aβ and hyperphosphorylated tau aggregation with changes in glutamate release, astrocytic uptake and receptor signalling in the glutamatergic tripartite synapse (comprising of a presynaptic terminal, a postsynaptic spine, and an astrocytic process), a key site for NMDARs dysregulation in AD (Rudy *et al.,* 2015). In addition, perturbances in NMDAR and calcium homeostasis, particularly as a result of accumulation of Aβ oligomers and PHF-tau, can lead to dysregulation in LTP particularly in the hippocampus (Selkoe, 2002; Walsh *et al*., 2002; Texidó *et al.,* 2011; Parsons *et al*., 2014). LTP was initially observed in glutamatergic synapses in the entorhinal cortex and dentate gyrus of the hippocampus, and is of particular importance in AD studies, where neurodegeneration, loss of NMDA transmission and accumulation of Aβ senile plaques and NFTs in the hippocampal region led similar outcomes to that of cholinergic impairment, such as LTP disruption, memory impairment and learning difficulties, all of which are features in AD development and progression (Chen *et al.,* 2000; Cooke and Bliss, 2006; Dehkordy and Bahrami, 2013).

1.3. Pathophysiology of type 2 diabetes

Type 2 diabetes mellitus (T2DM), formerly referred to as adult-onset and non-insulin-dependent DM, is one of the most common metabolic diseases in the modern world. It accounts for approximately 85% of all Diabetes Mellitus cases, in contrast with 10% for type 1 DM and 5% for additional subtypes, and affects approximately 370 million people worldwide, number projected to surpass 500 million by 2040 (Chatterjee *et al*., 2017; Zheng *et al*., 2018).

T2DM is characterised by three central metabolic disturbances: (1) insulin resistance (2) high levels of circulating glucose (hyperglycaemia) which, if sustained, result in (3) impaired insulin secretion. The development of insulin resistance is linked to excess body fat as well as genetic predisposition and is largely dependent on downregulation of insulin receptor substrates 1 and 2 (IRS1/2) in target tissues (Ye, 2013). It is observed years prior to hyperglycaemia and T2DM, although its consequences extend to metabolic imbalances such as dyslipidaemia and visceral adiposity, hypertension, increased inflammatory markers, endothelial dysfunction, amongst others (Freeman and Pennings, 2022). Insulin resistance is generally asymptomatic, although it is considered the first metabolic dysregulation in the development of diabetes (Kahn *et al.,* 2014).

In contrast, insulin secretion is dependent on intracellular glucose concentrations in β-cells of the Islet of Langerhans in the pancreas, which significant increases trigger a cascade of events leading to membrane depolarisation, activation of voltage-dependent Ca^{2+} channels and increase in intracellular Ca^{2+}, stimulating pulsatile insulin secretion and release (Wilcox, 2005). Additional stimuli, including BCAAs, fatty acids and neurotransmitters such as acetylcholine can also induce insulin secretion in β-cells (Bratanova-Tochkova *et al.*, 2002; Boucher *et al.,* 2014). Leucine, for instance, allosterically activates enzymes such as glutamate dehydrogenase (GDH) and stimulates intracellular Ca^{2+}, as well as activating the

mammalian target of rapamycin (mTOR) thus inducing protein synthesis (Yang *et al.,* 2010; Göhring and Mulder, 2012). And while acetylcholine may trigger insulin secretion via activation of phospholipases and protein kinase A and C (PKA/PKC), non-esterified fatty acids (NEFA) increase glucose output in hepatocytes via stimulation of gluconeogenesis, playing a role in glucose-stimulated insulin secretion (GSIS) (Amery and Nattrass, 2000; Wilcox, 2005).

However, overstimulation of insulin secretion and release (hyperinsulinaemia), as a result of insulin resistance and thus hyperglycaemia, eventually leads to loss in β-cell function and impact on insulin synthesis and secretion (hypoinsulinaemia) (Yang *et al.,* 2018). This ultimately results in hyperglycaemia and glucose intolerance and, as dysfunction progresses, the development of T2DM. The direct mechanisms through which insulin resistance and β cell dysfunction occur, however, require further investigation (Ramlo-Halsted and Edelman, 2000; Lenzen, 2014).

1.3.1. Nutrient-dependent regulatory mechanisms in T2DM

Autophagy is one of the main clearance mechanisms in the cell, although it plays a particular role under conditions of extreme nutritional deficit as an energy turnover process for cell survival. Under nutrient-rich conditions, however, autophagy is generally downregulated, which provides a curious link between T2DM and the accumulation of toxic aggregates in the pathogenesis of AD, once its inherited metabolic function is likely perturbed.

Intracellular kinases such as adenosine monophosphate (AMP)-activated protein kinase (AMPK) can activate autophagy by directly inhibiting the mTOR complex 1 (mTORC1) (Figure 5), or indirectly by activating tuberous sclerosis complex 1/2 (TSC1/2), which inhibits GTP-binding protein Rheb and prevents mTORC1 activation (Yue *et al.,* 2008; He *et al.,* 2018). mTORC1 is strongly

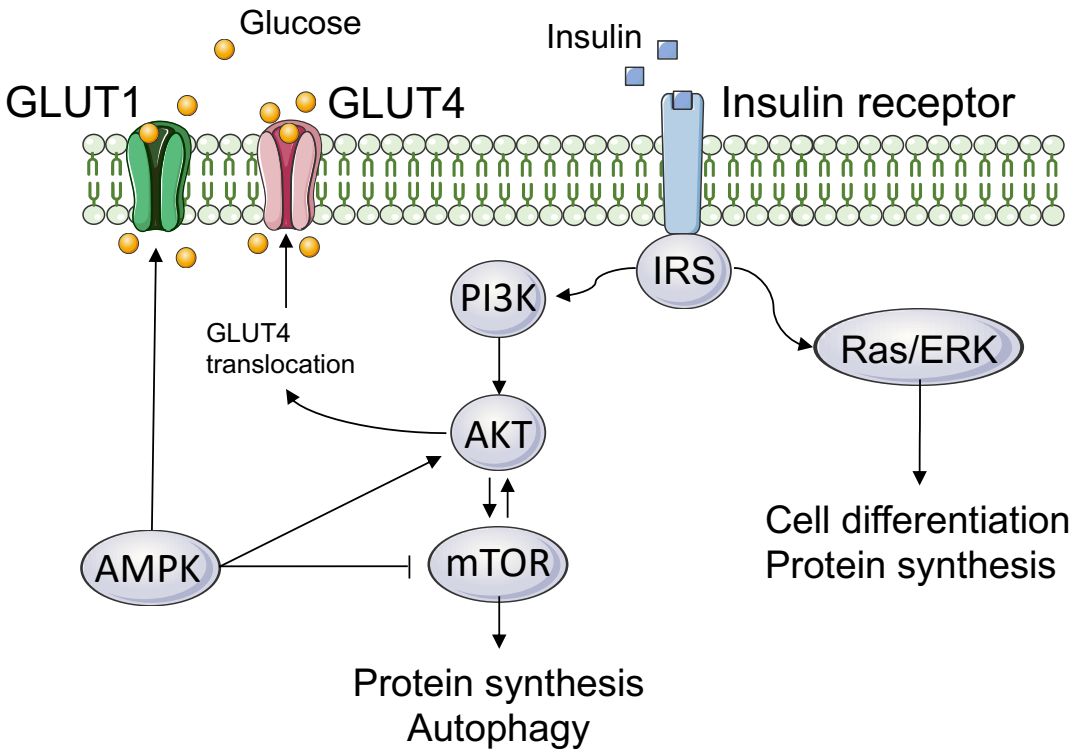

Figure 5. Insulin signalling and glucose uptake. Binding of insulin to its receptors in the cell surface stimulates phosphorylation of tyrosine kinase, followed by a cascade of events leading to the translocation of insulin-dependent glucose transporter 4 (GLUT4) from GLUT4-containing vesicles (GSVs) to the plasma membrane via targeted exocytosis. This process allows for GLUT4 expression in the extracellular membrane and subsequent glucose uptake into the cells, mediated by PI3K-dependent and -independent pathways (Chang *et al*., 2004). Alongside stimulation of GLUT4, insulin receptors can modulate neurotransmission via phosphorylation of NMDARs and binding of IRS-1/2 to PI3K (Akter *et al*., 2010). In addition, intracellular glucose uptake can occur via insulin-independent glucose transporter 1 (GLUT1), expressed in energy demanding tissues such as the brain and retina of the eye. However, it is also thought to mediate basal, insulin-independent glucose uptake in all cells throughout the body (Ismail-Beigi, 1993; Vannucci *et al*., 1994; Ebeling *et al*., 1998).

regulated by leucine and is involved in regulating mechanisms other than autophagy, including FA β-oxidation and synthesis, purine and pyrimidine metabolism, and insulin signalling (Kim and Guan, 2019; Son *et al.,* 2019). Differential phosphorylation of serine residues in the IRS1 by mTORC1/S6K1 can prevent the recruitment of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) to its docking site and further activation of protein kinase B (Akt), which acts to attenuate insulin responses such as glucose uptake and glycogen synthesis thus contributing towards insulin resistance (Yoon, 2016). This is important as not only are these mechanisms impacting on metabolic dysregulation and the subsequent development of T2DM, but insulin has also been shown to modulate synaptic plasticity by promoting LTP and memory consolidation in the hippocampus (Zhao *et al.,* 2019), while insulin resistance correlates with inhibition in LTP leading to memory impairment, one of the hallmarks in the development and progression of neurodegenerative conditions such as AD (Izumi *et al.,* 2003; Akter *et al*., 2010). Research suggests that basal levels of autophagy have important roles in the modulation of the inflammatory process in T2DM by inhibiting production of interleukin 1β (IL1β) (Crisan *et al.,* 2011), with others showing that autophagy inhibition, coupled with the already reduced insulin secretion, leads to mitochondrial dysfunction and ER stress, accelerating β-cell degeneration (Fujitani *et al.,* 2009). However, further studies are required to understand the precise role autophagy plays in dysregulated metabolic processes in T2DM.

1.3.2. Clinical presentation of metabolic dysregulation

Classical features of untreated T2DM include increased thirst and hunger, frequent urination, and extreme fatigue, with disease progression associated with long-term comorbidities such as hypertension, cardiovascular complications, damage to nerves (particularly in the retina of the eyes, kidney and peripheral nerves),

depression, thyroid gland disorder and chronic obstructive pulmonary disease (COPD). These clinical outcomes are suggested a result of dysregulation in multiple cellular pathways involved in cellular homeostasis, such as antioxidant systems regulating the production of ROS, advanced glycation end products (AGEs) and the autophagy pathway (Nowakowska *et al.,* 2019). Moreover, studies estimate that individuals with T2DM have a 60% higher risk of developing AD in later years, with dysregulation in insulin signalling and key metabolic pathways at the centre of attention (Kukull *et al*., 2002; Akomolafe *et al*., 2006). However, the exact link between these pathologies requires further exploration, and this project attempts to address some of the unanswered questions in relation to metabolic reprogramming in disease.

1.4. Metabolic pathways in health and disease

1.4.1. Branched chain and aromatic amino acids as powerful tools in insulin signalling and synaptic health

Dysregulated metabolism of BCAAs leucine, isoleucine and valine (Figure 6), and aromatic amino acids (ArAA) phenylalanine, tyrosine, and tryptophan (Figure 7), have been implicated in the development of AD as well as playing a role in insulin secretion and insulin resistance observed in T2DM (Izumi et al., 2003; McCormack *et al*., 2013; Würtz *et al.,* 2013; Hudd *et al.,* 2019). High levels of BCAAs (as well as fatty acids and triacylglycerols) have been identified as predictive biomarkers for the development of T2DM (McCormack *et al.,* 2013), with Boulet *et al.* (2015) showing that expression of hBCATm is significantly lower in the plasma of obese patients with insulin resistance, hyperglycaemia and high BCAA levels. BCAAs also act as key nitrogen donors and contributors to the production of downstream metabolites such as Acetyl-CoA, vital for processes such as the *de novo* synthesis of glutamate (Conway and Hutson, 2016), whilst ArAA metabolism is directed towards the

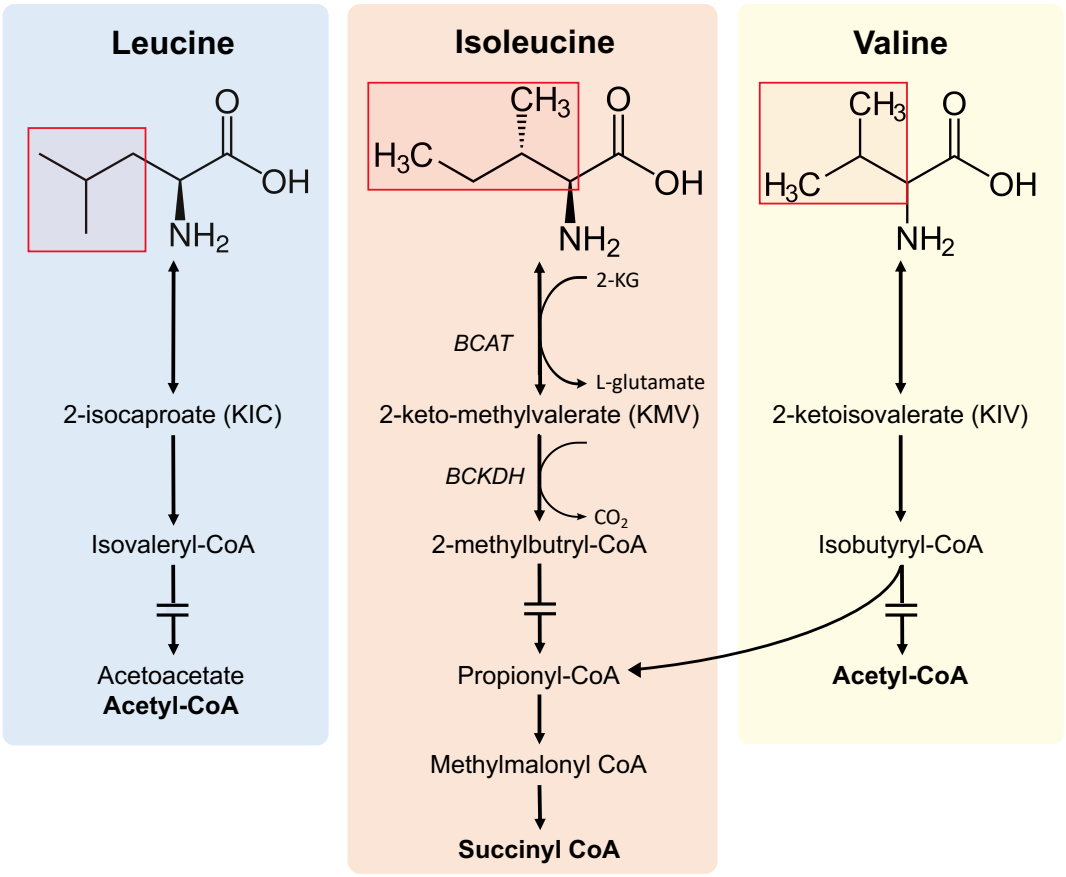

Figure 6. Overview of BCAA metabolism. In humans, the pyridoxal 5′-phosphate (PLP)-dependent branched-chain aminotransferase (BCAT, EC 2.6.1.42) protein catalyses the first step in the BCAA metabolism via its two isoforms, cytosolic (BCATc) and mitochondrial (BCATm). The reversible transamination of BCAA involves the transfer of the α-amino group from BCAA to α-ketoglutarate (α-KG), resulting in the production of L-glutamate and its respective branched chain α-keto acid (BCKA – ketoisocaproate, KIC; ketomethylvalerate, KMV; ketoisovalerate, KIV, respectively). While the reaction is reversible, it is predominantly directed towards the production of BCKAs and L-glutamate synthesis. BCKAs are subsequently irreversibly oxidised by the branched-chain α-keto acid dehydrogenase complex (BCKDC, EC 1.2.4.4) to produce TCA cycle intermediates such as acetyl-CoA and succinyl-CoA. L-glutamate can be recycled into α-KG via GDH (EC 1.4.1.4) or incorporated into the glutamate-glutamine cycle for neurotransmitter release (Torres *et al.,* 2001; Hull *et al.,* 2018).

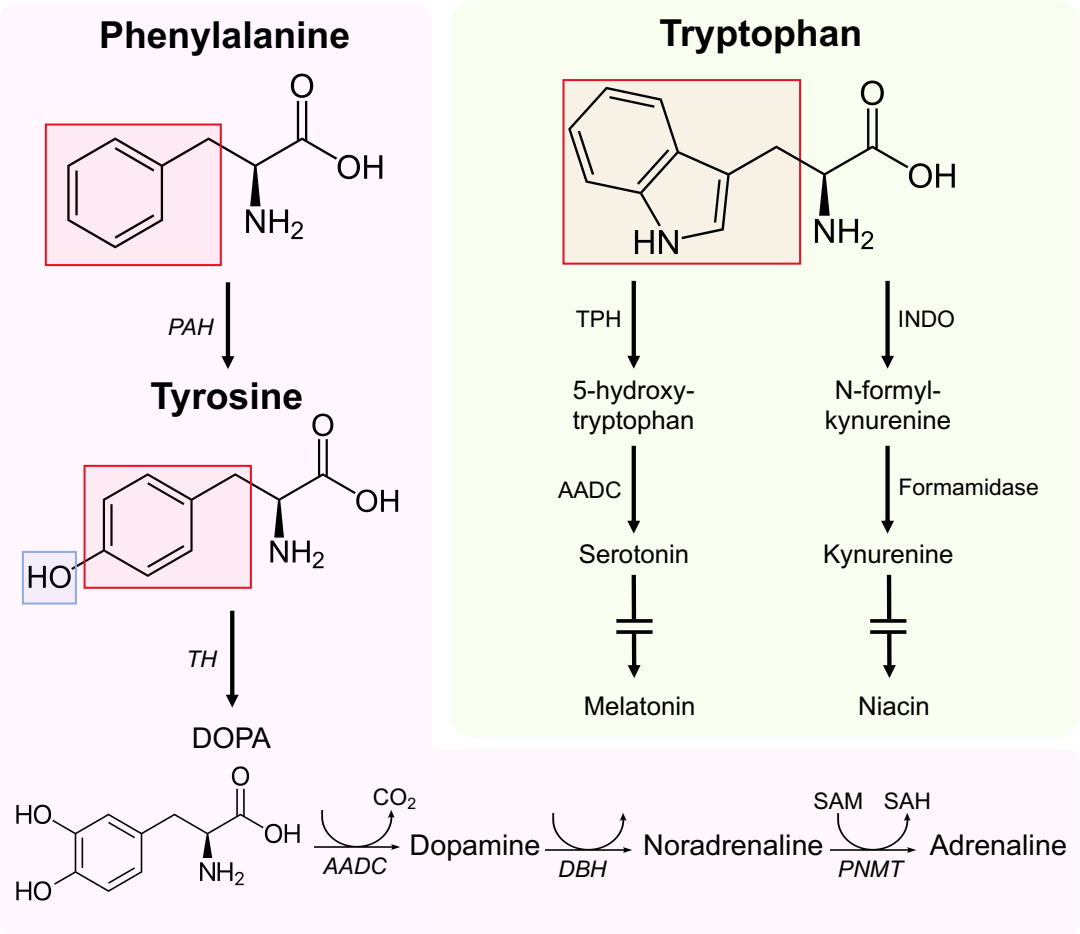

***Figure 7. Metabolism of aromatic amino acids*.** Hydroxylation of phenylalanine into tyrosine by phenylalanine hydroxylase (PAH, EC 1.14. 16.1) is the first step in a series of cascade reactions leading to the biosynthesis of neurotransmitters L-3,4- dihydroxyphenylalanine (L-DOPA), dopamine, adrenaline, and noradrenaline. Tryptophan catabolism, however, can occur via two distinct reactions. The hydroxylation step (via tryptophan hydroxylase – TPH, EC 1.14.16.4) synthesises neurotransmitters 5-hydroxytryptamine (5-HT/serotonin) and subsequent neurohormone melatonin, whereas deoxygenation (via indoleamine-2,3-dioxygenase - INDO, EC 1.13.11.52) produces kynurenine and niacin (vitamin B3) (Parthasarathy *et al.,* 2018).

production of vital neurologically active compounds (i.e., neurotransmitters dopamine and adrenaline) (Fitzpatrick, 2003). BCAAs and ArAAs, as well as the amino acids histidine and methionine, are readily transported across the blood brain barrier via L-type amino acid transporter 1 (LAT1 - SLC7A5), with leucine showing the highest amino acid influx in rat brain perfusion models (Wang and Holst, 2015; Scalise *et al.,* 2018). This is important as an increase in BCAA concentration may lead to a decrease in ArAA, methionine and histidine uptake, which can have severe consequences on processes such as neurotransmitter synthesis and the redox/antioxidant systems of the cell (Fernstrom, 2005; Martínez *et al.,* 2017; Holecek, 2020). In addition, as BCAAs and ArAAs have been shown to stimulate insulin secretion, it is clear that an imbalance in these metabolites is likely to contribute to dysregulation in cellular glucose uptake (Newgard *et al.,* 2009; Bloomgarden, 2018). Leucine in particular acts by serving as both a metabolic fuel and allosteric activator of GDH in β cells of the pancreas, enhancing glutamate turnover. Protein synthesis originating from BCAA metabolism requires abundant energy levels in tissues such as liver and muscles and normally increase glucose demand, potentially impacting on increased insulin signalling, GLUT4 expression and glucose uptake (Gannon *et al.,* 2018). Dysregulation in the metabolism of BCAAs and ArAAs can therefore be strongly associated with and contribute to the development and pathogenesis of T2DM and AD, although further understanding of the specific mechanisms impaired is required.

1.4.1.1. PLP-dependent branched-chain aminotransferase

The BCAT protein is central to the catabolism of BCAAs by mediating the reversible transamination of BCAAs and α-KG into BCKAs and glutamate under nutrient-rich, non-pathological conditions. BCKAs are further metabolised by the phosphorylation-dependent BCKD complex, whereas glutamate can be released

during excitatory neurotransmission or recycled by GDH via the glutamate-glutamine cycle (Hull *et al.,* 2018).

However, under stress conditions such as sepsis and trauma, the reaction can shift from protein synthesis to favouring the production of BCAAs, which can be subsequently utilised as an additional energy source with the production of glutamine and alanine (Holecek *et al.,* 1998). In humans, the mitochondrial isoform (BCATm) is ubiquitously expressed throughout the body, whereas the cytosolic isoform (BCATc) has been reported particularly in glutamatergic and GABAergic neurons in the CNS as well as in the placenta, testis, and ovaries (Suryawan *et al.,* 1998; Hull *et al.,* 2012). Moreover, Hull *et al.* (2012) showed that, in the human brain, BCATm is largely observed in endothelial cells, whilst BCATc is predominantly expressed in the neuronal cell body with some expression along axonal processes, suggesting its key contribution to the glutamate pool as well as neuronal excitation.

In addition to its transamination role, the BCAT protein has been implicated as a chaperone protein (Conway and Lee, 2015) and in redox regulation via its unique CXXC motif (Brüne and Mohr, 2001; Coles *et al.,* 2009). The thiol residues comprising the CXXC motif are vital for transamination, and have been shown to be conserved between BCATc (C335/338) and BCATm (C315/318); post-translational modifications such as S-nitrosylation and S-glutathionylation, as well as oxidation, can lead to reversible inactivation of the protein and impact on its key role in transamination (Conway et al., 2008) Oxidation of BCATm has been shown to induce the formation of a disulphide bond between the two cysteine residues (C315/318) and lead to a complete loss in enzyme activity. However, the subsequent addition of a reducing agent such as dithiothreitol (DTT) can halt oxidation and restore activity (Conway *et al.,* 2002).

Mutations in one or both the thiol groups can, however, also lead to irreversible loss in enzyme activity as demonstrated by Conway *et al.* (2004). Substitution of cysteine 315 to serine or alanine in BCATm reduced the enzyme activity by approximately 70%, rendering it insensitive to further oxidation. However, single mutation of C318, followed by exposure to H_2O_2, leads to irreversible oxidation and complete loss of enzyme activity. Here, C315 acts as redox sensor, whereas C318 operates as the resolving cysteine allowing reversible disulfide bond formation and preventing protein overoxidation under increased ROS conditions (Conway *et al.,* 2004).

In contrast to BCATm, BCATc was shown to have four additional cysteine residues (C335, C338, C221 and C242) which are vital for its physiological activity. Reversible redox regulation of the BCATc isoform is likewise governed by the thiol groups of the CXXC motif, C335 and C338. In this instance, C335 is suggested to act as the redox sensor, as substitution of the C338 residue increases sensitivity to hydrogen peroxide (Conway *et al.*, 2008). Furthermore, a study by Coles *et al.* (2009) showed that S-glutathionylation of BCATc increased under oxidising environments, a reversible process following exposure to redox repair proteins such as glutaredoxin (Grx) with the release of GSH adducts. This shows that S-glutathionylation could act as a mechanism to preserve BCATc under conditions of severe stress, as well as suggesting that BCATc may be implicated in regulation of cellular redox status.

More recently, unpublished data by Conway *et al.* showed that the BCAT protein can form associations with proteins involved in regulation of energy pathways via the N-terminal cysteines of its CXXC motif, offering insight into the protein's role in metabolic reprogramming. While in its reduced state BCAT was shown to bind enzymes in glycolysis such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hexokinase and enolase, oxidation decreased these associations and led to partnerships with additional glycolytic enzymes lactate dehydrogenase and

fructose-2,6-biphosphatase, as well as polyol pathway enzymes such as aldose reductase and (AR) and sorbitol dehydrogenase (SDH). This indicates that the reactive cysteines of its CXXC motif are an important tool for these associations to occur, and changes in the redox status of the cell, particularly under pathogenic conditions, are key contributors to its regulation (Conway *et al*., unpublished observations). Moreover, oxidation of native BCAT also influenced this binding kinetics. Collectively, these observations evidence that the BCAT proteins are intrinsically controlled and are likely to be affected under pathological conditions where the redox environment, as well as energy metabolism, are severely dysregulated.

1.4.1.2. Implications of the BCAT protein in AD pathogenesis

A study by Hull *et al.* (2015) showed a significant increase in the expression of the BCAT proteins in the brains of individuals with AD. Regional increase of BCATc, particularly in areas associated with AD pathology such as the hippocampus, suggest an important correlation with the accumulation of glutamate, leading to excitotoxicity and neurodegeneration. Hudd *et al.* (2019) showed that levels of BCAT and glutamate are significantly increased in the serum of patients with AD relative to matched controls, supporting the evidence that BCAA metabolism and BCAT dysregulation are involved in the development of AD. Furthermore, mutations in genes involved in the BCAA metabolism, particularly in the regulation of BCAT2 (BCATm) or the BCKD complex, can lead to extensive neurological damage such as mild memory impairment, headache and brain lesions, symptoms which can be improved with vitamin B6 supplementation, a co-factor for the transamination activity of BCAT (Lynch and Adams, 2014).

In light of the likewise impaired BCAA metabolism in T2DM as discussed previously, it is reasonable to infer that the expression of the BCAT protein is similarly dysregulated, which suggests a potential link between the two pathologies. Whether the protein is overexpressed in AD as a result of T2DM or otherwise, these results collectively show that the protein's role in homeostasis is key and requires further understanding, particularly in the context of metabolic reprogramming and its effects on energy pathways such as glycolysis and the TCA cycle.

1.4.2. Regulation of energy homeostasis via the glycolytic pathway

Metabolic reprogramming, in response to pathological levels of BCAAs, fatty acids (FAs) and glucose, contribute to the development of insulin resistance and are key features in the development of T2DM, as well as being widely implicated in the pathogenesis of AD where hyperinsulinaemia and insulin resistance (IR) in the early stages of the disease are reported to contribute to memory impairment. (Luchsinger *et al.,* 2004; Akomolafe *et al.,* 2006; Akter *et al.,* 2011; Mukherjee *et al.,* 2015).

In health, following insulin release and glucose transporter (GLUT) stimulation, glucose is transported intracellularly and phosphorylated into glucose-6-phosphate (G6P) by hexokinase (EC 2.7.1.1) and further metabolised via glycolysis into pyruvate, key metabolite linking a multitude of vital cellular pathways including the TCA cycle (Figure 8, hexokinase pathway) (Fry, 2010). However, under conditions of hyperglycaemia, such as in T2DM, a shift in glucose metabolism from glycolysis to the polyol pathway favours the production of sorbitol and subsequently fructose (Figure 8). Under normal physiological conditions only a small portion of glucose is metabolised via the polyol pathway, thought to be of particular importance in pathways which utilise fructose as main energy source (Hers, 1956; Murdoch and White, 1968; Frenette *et al.,* 2006). However, the polyol pathway is understood to be activated under hyperglycaemic conditions such as observed in T2DM, where it converts approximately 30% of the glucose in the body (Yan, 2018).

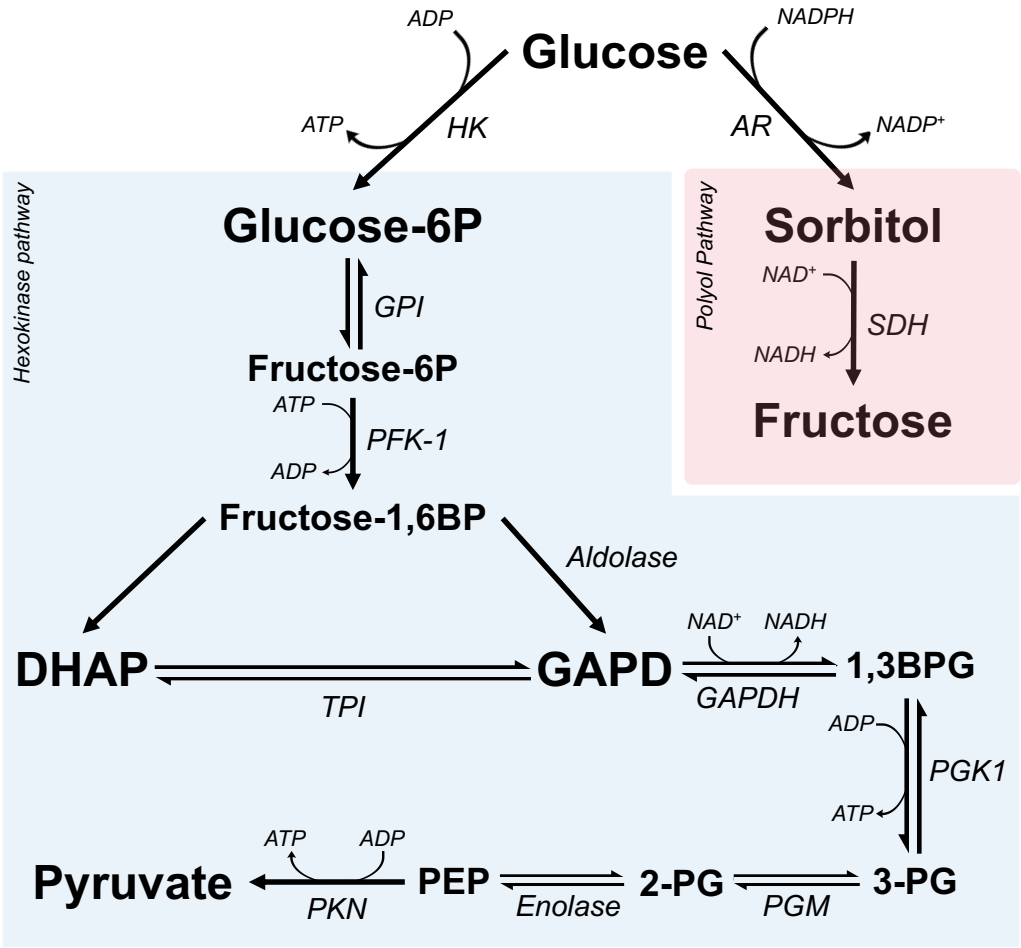

Figure 8. Glycolysis (blue) and the polyol pathway (red). Changes from glycolysis to the polyol pathway can be caused by two co-dependent processes: high levels of glucose increase the activity of HK and enhance G6P production which negatively regulates HK, decreasing glucose processing thus increasing its levels; and the activity of aldose reductase (AR, EC 1.1.1.21), the oxidoreductase responsible for the first step in converting glucose to sorbitol, which shows low affinity for glucose and therefore requires substantial levels to be activated (O'Brien and Schofield, 1983). In addition, glucose processed by AR into sorbitol is subsequently metabolised into fructose via sorbitol dehydrogenase (SDH, EC 1.1.1.14), however accumulation of sorbitol in T2DM can lead to increased osmotic pressure and oxidative stress, ultimately leading to cell death (Berg *et al*, 2002).

Accumulation of sorbitol is observed in tissues such as the retina and lens of the eye, kidneys, and peripheral nerves, where activity of SDH and AR are decreased and increased, respectively, impacting sorbitol production (Brownlee, 2005). This is of particular importance in the progression of diseases such as AD, where studies have observed an increase in sorbitol (3 – 4.2-fold) and fructose (3.9 – 5.7-fold) in AD brains relative to matched, healthy controls (Giacco and Brownlee, 2010; Xu et al., 2016). In uncontrolled T2DM, the damage generated from excessive levels of sorbitol ultimately lead to cell death, reflected as diabetic retinopathy, nephropathy, and neuropathy (Chung *et al.,* 2003). Studies using AR knockout mice and AR inhibitors have shown that deficiency in the enzyme can reduce the oxidative stress observed in the retina and nerve cells under diabetic states and protect the mice from functional deficits, suggesting AR plays a fundamental role in the early development of diabetic complications (Ho *et al.,* 2000; Cheung *et al.,* 2005).

Sorbitol is a highly hydrophilic, non-metabolising sugar which, unable to cross the cell membrane, accumulates and increases osmotic pressure intracellularly. The generation of sorbitol from glucose requires the reductant NADPH, a key substrate for the reduction of the antioxidant glutathione (GSH) from glutathione disulphide (GSSG). Depletion of GSH in the cell is directly correlated with ROS production, osmotic changes, and overall cellular damage (Mathebula, 2015). Increased sorbitol levels enhance fructose turnover via SDH, a reaction which releases NADH in the process and is suggested to play a role in the formation of ROS (Tang *et al*., 2012). In addition to decreasing GSH turnover, depletion of NADPH and NAD^+ pools impact on multiple reducing reactions throughout the body such as the production of myo-inositol, a key precursor for PI and subsequent 3-phosphoinositides (PIP) which are fundamental molecules implicated in insulin resistance and glucose homeostasis, vesicle trafficking, cell survival and growth, and neuronal and synaptic function. This is particularly important in AD, where it has been suggested that accumulation of Aβ

oligomers impact on the PIP signalling pathway, with emphasis to downregulation of phosphatidylinositol 4,5-bisphosphate (PIP_2) leading to the generation of toxic $A\beta_{42}$ (Landman *et al.,* 2006; Arancio *et al.,* 2008). In addition, myo-inositol has been associated with processes during foetal development such as modulation of Ca^{2+} channels in oocytes, meiotic maturation, and gametogenesis (Chau *et al.*, 2005; Kuşcu *et al.,* 2016).

High levels of fructose, as well as hyperglycaemia, oxidative stress, and inflammation, also affect the production of AGEs, molecules resulting from the non-enzymatic reaction of reducing sugars with free metabolites such as albumin, amino acids, fatty acids, and nucleic acids (Sasaki et al., 1998; Mathebula, 2015). Although AGEs production has been shown to occur under normal physiological conditions, pathological accumulation can lead to a variety of damaging cellular responses such as increased protein aggregation, ROS, overexpression of growth factors and subsequent modification of growth pathways, dysfunction in vasoregulation and cellular death (Stitt, 2001; Gkogkolou and Böhm, 2012). These mechanisms are mediated by AGE receptors such as RAGE, which have been shown to accumulate in diabetic tissues as well as being present in high levels in hippocampal neurons in AD brain, where the disease initiates (Li *et al.,* 1996; Wang *et al.,* 2009; Ramasamy *et al.*, 2011). Increasing levels of AGEs and subsequent activation of its receptor (RAGE) stimulate several pathways which are involved in the progression of AD, particularly APP processing, Aβ production and transport across the BBB (Deane *et al.,* 2003 and 2009). However, the specific events leading to such dysregulation remain to be elucidated, aspects of which will be studied in this project.

1.4.3. Reprogramming of the TCA cycle

Inadequate glucose and energy metabolism, resulting primarily from impaired mitochondrial function and oxidative stress, have been shown to occur in the early stages of AD progression and are associated with cognitive decline (Yin *et al.,* 2016). The TCA cycle is one of the central mitochondrial metabolic pathways in the cell for utilising substrates from multiple cellular reactions, with roles in the biosynthesis of nucleotides, lipids and proteins, the complete oxidation of pyruvate, the main product of glycolysis, and the generation of ATP with the generation of NADH. NADH can be incorporated into the electron transport chain and increase ATP production to meet the cell's requirement, showing both the TCA cycle and the electron transport chain are intrinsically linked and coordinate at several stages. Its initial substrate, Acetyl-CoA, can also be acquired via fatty acid β oxidation, ketone body oxidation, and (as well as succinyl-CoA) BCAA metabolism (Martinez-Reyes and Chandel, 2020).

Changes in intermediary pathways contributing to anaplerosis can directly impact the TCA cycle's normal function, mainly in the regulation of glucose and lipid metabolism. Moreover, tissues such as the brain, with remarkably high metabolic activity requiring a copious supply of oxygen and nutrients, is severely impacted by dysregulation in energy homeostasis (Sonnay *et al.,* 2017). For instance, a shift from the hexokinase to the polyol pathway, such as in T2DM, can directly decrease pyruvate output, while decreased fatty acid β-oxidation and impaired BCKA oxidation all contribute to dysregulation in the levels of Acetyl-CoA, succinyl-CoA, and oxaloacetate, as well as ATP/NADH (Monirujjaman and Ferdouse, 2014).

Oxaloacetate contributes to the production of aspartate, a key amino acid in the conversion of IMP to S-AMP in purine metabolism, and aspartate can be released into the cytosol from the nucleotide cycle as fumarate; fumarate can be converted back into oxaloacetate, reinitiating the cycle. However, deficiencies in this pathway can also decrease the overall output of fumarate produced from the reaction of S-

AMP to AMP in purine biosynthesis (Arinze, 2005). Dysregulation in the metabolite input and output in the TCA cycle can ultimately lead to impaired mitochondrial respiration, and increased ROS production leading to cellular damage. The resulting mitochondrial stress has been highly correlated with accelerated aging process and thus the progression of multiple age-related neurodegenerative diseases, particularly AD (Mancuso *et al.,* 2010; Simoncini *et al.,* 2015).

1.4.4. Synthesis and β-oxidation of fatty acids as key metabolic regulators

Fatty acids are the main components of phospholipids and glycolipids, vital molecules for biological membrane structure and integrity, as well as triacylglycerol synthesis (Berg et al., 2002). In addition, FAs can lead to protein modification/acylation such as S-palmitoylation and N-myristoylation, and act as hormones (i.e., prostaglandins and leukotrienes) and intracellular messengers (such as in the activation of protein kinase C, PKC) (Bhathena, 2006; Resh *et al.,* 2013).

Several studies have demonstrated an association between levels of insulin and BCAAs and dysregulation of lipid metabolism in disease (Newgard *et al.,* 2009; Lian *et al.,* 2015; White *et al.,* 2016; Gastaldelli *et al.,* 2017; Newgard *et al.,* 2017). In the early stages of T2DM, high levels of BCAA and hyperinsulinaemia can increase FA synthesis whilst decreasing β-oxidation, accelerating fat deposition and disease progression (Gastaldelli *et al*., 2017). Regulation of Acetyl-CoA carboxylase (ACC) activity, the enzyme involved in the first committed step in FA synthesis, is highly dependent on allosteric activation and inhibition by major circulating metabolites, particularly fatty acyl-CoA. Insulin and glutamate can act on protein phosphatases which directly decrease the fatty acyl-CoA pool and activate ACC, driving an increase in FA synthesis. Low levels of insulin lead to an increase in malonyl CoA, which can inhibit carnitine palmitoyl-CoA transferase I (CPT-1), increase fatty acyl-CoA

availability and inhibit ACC. In addition, glucagon and adrenaline can also inhibit ACC by enhancing AMP-dependent kinase activity (Brownsey *et al.,* 2006).

White *et al.* (2018) reported that ATP citrate-lyase (ACL), key enzyme in fatty acid synthesis, can be reversibly phosphorylated by the kinase (BCKDH kinase) and phosphatase (PPM1K) responsible for BCKD regulation, showing a vital integration between fatty acid synthesis and BCAA metabolism. Harris *et al.* (2020) showed that the cytosolic isoform of the BCAT protein can be translocated and, under oxidised conditions, bound to membranes in the cell via S-palmitoylation, in response to insulin levels, nutrient deprivation, and changes in the redox environment. This is of particular importance in the regulation of the autophagy mechanism, where membrane bound BCATc has been shown critical to the synthesis of autophagosomes which impacts on Aβ production and potentially in the progression of AD (Harris *et al.,* 2020). In addition, the BCAT protein has been shown to bind to proteins in FA metabolism, likely altering its substrates in health and impacting on the progression of disease (Conway *et al.,* unpublished observations). However, the exact mechanisms leading to dysregulation are not entirely understood and are therefore further explored in this project.

1.4.5. The cyclic nature of the s-adenosylmethionine pathway

S-adenosylmethionine (SAMe) is produced, utilised and regenerated via a cyclic process known as the SAMe cycle. It plays a key role as the main methyl donor group in the cell, polyamine and GSH synthesis (transmethylation, aminopropylation and transulfuration), as well as neurotransmitter synthesis and inactivation, cell membrane integrity, proliferation, differentiation, apoptosis, and cell death (Bottiglieri, 2002). SAMe daily requirement is directly correlated with the availability of methionine both *de novo,* resulting from methyltetrahydrofolate and vitamin B12, and via dietary intake (Froese *et al.,* 2019).

Recent studies showed that SAMe levels are significantly decreased in the CSF of individuals with AD, in addition to a decreased ratio of SAMe/s-adenosyl homocysteine (SAH) suggesting lower methylation capacity (Linnebank *et al.,* 2010). This correlates with additional evidence showing SAMe deficiency promotes the expression of PSN1, increasing γ-secretase and subsequent generation of toxic $A\beta_{42}$ peptides (Scarpa *et al.,* 2003). Similar to AD, individuals with T2DM were shown to have significantly decreased transmethylation and transsulfuration capacity and increased homocysteine levels alongside a decrease in clearance, relative to matched controls (Tessari *et al.,* 2005). The mechanisms leading to these imbalances in the pathogenesis of both AD and T2DM remain to be understood, however SAMe has been in clinical trials to treat several conditions since 1973, including AD. This comes as a result of several studies showing that metabolic pathways which depend on the SAMe cycle, particularly lipid metabolism, are impaired in the disease, as well as SAMe levels being reduced in cerebrospinal fluid (CSF) and other brain regions of individuals with AD (Molina *et al.,* 1998; Obeid and Herrmann, 2009).

Alongside the three main reactions in the SAMe cycle, methionine metabolism has also been suggested to occur via BCAT-dependent transamination of α-ketomethiolbutyric acid (KMTB). Several studies showed that like the metabolism of BCAAs (and ArAAs), leucine, isoleucine, valine, phenylalanine, and tyrosine are key amino donors for the transamination of KMTB and subsequent generation of methionine in several bacteria species (Mitchell and Benevenga, 1978; Berger *et al.,* 2003; Venos *et al.,* 2004). Of note is that, in humans, methionine shares the same cellular transporters as BCAAs and ArAAs which could directly affect its uptake into tissues, particularly across the blood brain barrier and into the brain, where its methylation step plays an important role in the synthesis and inactivation of neurotransmitter monoamines (Eriksson *et al.,* 1981; Bottiglieri, 2002).

Similar to leucine, high levels of methionine/SAMe activate mTORC1 and inhibit the autophagy pathway. SAMe acts by binding to and disrupting the association between SAMTOR and GATOR1 (GTPase activating protein for Rag1/2) to allow for the activation of mTORC1. Methionine starvation, however, has the direct opposite effect, increasing the SAMTOR-GATOR1 interaction inhibiting mTORC1, thus stimulating autophagy (Gu *et al.,* 2017). Studies in nutritional health suggest that BCAA, methionine and SAMe accumulation can impact on the ageing process via SAMTOR-GATOR1 disruption and mTORC1 activation, leading to a decrease in autophagy, increase in IR and potential acceleration in the development of age-associated conditions such as AD (Kitada *et al.,* 2019).

1.4.6. Nucleotide pathways beyond genetic regulation

Nucleotide metabolism has a cyclic relationship with the SAMe pathway and its substrates. Metabolites such as 10-formyltetrahydrofolate (10-FTHF) and 5,10-Methylenetetrahydrofolate (5,10-MTHF) have important roles in the synthesis and degradation pathways, and substrates derived from synthesis (particularly ATP) are vital for SAMe turnover, which is itself derived from the reaction between ATP and methionine. This suggests that any alterations in purine metabolism can directly impact on the normal function of the SAMe cycle (Ansoleaga *et al.,* 2015). In addition, the *de novo* synthesis of purines incorporates metabolites generated by biological pathways such as BCAA catabolism (glutamate/glutamine), with BCAA metabolism playing a major role in the production of glutamine by acting as an amino nitrogen donor in muscle cells. The glutamate generated from the catabolism of BCAAs contributes towards the production of glutamine, the primary amino acid in the rate-limiting step of purine synthesis, via the glutamate-glutamine shuttle, and can also react with the oxaloacetate produced via the TCA cycle to produce aspartate, amino acid of key importance in the steps leading to nucleotide synthesis (Arinze, 2005).

Perturbances in general metabolism can substantially affect purine and pyrimidine synthesis and impact on most cellular processes – from DNA and RNA synthesis to ATP generation and overall homeostasis (Nazki *et al.,* 2013). Recent studies have focused on assessing the levels of purine and pyrimidine metabolites in plasma and CSF of individuals presenting with T2DM and AD. Metabolites from purine catabolism, such as allantoin, have been shown to be indicative of increased risk of T2DM independently of additional risk factors, particularly in individuals carrying the transcription factor-7-like-2 (TCF7L2) rs7903146 polymorphism – which impairs β-cell function and hepatic insulin activity (Papandreou *et al.,* 2019). Similarly, nucleosides and nucleotides levels were elevated in the serum of patients with both T1DM and T2DM, which indicated that an increase in nucleoside availability likely originates from increased nucleotide degradation, a decrease in adenosine kinase activity or a change in the expression level of nucleoside transporters under T2DM conditions (Dudzinska *et al.,* 2014). Furthermore, in diseases such as AD, studies have shown that levels of the purine metabolites deoxyguanosine, xanthosine, dGMP and glycine, are decreased in different levels across the brain (frontal, parietal, and temporal) and more prominently observed in the early stages, prior to the development of Aβ plaques and NFTs (Ansoleaga *et al.,* 2015; Alonso-Andrés *et al.,* 2018). However, there is limited consensus on the changes in nucleotide metabolism that impact on T2DM and AD, and this project focuses on aspects which are likely interconnected.

1.5. Summary

Collectively, the presented evidence further substantiates that both T2DM and AD are intrinsically linked via their perturbed metabolic pathways and understanding the molecular mechanisms that are likely to initiate or participate in these pathways is essential. BCAA metabolism and the activity of its enzymes, BCAT and BCKD, have

been implicated in disease pathology, affecting pathways such as insulin, glucose and fatty acid metabolism, the SAMe cycle, purine nucleotide synthesis and the TCA cycle. Thus, this project aimed to show that BCAT dysregulation is a key feature of metabolic reprogramming in T2DM progression and in turn has a vital role in the development of AD.

1.6. Hypothesis

The central hypothesis in this project is that insulin resistance, paired with increasing levels of BCAAs, hyperglycaemia, and hyperlipidaemia, as observed in T2DM, contributes towards dysregulation of BCATc and thus energy homeostasis, leading to an increase in oxidative stress and subsequent changes in cellular redox environment, particularly because of disturbances in glucose metabolism and chronic activation of the polyol pathway. This in turn has a significant impact on the clearance mechanisms in the cell, predominantly autophagy, enhancing the accumulation of protein aggregates such as Aβ and NFTs from hyperphosphorylated tau and severely impacting on LTP, which as a result contributes towards the development of AD.

1.7. Aim and objectives

The aim of this project was to understand whether changes in expression and redox status of the BCATc protein dysregulate key metabolic energy pathways of the cell involved in T2DM which may additionally contribute to the development of AD.

Objective 1: To assess metabolite load, enzyme activity, glycolytic regulation and oxidative phosphorylation status in neuronal cell models following overexpression and knockdown of the BCATc protein and/or its thiol mutants.

Objective 2: To demonstrate that such metabolic dysregulation leads to an increase in oxidative stress, disruption in the autophagy mechanism and overexpression of APP, Aβ and PHF-tau, culminating to neuronal death.

Chapter 2

Materials & Methods

2.1. Materials

Gibco™ Dulbecco's Modified Eagle Medium/Ham F-12 (DMEM/F-12 – 31331028), Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM - 41090028), non-essential amino acids (NEAA – 11140035), foetal bovine serum (FBS – 15517589), 0.25% trypsin (25200056), Recovery™ Cell Culture Freezing Medium with DMSO (11560446) and Opti-MEM™ I Reduced Serum Medium (10149832), Invitrogen™ Subcloning Efficiency™ DH5α Competent Cells (18265017), Lipofectamine™ 2000 Transfection Reagent (10696153), Lipofectamine™ RNAiMAX Transfection Reagent (13778030) and Novex™ NuPAGE™ LDS Sample Buffer (4X) (11549166), GeneJET™ Plasmid Midiprep (K0481) and Miniprep (K0502) Kits, Thermo Scientific™ GelCode™ Blue Stain Reagent (10608494), Merck™ Luminata™ Western HRP Chemiluminescence Substrates, bovine serum albumin (BSA – BP9702-100), CHAPS (10274723), Thermo Scientific™ FastDigest DpnI (10809410) and dNTP Mix (10889390), Invitrogen™ Nuclease-Free Water (10429224), crystal violet (12926287), and standard chemicals were purchased from Fisher Scientific (Loughborough, UK).

Hydrogen peroxide solution (H1009), Immobilon-FL PVDF Membrane (IPFL00010), Thrombin (T6884), Acrylamide/Bis-acrylamide (9A3699), Phenylmethanesulfonyl fluoride (PMSF - P7626), Sodium fluoride (NaF - S7920), PhosSTOP™ Phosphatase inhibitors (4906845001), cOmplete™, Mini, EDTA-free Protease Inhibitor (11836170001), Bafilomycin B1 (11707), Chloroquine diphosphate salt (C6628), Poly-L-lysine (P4832), Rapamycin (R0395), Insulin (91077C), DL-Dithiothreitol (DTT - D9779), α-Ketoisocaproic acid (KIC - 68255), Dimethyl sulfoxide (DMSO - D2650), Bafilomycin A1 (SML1661), N,N,N′,N′-Tetramethylethylenediamine (TEMED - T9281), Ammonium persulfate (APS - A3678), L-leucine (L8000), L-valine (V0500), L-isoleucine (I2752), L-Glutamine (G3126), sodium borohydride (452882), 2x YT medium (Y2377), 2-mercaptoethanol (M3148), Ni Sepharose® 6 Fast Flow

(GE17-5318-01) and site-directed mutagenesis primers for hBCATc C335S, C338S and C335/8S were purchased from Sigma Aldrich/Merck (Dorset, UK).

Malate Dehydrogenase (MDH - ab183305), Glyceraldehyde-3-phsphate dehydrogenase (GAPDH - ab204732), Isocitrate Dehydrogenase (IDH - ab102528) and Enolase (ab241024) activity assays were purchased from Abcam (Cambridge, UK).

QuikChange II XL Site-Directed Mutagenesis Kit (200521), Seahorse XF base medium (103335-100), XF Cell Mito Stress Test Kit (103015-100), XF glycolytic rate assay kit (103344-100) and XFe24 FluxPak (102340-100) were purchased from Agilent (Cheshire, UK).

Revert 700 Total Protein Stain (926-11021) and Revert 700 Wash Solution (926-11012) were purchased from Li-cor Biosciences (Cambridge, UK).

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (G3580) and FuGENE® 6 Transfection Reagent (E2691) were purchased from Promega (Hampshire, UK).

PROTEOSTAT® Protein aggregation assay (ENZ-51023-KP002) and Protein aggregation standards (ENZ-51039-KP002) were purchased from Enzo Life Sciences (Exeter, UK).

VECTASHIELD® Hardset™ Antifade Mounting Medium with DAPI (H-1500) was purchased from Vector Labs (Peterborough, UK).

SH-SY5Y (CRL-2266™), IMR-32 (CCL-127™) and HeLa (CCL-2™) cells were obtained from ATCC® (Middlesex, UK).

siRNA for hBCATc (AAATGAAGATGGAGAAGAAGA) and hBCATm (AAGGAGATCCAGTACGGAATC) were ordered custom-made from Eurofins (Ebersberg, Germany).

pCS2+HyPer7-NES (Addgene plasmid #136467), pCS2+HyPer7-NLS (Addgene plasmid #136468) and pCS2+MLS-HyPer7 (Addgene plasmid #136470) were a gift from Vsevolod Belousov.

NADH, sodium pyruvate and lactate dehydrogenase (LDH) enzyme (Fisher Scientific, UK) were kindly supplied by Adele Ainger from our university.

Primary and secondary antibodies were as follows (Table 3 and 4).

Table 3. Primary antibodies.

Company	Code	Name	WB Dilution	ICC Dilution
Insight Biotechnology	Custom order	Polyclonal Rabbit-raised hBCATc	1:3,000	1:150
		Polyclonal Rabbit-raised hBCATm	1:3,000	
	ab7291	Anti-alpha Tubulin antibody [DM1A] - Loading Control	1:5,000	
	ab56416	Anti-SQSTM1 / p62 antibody	1:2,000	
	ab96193	Anti-MDH2 antibody	1:2,000	
	ab201060	Recombinant Anti-beta Amyloid 1-42 [mOC64]	1:1,000	1:250
	ab2072	Anti-APP	1:2,000	1:150
Abcam	ab155102	Anti-ENO1	1:3,000	
	ab14715	Anti-SDHA	1:1,500	
	ab172964	Anti-IDH1	1:1,500	
	ab15571	Anti-PRX1/PAG	1:1,000	
	ab13534	Anti-SOD	1:2,000	
	ab133524	Anti-TRX	1:5,000	
Cell Signaling	2118S	GAPDH (14C10) Rabbit mAb	1:5,000	
Invitrogen	MN1020B	Phospho-Tau (Ser202, Thr205) AT8	1:500	1:100
Agilent/Dako	A002401-2	Polyclonal Rabbit Anti-Human Tau A0024	1:500	
MBL International	PM036	Anti-LC3	1:1,500	
Protein Tech	15804-1-AP	GLRX	1:500	

Table 4. Secondary antibodies.

Company	Code	Name	Dilution
Li-cor Biosciences	925-32212	IRDye 800CW Donkey anti-Mouse	
	926-68073	IRDye 680RD Donkey anti-Rabbit	
Vector Labs	PI-1000	Goat Anti-Rabbit IgG Antibody (H+L), Peroxidase	Optimised according to primary antibody dilution
	PI-2000	Goat Anti-Mouse IgG Antibody (H+L), Peroxidase	1:100 to 1:10,000
Abcam	488	Alexa Fluor® Goat Anti-Rabbit IgG H&L	
	568	Alexa Fluor® Goat Anti-Mouse IgG H&L	

2.2. Methods

2.2.1. Validation of protein purification using BCATm and C318A mutant

BL21-DE3 cells from pET28a plasmids overexpressing wild-type or thiol mutant BCATm were grown overnight in 100 mL 2xYT broth (16 g/L Tryptone, 10 g/L Yeast Extract, 5 g/L NaCl) with 30 μM kanamycin sulphate at 37°C and 180 rpm. Following incubation, cells were transferred to 1 L 2xYT broth with 30 μM kanamycin sulphate and allowed to grow overnight at 25°C and 155 rpm. After 24 hours, 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to flasks and incubated for 4 hours at 37°C to induce expression of BCAT from the Lac promoter. Cells were subsequently centrifuged at 12,000 x g for 5 minutes, pellets were carefully weighted and stored overnight at -80°C.

Pellets were allowed to thaw on ice and gently resuspended in buffer A (10 mM Tris, 1 mM β-mercaptoethanol, pH 8.0). Suspensions were sonicated for 10 minutes with 30 seconds rest every 90 seconds and centrifuged at 8,000 x g for 10 minutes at 4°C. Supernatants were poured into 200 mL conical flasks and prepared to a final concentration of 4 M urea and pellets resuspended in buffer A for hBCATc (including thiol mutants), and buffer AU (10 mM Tris, 5 M urea, 5 mM β-mercaptoethanol, pH 8.0) for hBCATm (including thiol mutants). These were further sonicated for 10 minutes with 30 seconds rest every 90 seconds, and centrifuged at 8,000 x g for 10 minutes at 4°C. Supernatants were pooled, combined with nickel-NTA resin (pre-equilibrated in the appropriate buffer, approx. 15 mM, Ni Sepharose® 6 Fast Flow, GE Healthcare) and allowed to stir for 1 hour at 4°C. Following incubation, Ni-bound protein was poured into an empty column and washed with buffers A (10 mM Tris, 1 mM β-mercaptoethanol, pH 8.0) (hBCATc), AU (10 mM Tris, 5 M urea, 5 mM β-mercaptoethanol, pH 8.0) (hBCATm), B (10 mM Tris, 0.5 M NaCl, 20% glycerol, 5 mM β-mercaptoethanol, pH 7.4), C (10 mM Tris, 1.5 M NaCl, 20% glycerol, β-mercaptoethanol, pH 7.4), C50 (10 mM Tris, 0.75 M NaCl, 10% glycerol, 77 mM imidazole, β-mercaptoethanol, pH 6.0), respectively. The eluate was collected

at each step. Protein was finally eluted using buffer D (10 mM Tris, 0.75 M NaCl, 10% glycerol, 0.76 M imidazole, β-mercaptoethanol, pH 6.0). 1 mM EDTA was added to the final aliquot to chelate residual nickel resin. Samples were exchanged through a G25 column equilibrated with thrombin buffer (50 mM Tris, 150 mM NaCl, pH 7.5), and 100 units of thrombin were added to the collected protein to cleave the histidine tag, before incubation at 37°C for 1 hour. In the meantime, dialysis tubing was prepared by boiling in $NaHCO_3$ buffer (0.2 M NaHCO3, 5 mM EDTA), washing in deionised water three times and autoclaving at 120°C before storage at 4°C. Dialysis tubing was washed with stable buffer (150 mM NaCl, 50 mM Tris, 5 mM glucose, 1 mM EDTA, 1 mM KIC, 5 mM DTT, pH 7.5), knotted and clamped to avoid leaks. 100 mM α-ketoisocaproic acid (KIC) was then added to the samples and protein was carefully added to dialysis tubing, which was likewise knot-tied and clamped. Protein was incubated overnight in stable buffer at 4°C while stirring. Following incubation, proteins were stored in 30% glycerol at -20°C.

Aliquots at different experimental time-points were reserved for purity assessment (pellets, extract, elution (buffer D), thrombin buffer and final dialysed protein). Bradford assay (Bradford, 1976) was performed to determine protein concentration. Samples were prepared in loading buffer (Bromophenol blue, 8% SDS, 40% glycerol, 0.7 M β-mercaptoethanol, 200 mM Tris, pH 6.8), heated to 95°C for 10 minutes and separated on a 4% stacking (1% APS, 1% SDS, 0.06% TEMED, 4% acrylamide and 1.5 M Tris, pH 6.8) and 12% resolving (1% APS, 1% SDS, 0.06% TEMED, 12% acrylamide and 1.5 M Tris, pH 8.8) SDS-PAGE gel at 180V for 60 minutes in running buffer (25 mM Tris, 190 mM Glycine, 1% SDS, pH 8.3). Gel was Coomassie-stained (0.5% Coomassie Brilliant Blue G-250, 50% methanol, 10% acetic acid), washed in deionised water once, washed briefly in de-stain (40% methanol, 10% acetic acid), washed for an additional 20 minutes in de-stain and finally de-stained for 24 hours. Following incubation in de-stain, gel was washed three

times for 5 minutes in deionised water, and bands were visualised using an Odyssey FC (Li-cor Biosciences) at 700 nm wavelength.

2.2.3. Optimisation of siRNA and plasmid-DNA overexpression constructs for differential expression of the BCAT proteins

Knockdown (KD) siRNA for hBCATc and hBCATm were optimised for expression at 20 nM final concentration. Overexpression (OE) constructs were previously developed using Gateway® pDEST™26 (7.4 kb) by Dr Matthew Harris of our research group (see appendix). The plasmid DNA was transfected into our neuronal models at increasing concentrations ranging 0.5 to 1.5 µg in order to verify subtle metabolic changes depending on BCAT expression.

2.2.4. Expansion and purification of BCAT overexpression vectors

2.2.4.1. Transformation and expansion

Ampicillin (AMP)-resistant hBCATc- or hBCATm-OE DNA plasmid was mixed with NEB® 5-alpha High Efficiency Competent *E. coli* cells, incubated on ice for 30 minutes before heat shock for 20 seconds at 42°C and further incubated on ice for 2 minutes. Suspension was mixed with pre-warmed SOC medium (Super optimal broth with catabolite repression; 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and incubated for 1 h at 37°C and 225 rpm. Small volumes (approximately 50 µL) of final suspension were added to LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 100 µM AMP, and incubated overnight at 37°C. Following incubation, a single colony was removed from the plate and added to a flask containing fresh LB broth. Cells were subsequently incubated overnight at 37°C and 225 rpm.

2.2.4.2. Site-directed mutagenesis for the development of BCAT thiol mutants

BCATc (C335, C338, C335/8) and BCATm (C315S, C318S, C315/8S) thiol mutants relied on cysteine-to-serine point mutations and were generated via site-directed mutagenesis following the manufacturer's protocol for the QuikChange II Mutagenesis Kit (Agilent, Cheshire, UK); primers are listed in Table 4.

Table 5. Sense and antisense primers for the development of BCAT thiol mutants.

BCATc (8.561 kb)		
Thiol	**Sense (5' - 3')**	**Antisense (5' - 3')**
C335S	GGC TCT GGT ACA GCC TCT GTT GTT TGC CCA	TGG GCA AAC AAC AGA GGC TGT ACC AGA GCC
C338S	GGT ACA GCC TGT GTT GTT AGC CCA GTT TCT GAT ATA CTG	TAT ATC CCG AGA AAC TGG GCT AAC AAC ACA GGC TGT ACC
C335/8S	GGT ACA GCC TCT GTT GTT AGC CCA GTT TCT	AGA AAC TGG GCT AAC AAC AGA GGC TGT ACC
BCATm (8.579 kb)		
C315S	TCG GGC ACC GCT TCC CAG GTC TGC CCA	TGG GCA GAC CTG GGA AGC GGT GCC CGA
C318S	GCT TGC CAG GTC TCC CCA GTG CAC CGA	TCG GTG CAC TGG GGA GAC CTG GCA AGC
C315/8S	GGC ACC GCT TCC CAG GTC TCC CCA GTG CAC CGA	TCG GTG CAC TGG GGA GAC CTG GGA AGC GGT GCC

Briefly, template BCATc/m overexpression expressed in pDEST™26 Gateway vector plasmid (100 ng) and forward (FWD) and reverse (REV) mutant primers (125 ng) were incubated with polymerase-chain reaction master mix (1 x Reaction buffer, 200 µM dNTP, 2.5 U/µL *PfuUltra* HF DNA Polymerase in Quik Solution and nuclease-free water) and PCR-amplified following table 5. Elongation

times were calculated based on plasmid size. Methylated parental DNA was digested using methylation-dependent endonuclease Dpn I according to the manufacturer's protocol.

Table 6. PCR amplification times.

Step	Time (s)	Temperature (°C)	Repeat (X)
1	60	95	1
2	50 50 480	95 60 68	12
3	7	68	1

2.2.4.3. Transformation and expansion

Mutants were transformed into XL10-Gold cells following the manufacturer's protocol (Agilent, Cheshire, UK). Cells were thawed on ice and 45 uL added to a round-bottom tube containing 2 µL of β-ME and incubated for on ice 10 minutes with a gentle swirl every 2 minutes. Following from that, 2 µL of Dpn I-treated DNA were added to each tube, gently mixed and samples were heat-treated for 30 seconds at 42°C with subsequent incubation on ice for 2 minutes. 0.5 mL of pre-heated SOC medium was added to the tubes, and samples were incubated at 37°C for 1 h at 225 rpm. Following incubation, transformed cells were plated in LB-Agar plates containing 50 µg/mL ampicillin and incubated at 37°C overnight (>16 h). The following day, colonies were visually assessed and two colonies from each plate/transformation variant were added to LB-Amp to expand overnight at 37°C and 225 rpm.

2.2.4.4. DNA preparation and purification

Mini/Midiprep was performed using GeneJET™ Plasmid Mini/Midiprep Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA,

USA). In brief, incubated cells were centrifuged at 5,000 x g for 10 minutes and supernatant discarded. Pellet was initially resuspended by vortexing in resuspension solution containing RNAse A (2 mL) until homogenous. Lysis solution (2 mL) was subsequently added, suspension was mixed by inversion until viscous and clear and incubated for 3 minutes at RT. Neutralisation solution (2 mL) was added and mixed by inversion prior to adding endotoxin binding reagent (0.5 mL), mixing by inversion, and incubating for 5 minutes at RT. Next, 96% ethanol (3 mL) was added to solution and mixed by inversion. Suspension was centrifuged at 4,500 x g for 40 minutes and supernatant carefully transferred to a fresh tube. Further 96% ethanol (3 mL) was added and mixed by inversion.

Suspension (approximately 5.5 mL) was added to silica-based membrane columns and centrifuged at 2,000 x g for 3 minutes using a swinging bucket rotor. Flow-through was discarded and process repeated until suspension was completely through the column. Wash solution I in 25% isopropanol (4 mL) was subsequently added to column and centrifuged at 3,000 x g for 2 minutes. Flow-through was discarded and wash solution II in 62.5% ethanol (4 mL) added, with another centrifugation step at 3,000 x g for 2 minutes. This step was repeated twice. To remove any excess ethanol from the final column, a final centrifugation at 5,000 x g and 5 minutes was performed. The column was transferred to a fresh collection tube and elution buffer (0.35 mL) was added to the centre of the purification column prior to incubation for 2 minutes at RT. The column was subsequently centrifuged at 3,000 x g for 5 minutes to elute plasmid DNA, and final DNA suspension was stored at -20°C until further required.

2.2.5. Routine culturing of cell models

SH-SY5Y cells were cultured in 25 cm^2 flasks with 5 mL of complete DMEM/F-12 (1% NEAA and 10% FBS) and transferred to a 75 cm^2 flask. Cells were routinely

passaged and maintained at sub-confluence in an incubator at 37°C and 5% CO_2, unless otherwise stated. For cell passage, medium was removed, and cells washed with phosphate buffer saline (PBS) prior to incubation with 0.25% trypsin to allow cells to detach. Trypsin was inactivated with medium, cells centrifuged at 1,000 rpm for 5 minutes and resuspended in fresh medium and transferred to a new flask and incubated as described above.

2.2.6. Differentiation of SH-SY5Y cells

Cells were seeded and incubated in DMEM/F-12 or according to experimental design and allowed to grow to approximately 60% confluency. Media was subsequently removed, and cells were incubated with low-serum DMEM/F12 containing retinoic acid (1% NEAA, 2% FBS, 10 µM retinoic acid) for 72 to 96 hours and subsequently assayed as required.

2.2.6.1. Protein overexpression with Lipofectamine™ 2000

All experiments were performed with cultured cells in parallel to confirm successful KD/OE transfection. BCAT was knocked down and/or overexpressed following the transfection reagent's manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). In brief, cells were allowed to attach and reach approximately 80% confluency prior to OE transfection (forward transfection) or transfected upon seeding for KD with an additional transfection 24 h after (modified reverse transfection). Lipofectamine™ 2000 (Table 6) and siRNA/DNA plasmid were diluted in Opti-MEM (Gibco) in separate tubes, mixed to a 1:1 dilution, and incubated for 5 minutes at RT. Final siRNA- or DNA-lipid complex (20 nM or 0.5, 1 or 1.5 µg, respectively) was thereafter added to cells and incubated for 72 to 96 h (KD) or 48 h (OE), with a media change 24 h after transfection.

Table 7. Lipofectamine™ 2000 transfection volumes.

Culture vessel (plate)	Experiment type	Lipofectamine™ 2000 volume (per well)	Total complex volume (per well)
96-well	Cellular viability and proliferation (crystal violet/MTS), ROS-DCFDA	0.5 µL	10 µL
24-well	Confocal microscopy, Seahorse assay	1.5 µL	62.5 µL
12-well	Western Blot analysis, enzyme assays	3 µL	125 µL
6-well	Western Blot analysis (for large concentrations of protein)	5 µL	250 µL

2.2.6.2. siRNA Knockdown with Lipofectamine™ RNAiMAX

The BCAT protein was knocked down using 20 nM siRNA following the transfection reagent's manufacturer's protocol for reverse and forward transfection. In brief, Lipofectamine™ and siRNA were diluted in Opti-MEM in separate microcentrifuge tubes, mixed and incubated for 10 minutes at RT. Final siRNA-lipid complex was subsequently added to freshly-plated cells to a final concentration of 20 nM and incubated for a period of 48 to 96 h. Re-transfection of the siRNA-Lipid complex was performed in similar fashion 24 h post-transfection, with a media change an additional 24 h after.

2.2.6.3. DNA overexpression with FuGENE® 6

BCAT was overexpressed following the transfection reagent's manufacturers' protocol. In brief, cells were cultured as described in section 2.2 and allowed to attach overnight. Transfection mixture was prepared by diluting 5 µg FuGENE® 6 in Opti-MEM and incubating for 5 minutes at RT. 1.0 µg of BCAT plasmid DNA was

subsequently added to the mixture and incubated for a further 15 minutes at 37°C. Cells were incubated for 48 h with the DNA-lipid complex, with a media change 24 h post-transfection.

2.2.7. Optimised cellular treatments for metabolic assessment

Prior to treatments, cells were nutrient deprived in EBSS for 2 hours, and treated accordingly in EBSS or DMEM supplemented with 1% FBS for prolonged (> 16 h) treatments (Pirkmajer and Chibalin, 2011). Treatments included 100 nM insulin, 100 nM rapamycin, 100 nM Bafilomycin B1, or 50 μM chloroquine for 30 minutes; 25 mM glucose, or 10 mM BCAA for 18 hours. Cells were subsequently analysed using the assays described below.

2.2.8. Cellular viability and proliferation using MTS and crystal violet assays

1.5 x 10^4 cells were seeded into a clear 96-well plate and allowed to attach overnight, or immediately transfected as described (Section 2.3). Following relevant treatments (Section 2.4), 100 μL of fresh medium and 20 μL of the CellTitre Aqueous One Solution Reagent were added and cells incubated for 90 minutes in the dark. Absorbance was measured at 490 nm using a FLUOStar Omega (BMG Labtech). In separate, clear wells, 100 μL of media and 20 μL of the CellTitre Aqueous One Solution Reagent were added and incubated to serve as normalisation control for the experiment. For crystal violet, cells were fixed with 4% paraformaldehyde for 30 minutes, washed three times in PBS and stained with 0.05% crystal violet. Cells were subsequently washed three times with PBS and incubated with 1% SDS in PBS for one hour, after which absorbance was taken using a FLUOStar Omega (BMG Labtech) at 595 nm.

2.2.9. Assessment of reactive oxygen species using Hyper7 molecular probe

ROS production resulting from BCAT dysregulation was analysed using the molecular probe Hyper7 (gift from Vsevolod Belousov). HyPer7 is a genetically encoded fluorescent probe for ultrafast sensing of intracellular hydrogen peroxide. It consists of a circularly permuted yellow-fluorescent protein (cpYFP) transformed into N. meningitidis OxyR protein, an ultrasensitive H2O2 sensing domain (Belousov et al., 2006; Pak et al., 2020). Detection of the Tyr residues of the YFP chromophore at excitation 420 nm (protonated) and 500 nm (charged), and emission at 510-550nm, allow for ratiometric analysis (420/500) to correct for cellular movement and probe expression level between cells (Mishina et al., 2013).

1.5 x 10^4 cells were seeded into a black-wall, clear-bottom 96-well plate and left to attach overnight, or immediately co-transfected with BCAT constructs and pCS2+HyPer7-NES (cytosolic) or pCS2+MLS-HyPer7 (mitochondrial matrix) probes as previously described. Cells in a single row were treated with 100 μM H_2O_2 to validate assay sensitivity. To assess ROS levels with Hyper7, cells were visualised using fluorescence and confocal microscopy prior to having fluorescence read at 420/500 nm excitation and 510 nm emission, and the ratio from 420 to 500 nm calculated. Cell viability was measured with crystal violet and absorbance read at 595 nm to control for differences in cell number.

2.2.10. Fluorescence and confocal microscopy measurement of ROS using Hyper7

Cells were seeded in 18- and 8-well chambers at 1.0 x 10^4 and 1.5 x 10^4, respectively, and allowed to attach overnight prior to transfection with OE plasmids; for KD, cells were immediately transfected. Following transfections, cells were incubated overnight, and media changed to differentiation media and incubated for 72 – 96 hours. For fluorescence, cells were visualised under 10 x magnification with Nikon Eclipse TE300 at 516/405 nm emission/excitation using Andor IQ software.

Prior to confocal imaging, wells were incubated for 30 minutes with 1 µg/mL Hoechst 33342 and images and short videos were captured at 62-64 x magnification using a Leica TCS-SP8 microscope with Leica LAS X Software at 300-405 nm.

2.2.11. Enzyme activity optimisation and assay development

Enzyme assays were performed following the manufacturer's protocols (Abcam, Cambridge, UK). To optimise the number of cells required for each enzyme activity assay, cells were seeded in 12-well plates at a starting concentration of 1.0 x 10^6 cells/well and allowed to attach overnight. Cells were harvested using 0.25% trypsin-EDTA and pelleted to final volumes of 0.2, 0.4, 0.6 and 0.8 x 10^6, washed twice with PBS and resuspended with assay buffer (GAPDH, MDH, IDH, enolase or LDH). Samples were homogenised thoroughly and incubated on ice for 10 minutes, prior to centrifugation for 5 minutes at 10,000 x g and 4°C. Supernatant was collected and transferred to fresh, pre-chilled tubes and incubated on ice until further use.

2.2.11.1. GAPDH Activity assay

The GAPDH assay kit (ab204732) is based on the conversion of glyceraldehyde-3-phosphate and NAD^+ to 1,3-bisphosphoglycerate and NADH. Upon the reaction of NADH with a developer, the absorbance of the generated coloured product can be measured at 450 nm. Samples were prepared with GAPDH assay buffer, developer and substrate and added to wells in duplicate alongside positive control and background control samples (assay buffer and developer only). Absorbance (450 nm) was read immediately in kinetic mode at 37°C every 2 minutes for a total of 10 minutes. Two time-points within the linear range of previously calibrated NADH (nmol) standard curve were used to estimate GAPDH activity.

2.2.11.2. MDH activity assay

The MDH assay kit (ab204732) is based on the oxidation of malate into oxaloacetate using the reduction of NAD^+ to NADH. NADH reacts with a developer which gives a strong absorbance at 450 nm. Assay mix (MDH assay buffer, enzyme mix, developer, and substrate) were added to samples and positive control. Samples were added to wells in duplicate alongside background control (assay buffer, enzyme mix and developer only). Absorbance (450 nm) was measured in kinetic mode at 37°C for 20 minutes. MDH activity in samples was estimated using two time-points within the linear range of previously calibrated NADH (nmol) curve.

2.2.11.3. IDH activity assay

The IDH activity assay kit (ab102538) can measure NAD^+- (cytosolic) and $NADP^+$- (mitochondrial) dependent NADH/NADPH generation in the cell following oxidative decarboxylation of isocitrate. The reaction of NADH/NADPH with a specific developer produces a coloured product which absorbance can be measured at 450 nm. Assay mix (IDH assay buffer, developer, substrate, and NAD^+/$NADP^+$ probe) was added to samples and positive control and samples were added to wells in duplicate alongside background control (assay buffer and developer only). These were incubated for 3 minutes, and absorbance was measured in kinetic mode at 450 nm 37°C every 5 minutes for 30 minutes. IDH activity was estimated using two time-points within the linear range of previously calibrated NADH (nmol) curve.

2.2.11.4. Enolase Activity Assay

Enolase assay kit (ab241024) is based on the conversion of 2-phosphoglycerate to phosphoenolpyruvate and H_2O, which intermediate reacts stoichiometrically with a probe to generate colour observed at λ = 570 nm. Reaction mix (Enolase assay buffer, substrate mix, convertor, developer, and probe) was

added to samples and positive control and samples were added to wells in duplicate alongside background control (no substrate mix). Absorbance was measured in kinetic mode at 570 nm and 25°C every 3 minutes for 40 minutes. Enolase activity was estimated using two time-points within the linear range of previously calibrated H_2O_2 (pmol) curve.

2.2.11.5. LDH Activity Assay

The LDH assay was based on the conversion of pyruvate to lactate alongside oxidation of NADH to NAD^+. The reaction was measured at λ = 340 nm. Reaction mix (0.2 M Tris-HCl, 6.6 mM NADH, 30 mM sodium pyruvate, pH 7.3) was added to a 96-well plate and incubated in the spectrophotometer for 5 minutes for temperature equilibration and measurement of blank at 25°C. Following incubation, samples were individually added, and absorbance read at 340 nm every half a minute for 7 minutes or until plateau. NADH standard was measured separately to allow calculation of total NADH oxidation in samples.

2.2.12. Assessment of glycolytic rate and mitochondrial stress using the Seahorse assay

Method was performed according to the manufacturer's protocol (Agilent, Cheshire, UK). The glycolytic rate assay measures basal glycolysis prior to inhibition of oxidative phosphorylation with Rotenone/Antimycin A (Rot/AA). Compensatory glycolysis is subsequently estimated using total proton efflux rate (PER), with 2-deoxy-D-glucose (2-DG) added to inhibit hexokinase and glycolysis. This provides the PER prior to injection, and thus glycolysis-dependent proton efflux rate (glycoPER)/glycolytic rate using the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). While ECAR measures acidification rate following lactate release in the cytosol, OCR shows proton release from oxidative

phosphorylation. In contrast, the mitochondrial stress test measures oxidative phosphorylation using OCR. Oligomycin inhibits Complex V and decreases electron transport and respiration, while FCCP disrupts mitochondrial membrane potential and Rot/AA inhibit complex I and III to stop mitochondrial respiration allowing for the calculation of non-mitochondrial respiratory events.

Cells were cultured in Agilent 24-well plates at a density of 2.5 x 10^4 cells/well, allowed to attach overnight for overexpression or immediately transfected with 20 nM siRNA for knockdown of the hBCAT proteins, and incubated for 72 to 96 h. Cells were washed three times with basal medium (2 mM L-glutamine, 10 mM glucose, 1 mM sodium pyruvate, pH 7.4) and incubated in basal medium for 1 hour at 37°C. During this time, appropriate assay compounds for mitochondria stress test (oligomycin, FCCP, rotenone/antimycin A), glycolytic flux/glycolytic rate (rotenone/antimycin A, 2-deoxy-D-glucose, 2-DG) were added to previously calibrated cartridges into respective injection ports. Following incubation, mitochondrial stress and/or glycolytic flux were measured using a Seahorse XFe24 Analyser (Agilent, Cheshire, UK). Crystal violet was performed for cell number normalisation.

2.2.13. Assessment of AD-related protein expression using immunocytochemistry

Solutions were prepared in 0.1 M sodium cacodylate in 0.1 M sucrose (pH 7.4) and washes were performed in sodium cacodylate buffer unless otherwise stated. Glass coverslips were added to a 12-well plate and coated with poly-L-lysine for 20 minutes, before three brief washes with distilled water. SH-SY5Y were seeded at 5 x 106 cells/well, transfected as described (Section 2.3) and incubated for 48 h (overexpression) to 72 h (knockdown). Cells were subsequently washed twice and fixed with 0.25% glutaraldehyde for 20 minutes. Following from two washes, cells were incubated with 100 mM glycine for 5 minutes, subsequent 5 minutes in 10% FBS and washed twice. Cells were incubated with 0.2% Triton X-100 for 20 minutes, washed twice and incubated 4 times with 1 mg/mL sodium borohydride. After two

more washes cells were blocked overnight with 3% BSA at 4°C, prior to incubation with primary antibodies for 1 hour (Table 2), two washes with sodium cacodylate and an additional incubation with fluorescent secondary antibody AlexaFluor® 488 (mouse) and AlexaFluor® 568 (rabbit) (Table 4) for 1 hour. Following from two washes, coverslips were mounted in glass slides using Vectashield hard set mounting medium with DAPI and imaged using a Nikon Eclipse TE300 at 4, 10 and 20 x magnification.

2.2.14. Metabolomics using Liquid and Gas Chromatography Mass Spectrometry (LC-MS/GC-MS)

2.2.14.1.LC-MS/MS (University of Cambridge)

Cells were cultured in 6-well plates at 1.5 x 10^6 cells/well, immediately transfected with knockdown siRNA, or allowed to attach overnight before transfection with overexpression vectors for BCATc. Cells were incubated for 48 to 96 h, and one plate used for cell count with a Luna^{TM} FI (Logos Biosystems, South Korea) and extraction with RIPA buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM NaF, 1 mM PMSF, 1 x phosphatase inhibitor, 1 x protease inhibitor, pH 7.6). Extracted cells were submitted to Western Blot analysis to confirm overexpression and knockdown of the BCAT proteins (see section 2.16). Cell culture media in remaining plates was removed and centrifuged at 17,000 x g for 5 minutes at 4°C to remove debris, and 50 µL transferred to fresh tubes containing extraction solution (50% methanol, 30% acetonitrile, 20% ultrapure water, 5 µM d8-valine). Suspension was vortexed for 5 seconds and incubated for 15 minutes at 1,000 rpm. Lysates in extraction buffer were snap-frozen in dry ice and sent to our colleagues in the Frezza's Laboratory, MRC Cancer Unit at the University of Cambridge for full metabolic profile analysis using quadrupole LC-ESI MS/MS. Data was analysed by dividing individual metabolite ion intensity (arbitrary unites - AU) by the total ion count

(TIC) for each condition and calculating the relative ion intensity. T-test was performed for individual metabolites.

2.2.14.2. ^{13}C-glucose tracing via GC-MS (University of Bristol; McGill University)

Cells were washed in PBS and pulsed with 10 mM ^{13}C-glucose in glucose- and pyruvate-free media for 8 hours. Following incubation, plates were kept on ice and washed with ice-cold saline (9 g/L NaCl) prior to scraping with dry ice-cold 80% methanol. Cells were centrifuged for 10 minutes at 20,000 x g and 4°C, supernatant collected and stored at -80°C. Samples were subsequently sent to our collaborator Dr Emma Vincent at Bristol University and McGill University in Canada for GC-MS analysis.

2.2.15. BCAT localisation and autophagy impact - subcellular fractionation

Cells were washed in ice-cold PBS twice and extracted by gently scraping in ice-cold PBS. Lysates were centrifuged at 900 x g for 5 minutes at 4°C, supernatant removed, and pellet resuspended in Tris buffer (50 mM Tris, 150 mM NaCl, 1 x protease inhibitor, pH 7.5). Suspension was passed through a G25 needle 10 times and incubated on ice for 30 minutes. Nuclear fraction was removed by centrifugation at 900 x g for 5 minutes at 4°C and the pellet resuspended in RIPA buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM NaF, 1 mM PMSF, 1 x phosphatase inhibitor, 1 x protease inhibitor, pH 7.6) which was frozen at -20°C. The remaining supernatant was centrifuged at 20,000 x g for 30 minutes at 4°C and the supernatant containing the cytoplasmic fraction was frozen at -20°C. The pellet was resuspended in MES-buffered saline (25 mM MES, 150 mM NaCl, 1% triton X-100, 1 x protease inhibitor, pH 6.5) and incubated on ice for 60 minutes. The non-raft membrane fraction was isolated by centrifuging at 20,000 x g for 30 minutes and the supernatant frozen at -20°C.

2.2.16. Quantification of protein in cell lysates using the Bradford Assay

Protocol was adapted from Bradford (1976). Bradford dye (5x: 2% Coomassie Brilliant Blue, 23.5% methanol, 50% phosphoric acid) was diluted to a concentration of 1x working dye prior to the start of the experiment. Standards were prepared using 1 mg/mL BSA, with concentrations (in mg/mL): 0, 0.2, 0.4, 0.6, 0.8 and 2.0, and 5 µL were added in triplicate to a 96-well plate alongside 5 µL triplicates of unknown sample. Following from that, 200 µL working dye were added to the wells and plate was incubated for on a rocker at medium speed for 5 minutes at RT. Absorbance change was monitored at 595 nm using a FLUOStar Omega (BMG Labtech) and protein concentration of unknown sample was estimated from the BSA standard curve generated, in mg/mL.

2.2.17. Western Blot analysis

Cells were cultured, transfected, and treated as described above (Sections 2.2.3, 2.2.4 and 2.2.5). Following from treatments and extraction of proteins with RIPA buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM NaF, 1 mM PMSF, 1 x phosphatase inhibitor, 1 x protease inhibitor, 0.01% Triton X-100, pH 7.6), cells were sonicated and centrifuged at 20,000 x g for 10 minutes at 4°C and quantified following section 1.2.17. Protein samples (5 - 20 µg) were prepared in loading buffer (Bromophenol blue, 8% SDS, 40% glycerol, 0.7 M β-mercaptoethanol, 200 mM Tris, pH 6.8), heated to 95°C for 10 minutes and separated on a 4% stacking (1% APS, 1% SDS, 0.06% TEMED, 4% acrylamide and 1.5 M Tris, pH 6.8) and 15% resolving (1% APS, 1% SDS, 0.06% TEMED, 15% acrylamide and 1.5 M Tris, pH 8.8) SDS-PAGE gel at 100 V for 20 minutes and 160 V for 60 minutes in running buffer (25 mM Tris, 190 mM Glycine, 1% SDS, pH 8.3). Proteins were subsequently

transferred to a 0.45 μM PVDF membrane for 2 hours at 60 V or overnight at 30 V in transfer buffer (20% Methanol, 25 mM Tris, 190 mM Glycine, pH 8.3).

Following from transfer, the membrane was washed three times with 1x Tris buffered saline with Tween (TBST: 0.1% Tween, 200 mM NaCl, 2 mM Tris, pH 7.5) for 5 minutes each and blocked with blocking solution (5% non-fat milk or 5% BSA) for 2 hours, prior to overnight incubation at 4°C with primary antibodies (Table 2) in blocking solution. After three 5-minute washes with 1 x TBST, secondary antibody (Table 3) in blocking solution was added for 1 hour and incubated in the dark. Following three 5-minute washes with 1 x TBST, chemiluminescent HRP substrate was added to HRP-incubated membranes and developed using an Odyssey FC (Li-cor Biosciences, Lincoln, NE, USA). Densitometry analysis was performed in Image Studio Lite (Li-Cor Biosciences, Lincoln, NE, USA), using α-tubulin as loading control for expression normalisation. For statistical analysis, treatments were collectively compared with control samples using One-Way ANOVA, and values with $p < 0.05$ were considered significant and added to the final results.

Chapter 3

Development and validation of BCAT constructs

3.1. Introduction

Expression of the BCAT protein has been shown significantly upregulated in the brain of AD individuals in relation to age-matched controls (Hull *et al.,* 2015), while a single nucleotide polymorphism in the BCAT1 gene has been associated with the development of T2DM (Alfaqih *et al.* 2022). The development of molecular tools to create models displaying similar characteristics (i.e., BCAT overexpression in neuronal cells *in vitro*) and understand the impact of such dysregulated mechanisms is therefore a valuable asset.

Recombinant DNA technology is one such tool, a turning point in studying modifications in the expression of target genes in health and disease, giving rise to technique such as site directed mutagenesis for protein engineering (Khan *et al.,* 2016). Site-directed mutagenesis works to mutate, insert and/or delete base pairs and/or codons in a DNA sequence using oligonucleotide primers with the specific altered sequence (Edelheit *et al.,* 2009). Mutated DNA cloned into mammalian vectors can be inserted into cellular models for protein overexpression via transient cationic lipid-mediated transfection, for instance, which utilises liposomes as a vehicle for intracellular nucleic acid delivery (Sork *et al.,* 2016). Mammalian expression vectors are widely utilised in the field as these offer conserved mechanisms between eukaryotic systems particularly in the context of DNA transcription and translation, protein stability and post-translational modifications (Kaufman, 2000). Gene knockdown and/or silencing, in contrast, relies on post-transcription messenger RNA (mRNA) degradation, most commonly using RNA interference (RNAi) techniques. Small interfering RNA (siRNA) is delivered intracellularly via lipofection and incorporated into an RNA-induced silencing complex (nuclease complex), leading to targeted mRNA degradation and subsequent gene knockdown and/or silencing (Haiyong, 2018).

The aforementioned techniques have been widely explored in the study of health and disease, offering further understanding of the impact of changes in gene and protein expression in homeostasis disturbance. For the purpose of assessing the role of BCAT in the pathogenesis of AD and T2DM, this chapter focused on the optimisation of overexpression (OE) and knockdown (KD) constructs of the protein, with particular focus on the cytosolic isoform (BCATc). In addition, the thiol mutants at position C335, C338, and C335/338 were developed using site-directed mutagenesis and sequencing analysis. The results presented hereafter outline the processes in further detail.

3.2. Aims

3.2.1. Specific aims

Specific aim 1: To validate the vectors for overexpression and knockdown of the BCAT protein in neuronal models.

Specific aim 2: To assess whether the expression of BCATc and BCATm changes upon differentiation of these models.

Specific aim 3: To develop the BCATc thiol mutants C335S, C338S and C335/8S.

3.3. Results

3.3.1. Lipofectamine™ 2000 was chosen for transfection efficiency

To confirm non-interference of the transfection reagent on BCAT overexpression or knockdown, cells were transfected with several lipofection reagents (n = 3) and the expressions of multiple proteins such as BCATc, BCATm, succinate dehydrogenase, peroxiredoxin, cytochrome C, GAPDH, β-actin and α-tubulin were taken into consideration during statistical analysis (One-Way ANOVA). FuGENE® 6 (Plasmid DNA), Lipofectamine™ RNAiMAX (siRNA), or Lipofectamine™ 2000 were compared with media-only controls and showed no significant differences in protein expression, thus validating the approaches. However, Lipofectamine™ 2000 was chosen for both knockdown (KD) and overexpression (OE) to minimise transfection variation (Figure 9).

3.3.2. BCAT constructs significantly overexpressed or knocked down the protein in neuronal cell models

To assess whether the BCATc and BCATm DNA overexpression plasmids and siRNA constructs were performing successfully, neuronal models were transfected with 1.0 µg of plasmid DNA and incubated for 48 h, or 20 nM siRNA constructs with 96 h incubation. Western blot analysis showed that both the cytosolic and mitochondrial isoforms of the BCAT protein were significantly overexpressed ($p < 0.0047$ and $p < 0.0001$, respectively). In contrast, although BCATc and BCATm were equally knocked down ($p < 0.0001$), the expression of BCATc was decreased to approximately 55% while BCATm was decreased to less than 40% (Figure 10).

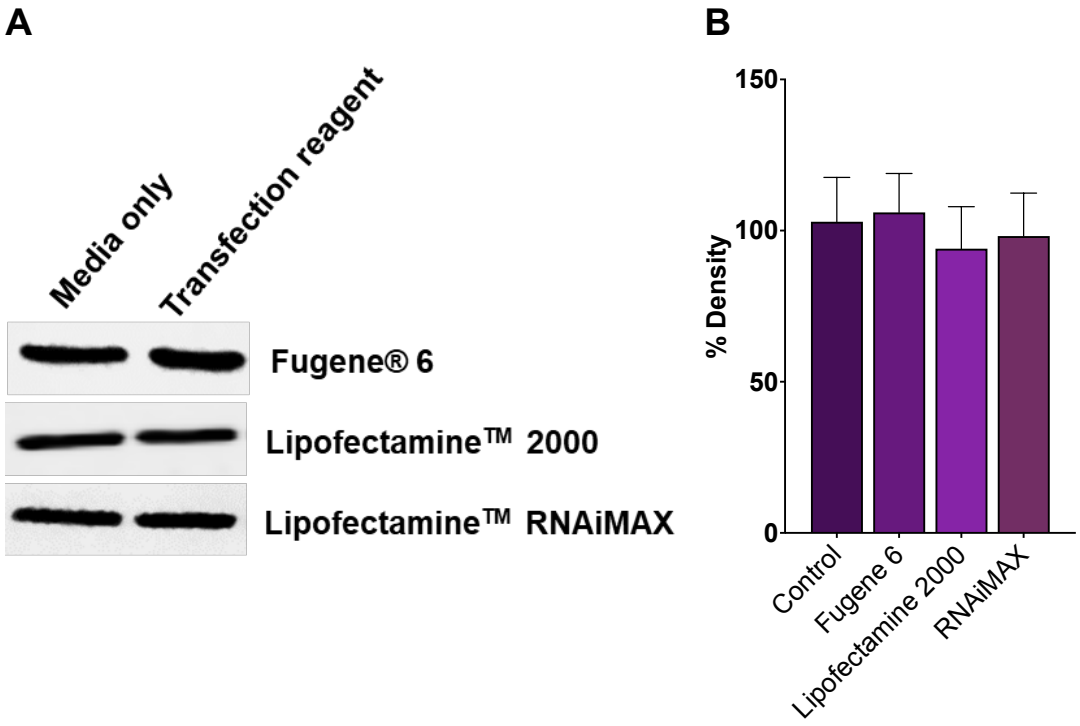

Figure 9. Transfection reagents showed no effects on the expression of several proteins. Cells were incubated with Fugene 6, Lipofectamine 2000 and RNA iMax for 48 hours and the expression of multiple cytosolic and mitochondrial proteins was assessed in relation to control. (A) Representative Western Blot analysis showing no difference in protein expression between cells treated with media only or transfection reagent. (B) Respective densitometry analysis showing percentage density change ± SEM (n = 6).

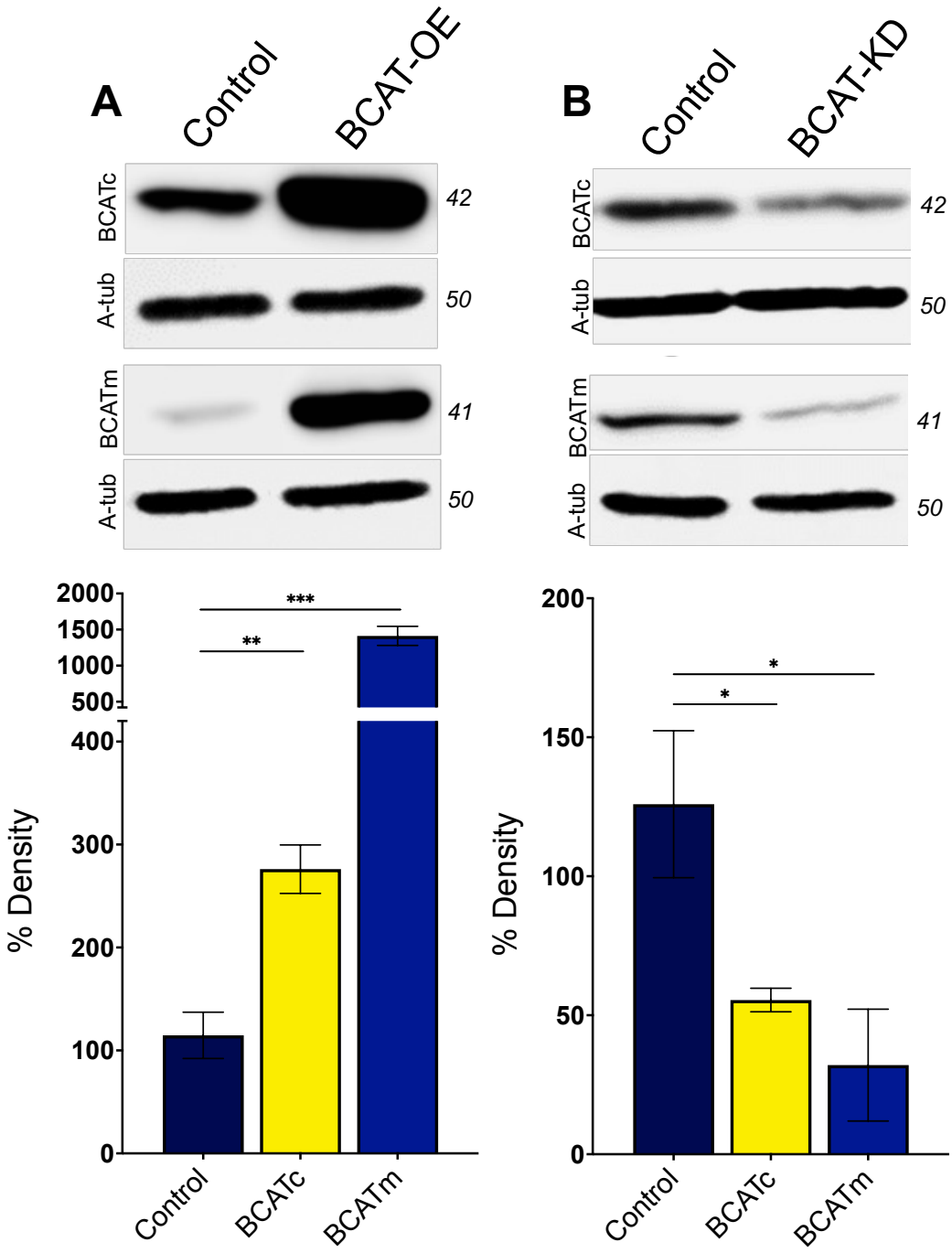

Figure 10. Validation of knockdown and overexpression of the BCAT proteins in SHSY-5Y. Cells were incubated with (A) 1.0 µg hBCATc or hBCATm DNA plasmid for 48 h or (B) 20 nM BCAT1 or BCAT2 siRNA for 96 h, prior to extraction and Western Blot analysis. Densitometry analysis represented as percentage density change ± SEM (One-way ANOVA, n = 3) *p < 0.05, **p < 0.01, ***p < 0.001.

3.3.3. BCATm expression is significantly decreased in differentiated cell models

To evaluate whether differentiated, dopaminergic-like SH-SY5Y cells had significantly reduced expression of the mitochondrial isoform of the BCAT protein (BCATm), cells were transfected with overexpression plasmids for BCATc and BCATm and differentiated for 3, 5 and 7 days in 2% FBS and 10 μM retinoic acid. Whereas 5 and 7 days showed no differences in overexpression, likely as a result of incubation period, cells differentiated for 3 days showed consistent BCATc overexpression (Figure 11A), whereas BCATm expression at baseline was significantly decreased to below detection levels (Figure 11B). This shows that although the undifferentiated, neuroblastoma-like model expresses both isoforms of the BCAT protein, differentiation leads to a similar pattern of protein expression to what is observed in the human brain, with only BCATc expressing in neurons. It provides a robust method of assessing the impact of BCATc in neurons without a compensatory mechanism by BCATm.

3.3.4. Thiols 335 and 338 were successfully mutated following site-directed mutagenesis

To allow the study of the redox element of the BCATc protein in neuronal cells, N-terminal (335) and C-terminal (338) cysteine residues were individually or double substituted with serine, an amino acid of similar chemical structure to cysteine, using site-directed mutagenesis. The method required previously optimised BCATc overexpression constructs as template and relied on oligonucleotides and their complementary sequences containing the mutations, namely C335S (5′-GGC TCT GGT ACA GCC TCT GTT GTT TGC CCA-3), C338S (5′-GGT ACA GCC TGT GTT GTT AGC CCA GTT TCT GAT ATA CTG-3’) and

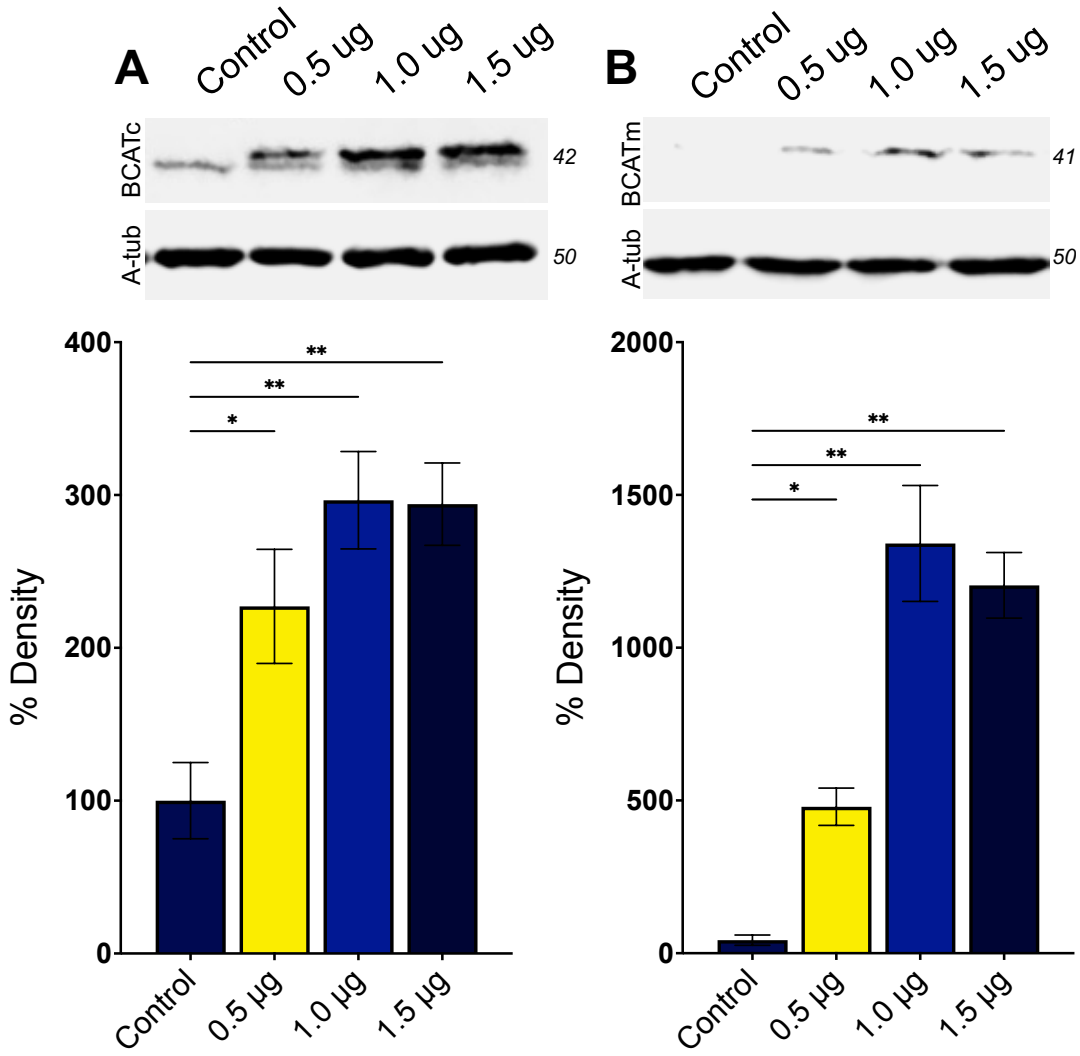

Figure 11. Expression of BCATm decreases significantly when SH-SY5Y cells are differentiated. Neuronal models were differentiated with DMEM/F12, 2% FBS and 10 µM retinoic acid. The optimal overexpression, considering the transient effects of lipofection, was chosen at 3 days. (A) Overexpression of BCATc showing a significant increase in relation to non-transfected cells. The double banding observed likely results from residual His-tag following protein purification. (B) Endogenous BCATm expression was significantly decreased, showing only a mild increase when overexpression was induced. Densitometry analysis represented as percentage density change ± SEM (n = 3) *p < 0.05, **p < 0.01.

C335/8S (5′-GGT ACA GCC TCT GTT GTT AGC CCA GTT TCT-3′).

Following successful transformation and purification of mutated DNA, these were submitted for Sanger sequencing at Source Bioscience (Cambridge, UK) and the results showed successful mutation at position 335 (G – C) and 338 (T - A) in both the single and double mutants (Figure 12A). To further confirm overexpression of the thiol mutants, cells were transfected following transient lipofection protocols (Lipofectamine 2000^{TM}) and submitted to Western Blot analysis, showing the constructs were overexpressing in a similar fashion to BCATc wild type (Figure 12B).

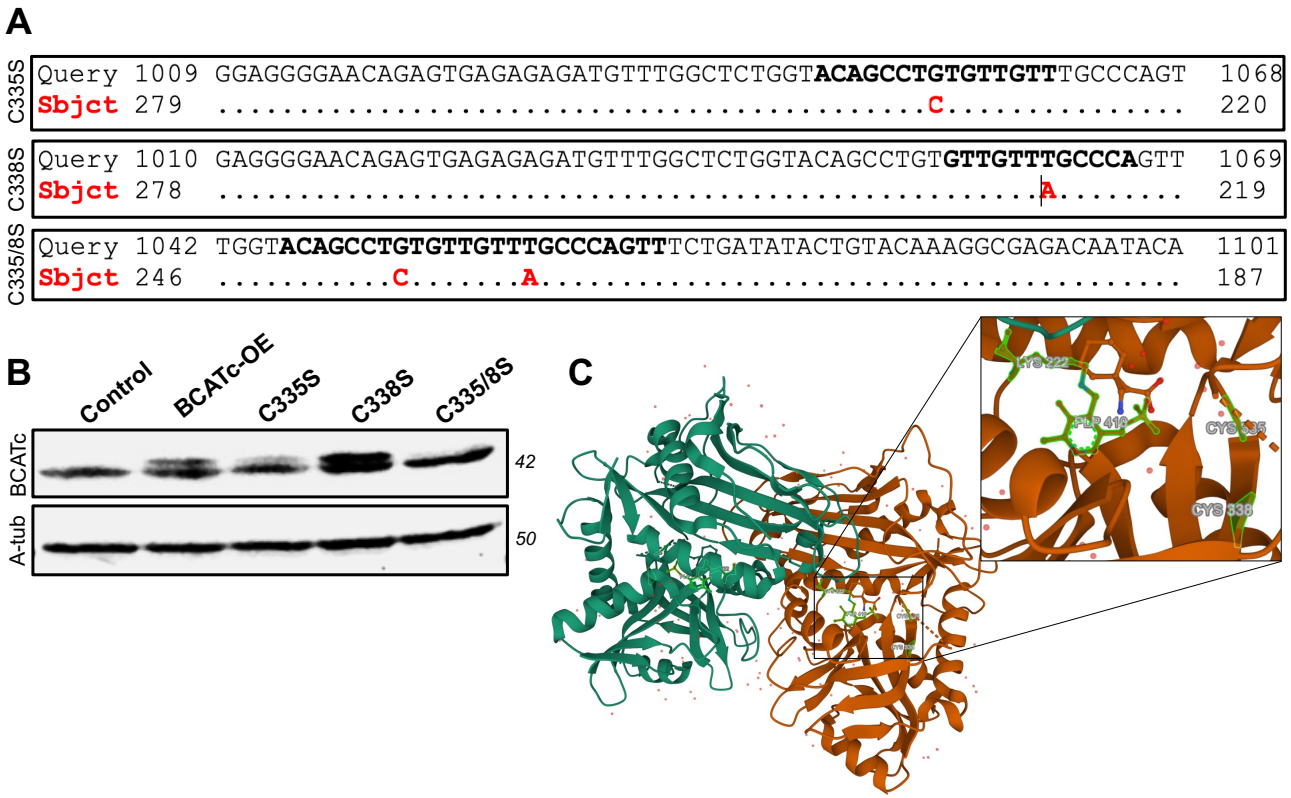

Figure 12. Sequencing and expression of the BCATc thiol mutants. BCATc overexpression plasmids were used as templates for the site directed mutagenesis of thiols C335, C338 and C335/338. (A) Purified plasmids were sent for Sanger sequencing showing that cysteines at positions 335, 338 and 335/338 were successfully substituted by serine residues. (B) Western blot analysis showing cells transfected with the thiol mutants were overexpressing the desired mutation. (C) BCATc crystallography structure showing the position of the thiol mutants in relation to the lysine 222/PLP active binding site (Adapted from Goto *et al*., 2005).

3.4. Discussion

Despite strong debate over reliability and efficiency of recombinant DNA technology, with many *in vitro* deliveries not applicable *in vivo*, it remains a powerful tool for analysing and characterising changes in complex biological organisms. Site-directed mutagenesis in particular is crucial to understand the potential effects of altering gene and protein expression in metabolic dysregulation. Here, the validation and optimisation of previously developed overexpression constructs in mammalian vector pDEST26™ (Harris, 2016) and siRNA sequences for the overexpression and knockdown of the BCAT protein, respectively, were performed. This created a tool for assessing changes in BCAT expression following differentiation of SH-SY5Y models, in addition to providing the backbone to the development of the BCATc thiol mutants C335S, C338S and C335/8S.

3.4.1. Differentiated SH-SY5Y are robust models for overexpression and knockdown of the BCATc protein

The findings demonstrate that BCATc can be successfully overexpressed and knocked down in the SH-SY5Y models, and that its expression remains following differentiation of the models into dopaminergic-like neurons. In contrast, although BCATm overexpression and knockdown were likewise achieved in undifferentiated cells, its endogenous expression following cellular differentiation decreased to below detection levels. Unlike the distribution of the protein's isoforms in mice (BCATc in neurons, BCATm in astrocytes/microglia) (Bixel *et al.,*1997; Bixel *et al.,* 2001), the human BCATc protein is localised to neurons whereas BCATm is mainly observed in endothelial cells (Hull *et al.,* 2014). Under normal physiological conditions, proteins responsible for performing similar functions are unlikely to be localised to the same cellular type, although genetic redundancy is a common feature in cancer subtypes (Cereda *et al.,* 2016). This raised the question as to the mechanisms leading to the

expression of both isoforms of the BCAT protein in this particular model, and the reason surrounding BCATm complete downregulation following differentiation. Considering the model's neuroblastoma origin, it is not surprising that both isoforms would be simultaneously expressed, contributing to an increase in BCAA catabolism, energy output and overall metabolism – driving cancer progression. As previously mentioned, redundancy in mechanisms governing cancer is not unheard of; in fact, it may exacerbate resistance pathways and contribute to migration and proliferation (Lavi, 2015). Differentiation of SH-SY5Y models therefore leads to the generation of a more stable, homogenous population of cells with a similar phenotype to dopaminergic neurons (Shipley *et al.,* 2016). Thus, the expression of the mitochondrial isoform of the protein is no longer necessary. This is important as downregulation of the BCATm protein following differentiation decreases the likelihood of a compensatory mechanism, for instance in BCATc knockdown models, and provides a more robust translational model for assessing the role of BCATc in metabolic reprogramming, the central hypothesis in this project.

3.4.2. BCATc thiols can be successfully mutated to provide understanding of the impact of redox changes in disease

With the successful validation of models for up and downregulation of BCATc expression, developing its thiols mutants to assess the impact of changes in its redox status was the logical subsequent step. Oxidative damage plays a major role in T2DM, contributing to dysregulated mechanisms such as overactivation of the hexosamine pathway and triggering of the polyol pathway, formation of AGEs and activation of PKC isoforms such as Ca^{2+}-associated PKCα (Giacco and Brownlee, 2010). It has also been consistently associated with AD development and progression, which alone implies a strong link between the pathogenesis of these two diseases (Butterfield and Halliwell, 2019). Regulation of the BCAT protein is highly

dependent on the redox status of the cell, where a reducing environment is required for its optimal activity (Davoodi *et al.,* 1998). Oxidising conditions leading to loss of either thiol in its redox-active CXXC motif, C335 or C338, have a significant impact on BCAT activity (Conway *et al.,* 2021), although the protein remains active following these mutations to approximately 60% (Conway *et al.,* 2008). Considering the upregulation of the BCAT protein in the AD brain (Hull *et al.,* 2015), and its previous association with increased risk of obesity and T2DM (Chen *et al.,* 2013; Alfaqih *et al.,* 2022), developing constructs to represent the effects of changes in its redox status was a fundamental step in this project. It is important to note, however, that although these mutants have been previously shown enzymatically active in pure protein assays (Conway *et al.,* 2008), validation of their enzyme activity in cell lines requires further assessment.

Overall, the data obtained from these experiments show that differentiated SH-SY5Y offer a promising model for the study of AD pathogenesis in the context of metabolic dysregulation and redox imbalance. The ability to directly alter the expression of the BCATc protein and its thiol mutants within the models will offer future insights into the disrupted mechanisms involved in AD, and which of those are related with T2DM pathology.

Chapter 4

Metabolic reprogramming mediated by redox-regulated BCATc expression

4.1. Introduction

Insulin resistance and chronic hyperglycaemia, in combination with high levels of fatty acids and BCAAs are key features in the progression of T2DM (Campos, 2012; Laakso, 2019). BCAAs have been associated with the development of insulin resistance likely via activation of mTORC1 (Bloomgarden, 2018), and may decrease glucose oxidation while driving fatty acid oxidation via inhibition of pyruvate dehydrogenase (Li *et al.,* 2017). More recently, these metabolites have been associated with neurotoxicity linked to AD (Schönfeld and Reiser, 2017; Li *et al.,* 2018; Tournissac *et al.,* 2018; Siddik *et al.,* 2019; Zhang, *et al.,* 2021). Perturbed BCAA metabolism may also be linked to the dysregulated expression of the BCAT protein, the first enzyme in BCAA catabolism, which was found to be significantly overexpressed in the brains of subjects with AD (Hull *et al,* 2015).

Conway *et al.* (unpublished observations) showed that changes in the redox status of the BCAT protein led to its redox-mediated binding with several proteins of the glycolytic pathway. In its reduced, active form, BCAT has been shown to bind proteins such as hexokinase, GAPDH, enolase and pyruvate kinase. In contrast, when oxidised and thus inactive, the protein was not only unable to bind previous partners but also associated with proteins of the polyol pathway such as AR and SDH. These results indicate that the BCAT protein acts as a glycolytic regulator and its overexpression and related redox state may play a role in metabolite reprogramming linked to conditions such as diabetes and AD. Ultimately, we hypothesise that hyperglycaemia will dominate and trigger a chain of events potentially leading to the development of advanced glycation end products (particularly from non-metabolising sugars such as sorbitol), accumulation of toxic peptide aggregates and oxidative stress, all of which are similarly observed in T2DM and AD.

The focus of this chapter was to evaluate the impact of overexpression of BCATc on the metabolic profiles of SH-SY5Y cells, with particular focus on

metabolites of amino acid, fatty acid metabolism and energy pathways such as glycolysis and the TCA cycle. In order to establish the protein's role as a metabolic regulator, the activity of key enzymes in the glycolytic pathway and TCA cycle were also assessed, and the functional impact of enzymatic perturbation was demonstrated by estimating glycolytic rate and mitochondrial stress. The effects of changes in the redox status of BCAT on metabolite reprogramming was also considered under similar conditions. Overall, the results support a key role for BCATc in metabolic reprogramming, and changes in its expression as a result of early events such as chronic high levels of BCAAs are likely to contribute to the development of neurodegenerative conditions such as AD.

4.2. Hypothesis and Aims

4.2.1. Hypothesis

Changes in BCATc expression affect metabolic enzymes and dysregulate its associated metabolic pathways, contributing to metabolite reprofiling, an increase in oxidative stress, and decreased neuronal viability, linked to markers of AD pathology.

4.2.2. Specific aims

Specific aim 1: To identify the metabolic pathways affected by overexpression of BCATc and determine the effect on the respective metabolic profiles

Specific aim 2: To investigate the role of BCATc as a glycolytic regulator.

Specific aim 3: To evaluate how changes BCATc redox status alter its role in metabolic reprogramming

4.3. Results

4.3.1. BCATc overexpression regulates metabolic profiles of key energy pathways

4.3.1.1. Essential and non-essential amino acids

Amino acids are organic compounds with vital roles as protein building blocks and in neurotransmitter synthesis and are classed as essential and non-essential based on its *de novo* synthesis (Church *et al.,* 2020). To evaluate the impact of BCATc overexpression on the amino acid pool and thus determine the impact of protein dysregulation in cellular homeostasis, BCATc was overexpressed in neuronal models and metabolite profile was assessed using LC-MS/MS. In response to increased BCATc expression (> 200% expression, Figure 13) there was a significant increase in levels of transamination-dependent amino acids alanine, aspartate, glutamine and histidine (p=0.0002, 0.0499; 0.0176; 0.0028, respectively) (Figure 14). Amino acids such as glutamate and methionine, however, shower no significant difference between groups.

Of the BCAAs, valine was the only which showed a significant increase (p=0.013). Leucine and isoleucine, although not significantly, showed the opposite trend with decreasing levels (Figure 15). Aromatic amino acids tyrosine and its precursor phenylalanine also showed a significant decrease when BCATc was overexpressed (p=0.009; p=0.038, respectively) (Figure 16). One possibility for this is that the amino acid SLC1 transporters may favour BCAAs transport intracellularly, thus leading to a decrease in ArAAs. Tryptophan was the only ArAA which showed no significant changes, although levels of its degradation product kynurenine were significantly decreased (p=0.006) (Figure 16).

4.3.1.2. Fatty acids, their substrates, and products

The roles of fatty acids in cellular health, particularly cell membrane structure, hormonal signalling and as transport carriers have been well established (Calder,

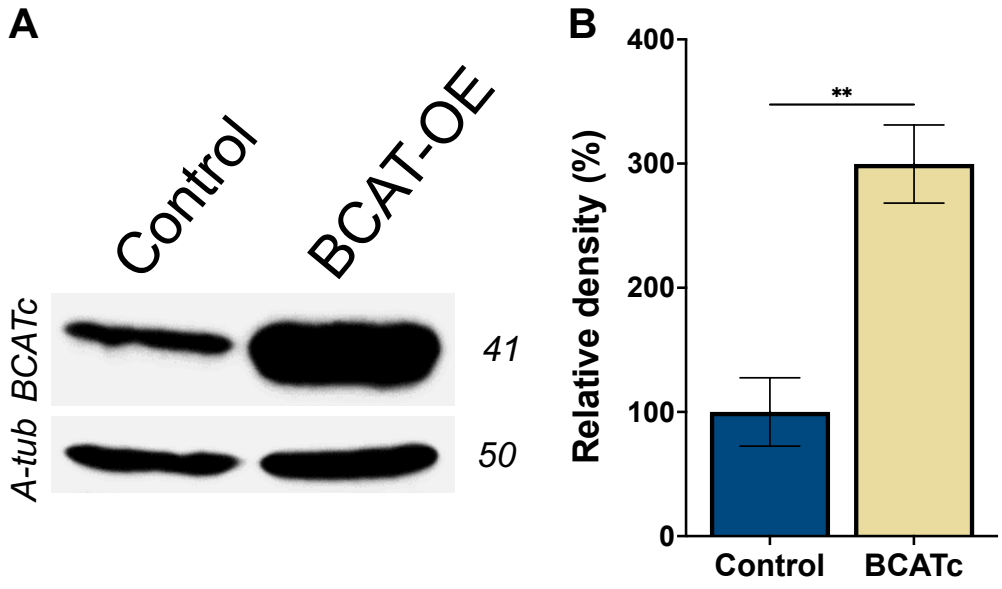

Figure 13. Validation of BCATc overexpression for untargeted metabolomics analyses. Undifferentiated neuronal cells were transfected with 1.0 µg of the BCATc overexpression for 48 hours and protein expression subsequently assessed. (A) Western blot analysis showing successful overexpression of the BCATc protein. (B) Respective densitometry analysis showed as percentage relative density ± SEM (Unpaired t-test, n = 3). **p < 0.05.

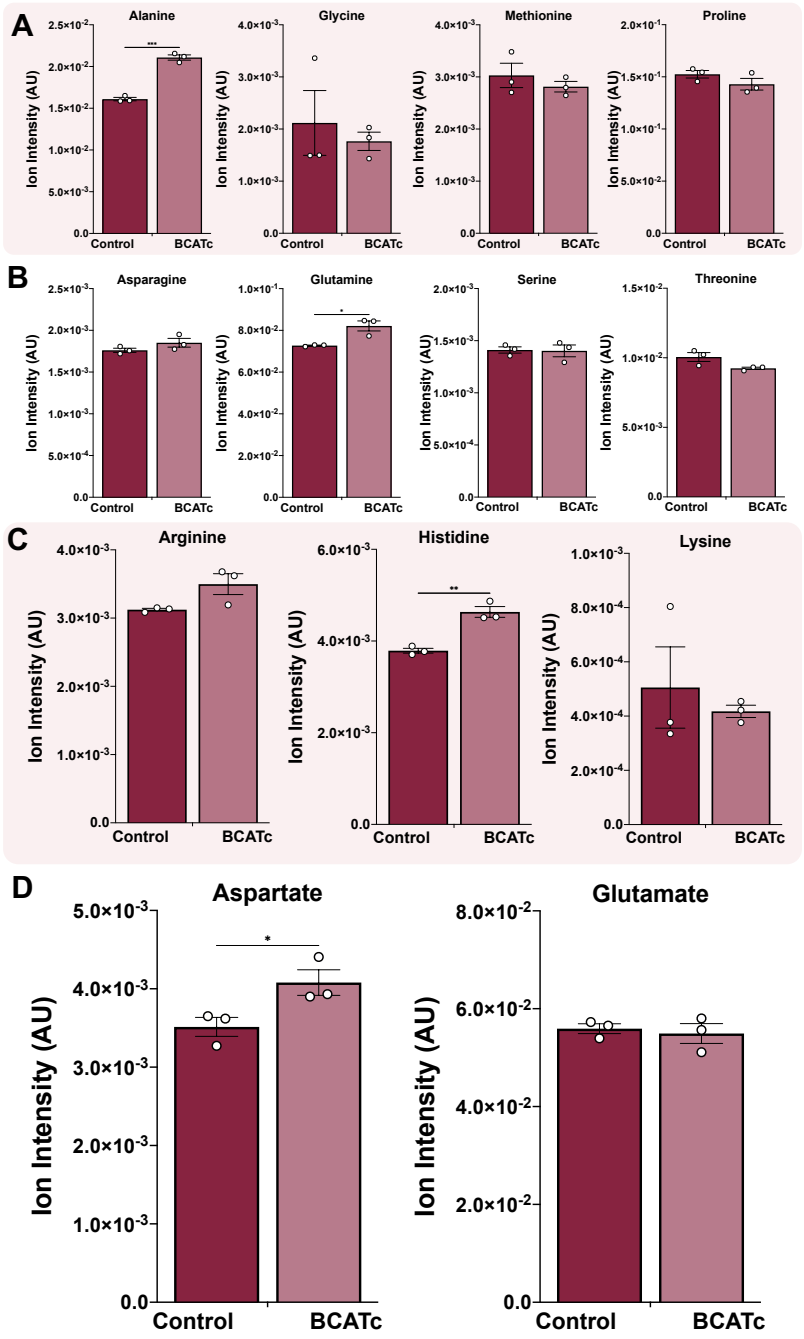

Figure 14. Amino acid levels were mildly affected by BCATc dysregulation. Overexpression of BCATc was induced in undifferentiated SH-SY5Y cells and amino acid levels were assessed using untargeted metabolomics. (A) Non-polar amino acids alanine, glycine, methionine and proline. (B) Polar amino acids asparagine, glutamine, serine and threonine. (C) Amino acids with positively charged side-chains arginine, histidine and lysine. (D) Negatively charged side chain amino acids aspartate and glutamate. Ion intensity shown as arbitrary units (AU) ± SEM (Unpaired t-test, n = 3) ***p < 0.001, **p < 0.01, *p < 0.05.

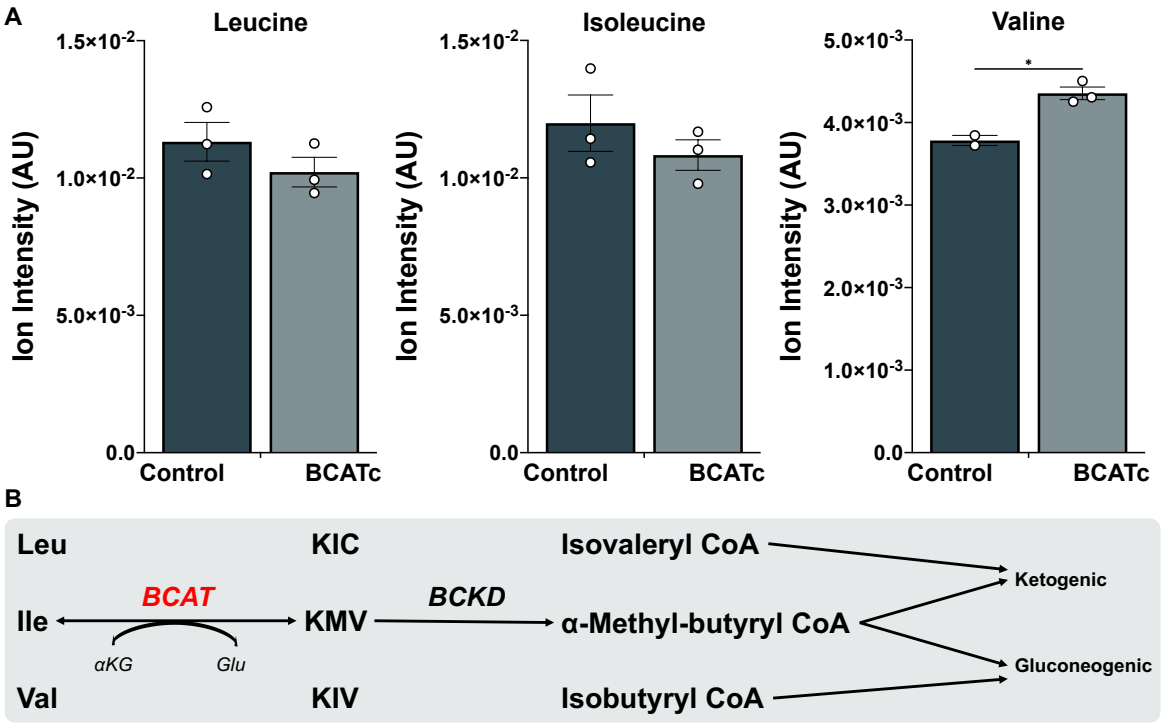

Figure 15. BCATc overexpression decreases leucine and isoleucine while increasing valine levels in neuronal models. SH-SY5Y models were overexpressed with 1.0 µg of the BCATc protein and submitted to untargeted metabolomics. (A) Levels of BCAAs leucine, isoleucine and valine were estimated and contrasted with controls. (B) Diagram illustrating BCAA catabolism via BCATc transamination and resulting downstream metabolites. Ion intensity shown as arbitrary units (AU) ± SEM (Unpaired t-test, n = 3) ***p < 0.001, **p < 0.01, *p < 0.05.

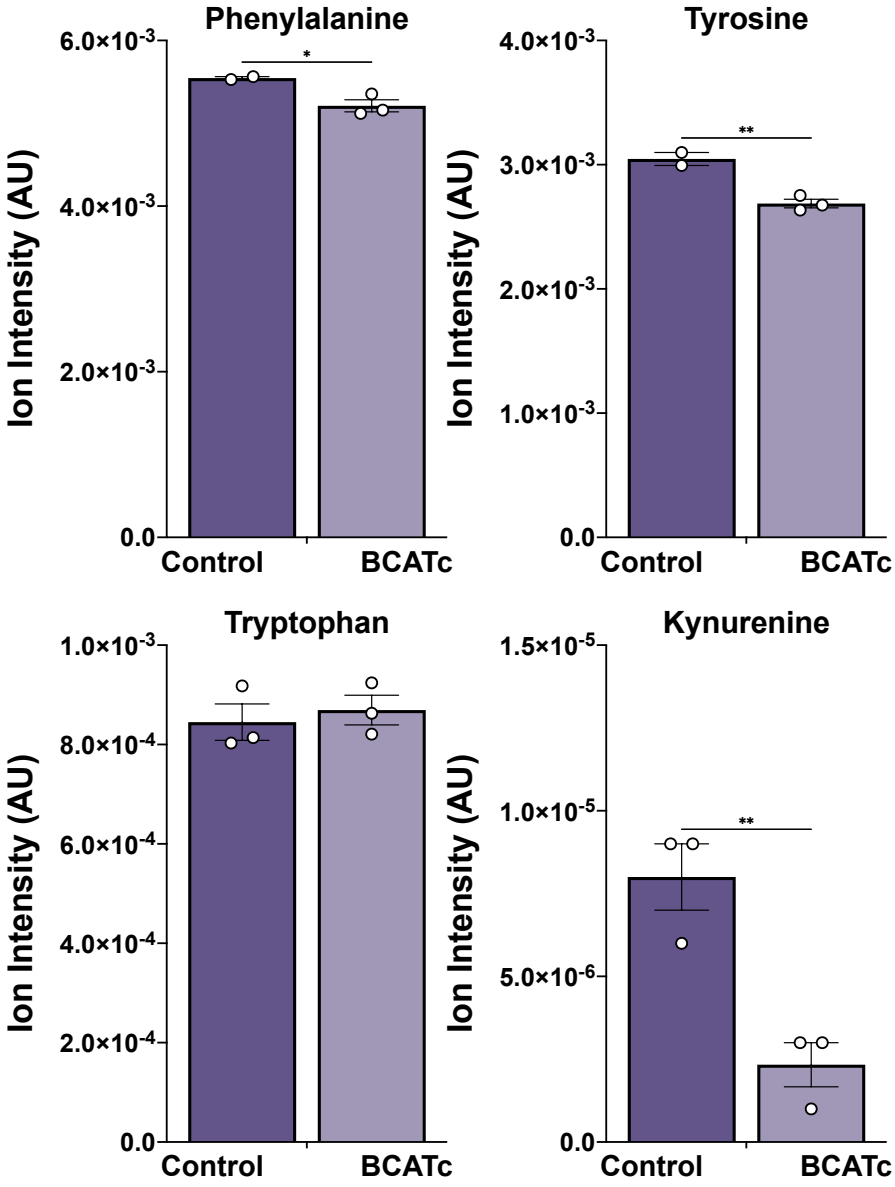

Figure 16. ArAA and its associated metabolite levels are decreased under BCATc dysregulation. BCATc was overexpressed in neuronal cell models and extracted following LC-MS/MS protocol. Levels of phenylalanine, tyrosine, tryptophan, and kynurenine were measured using untargeted metabolomics. Ion intensity shown as arbitrary units (AU) ± SEM (Unpaired t-test, n = 3). **p < 0.01, *p < 0.05.

2015). Recent studies suggest high levels of fatty acids may also be a causative factor in the development of insulin resistance and T2DM (Shetty and Kumari, 2021). In order to evaluate the effects of BCATc overexpression on fatty acid metabolism, levels of metabolites from β-oxidation to fatty acid synthesis were assessed (Figure 17). BCATc overexpression showed that while levels of fatty acids palmitic and stearic acid were increased, only stearic acid was significantly impacted (p=0.047).

B-oxidation intermediate myristoylcarnitine was significantly increased (p=0.006). However, most importantly, dysregulation of BCATc significantly decreased levels of isobutyrylcarnitine (p=0.0402), by-product of valine catabolism. This directly links with the observed high levels of valine and suggests BCAT and thus valine dysregulation prevent the synthesis of downstream metabolites such as C4-acylcarnitine, affecting FA B-oxidation, Acetyl-CoA production and ultimately ATP generation. In addition, overexpression significantly increased levels of fatty acid amides stearamide (p=0.003), palmitamide (p=0.009) and oleamide (p=0.034), vital bioactive compounds particularly in mammalian cell signalling in the brain.

4.3.1.3. Neurotransmitters and its precursors

Dysregulated amino acid metabolism, particularly BCAAs and ArAAs, impair neurotransmitter synthesis and damage its receptors, leading to severe consequences in memory and learning (Dalangin *et al.*, 2020). Accumulation of toxic Aβ and hyperphosphorylated tau tangles have been associated with dysregulation in neurotransmission in AD (Kaur *et al.,* 2019), with a recent study demonstrating that mice models of T2DM also present with decreased dopamine levels relative to controls (Pérez-Taboada *et al.,* 2020). To evaluate the impact of BCATc overexpression in neurotransmission, metabolites associated with this pathway were assessed using untargeted metabolomics.

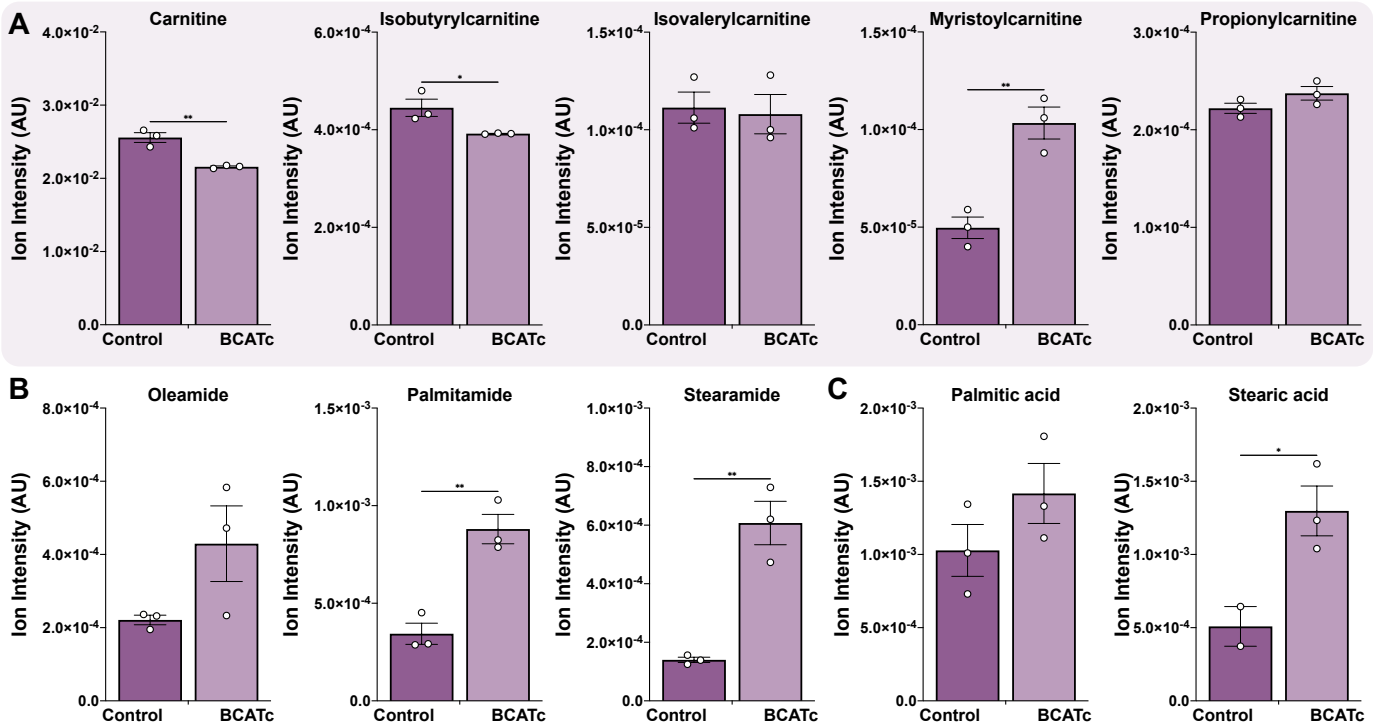

Figure 17. Metabolites in fatty acid metabolism are significantly impaired when BCATc is dysregulated. Following overexpression of the BCATc protein in SH-SY5Y neuronal models, metabolites in the fatty acid pathway were measured using LC-MS/MS untargeted metabolomics. (A) Long-chain fatty acid transporter carnitine and its deriving compounds Isobutyryl-, Isovaleryl-, myristoyl- and propionyl-l-carnitine. (B) Fatty acid amides oleamide, palmitamide and stearamide. (C) Fatty acids palmitic and stearic acid. Ion intensity shown as arbitrary units (AU) ± SEM (Unpaired t-test, n = 3). **p < 0.01, *p < 0.05.

The results acquired showed that although levels of choline-containing phospholipid choline alphoscerate (α-GPC) were significantly increased when cells were overexpressed with BCATc (p=0.0198), its downstream metabolites choline and major neurotransmitter acetylcholine were significantly decreased (p=0.0017 and p=0.0074, respectively) (Figure 18). This is important as while α-GPC synthesis relies on the availability of fatty acids, which have been previously shown increased (see section 4.3.1.2), choline requires acetyl-CoA to produce acetylcholine. Similarly, levels of N-acetylaspartate, precursor to the third most abundant peptide in the brain N-acetyl-aspartyl-glutamate, were significantly decreased (p=0.0063) (Figure 18). This, paired with increased levels of aspartate, may indicate that synthesis of the metabolite is likewise impaired, most likely as a result of a decrease in Acetyl-CoA pool.

4.3.1.4. Glycolytic regulation and the TCA cycle

The human brain utilises approximately 20% of glucose-derived energy solely to maintain its resting state (Mergenthaler *et al.,* 2013). Glucose imbalance is a key aspect of T2DM, and it has been suggested to play an important role in the development of AD via metabolic and mitochondrial dysregulation, and changes to post-translational modifications (Gonzalez *et al.,* 2022). To evaluate whether BCATc plays a role as a glycolytic regulator, levels of glycolysis metabolites such as glucose, fructose and sorbitol were assessed. Analyses showed that overexpression of BCATc significantly increased the level of glucose (p=0.024) and fructose (p=0.499) (Figure 19A). Sorbitol levels, however, were substantially decreased (p<0.001) (Figure 19A). This suggests that although hyperglycaemia may drive activation of the polyol pathway and initially increase sorbitol levels, it may be rapidly oxidised by sorbitol dehydrogenase into fructose, explaining the higher intracellular levels of the metabolite. Alternatively, BCATc overexpression may also regulate enzymes of the

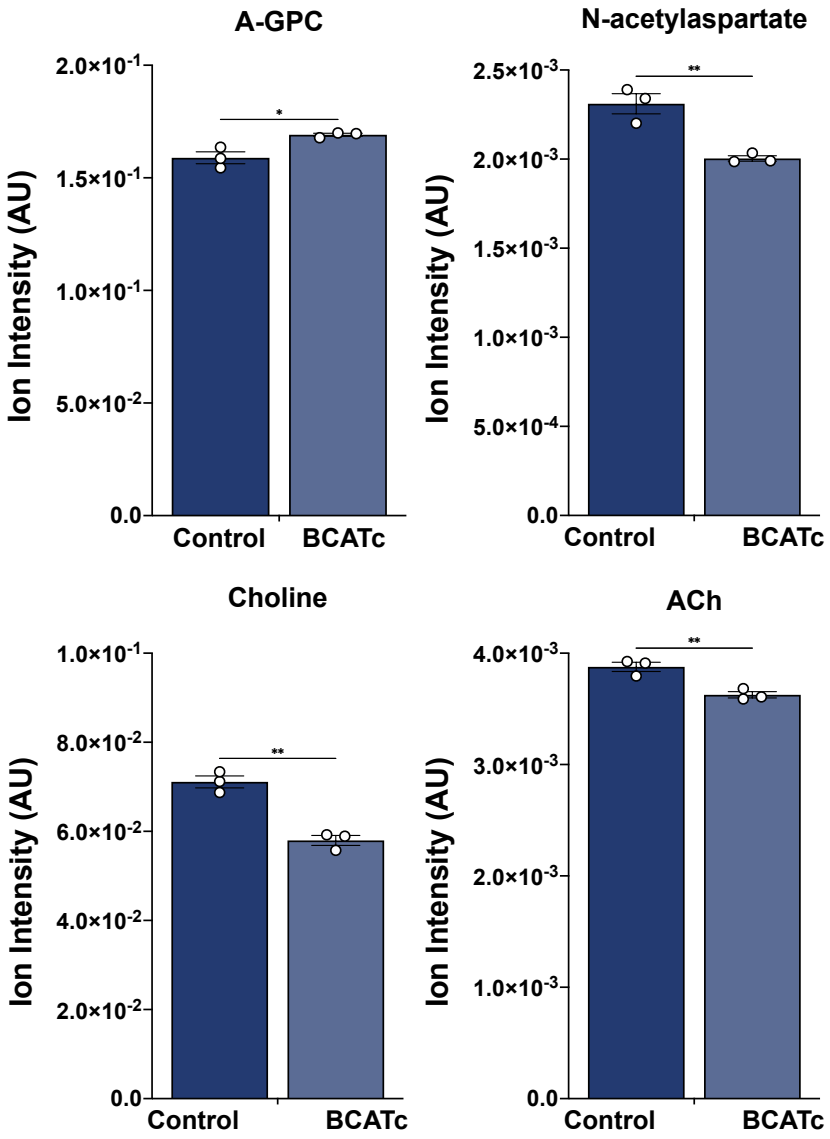

Figure 18. BCATc overexpression decreases vital neurotransmission metabolites. Following overexpression of the BCATc protein in SH-SY5Y neuronal cell models, levels of choline alphoscerate (α-GPC), its derivatives choline and acetylcholine, and N-acetylaspartate (NAA) were measured using untargeted metabolomics. Ion intensity is shown as arbitrary units (AU) ± SEM (Unpaired t-test, n = 3). **p < 0.01, *p < 0.05.

polyol pathway such as aldose reductase, impairing glucose reduction into sorbitol. In contrast, the impact on TCA cycle metabolites was relatively benign apart from an increase in succinate (p=0.027) and a significant decrease in ATP (p=0.036) (Figure 19C), suggesting that although oxidative phosphorylation may be only mildly impacted, ATP production and output is still compromised, likely as a result of glycolytic disruption.

4.3.2. ^{13}C-glucose tracing

As previously discussed, BCATc was shown to have redox-mediated interaction with proteins important in glucose regulation. To ascertain if an intact redox active CXXC mutant is important to the role of BCATc as a glycolytic regulator, we next explored the effect of mutating the redox resolving cysteine on metabolic profiling using ^{13}C-glucose tracing analyses. Following incubation of SH-SY5Y cells with the labelled substrate (in this instance, ^{13}C-glucose), flux distribution was assessed to show changes in metabolite distribution over time. This analysis included amino acids: alanine, asparagine, aspartate, cysteine, glutamine, glycine, methionine, proline, serine, threonine, branched chain and aromatics; glycolytic products glycerol-3-phosphate, pyruvate and lactate; and TCA intermediates citrate, αKG, succinate, fumarate and malate.

While there were no significant changes observed in flux distribution when BCATc was overexpressed in cell models, apart from a small decrease in lactate and pyruvate (ns) (Figure 20), mutations in the thiol 338 had an impact on glycolytic products as well as TCA intermediates, particularly citrate and αKG by decreasing ^{13}C-glucose flux (Figure 21). This suggests BCATc overexpression is likely impacting glycolytic rate by binding to and changing the conformation and/or activity of enzymes such as GAPDH and enolase, affecting glycolytic output. Although mutation of thiol 338 may still lead to the protein interacting with similar binding partners this association cannot be resolved, impacting on further downstream metabolites.

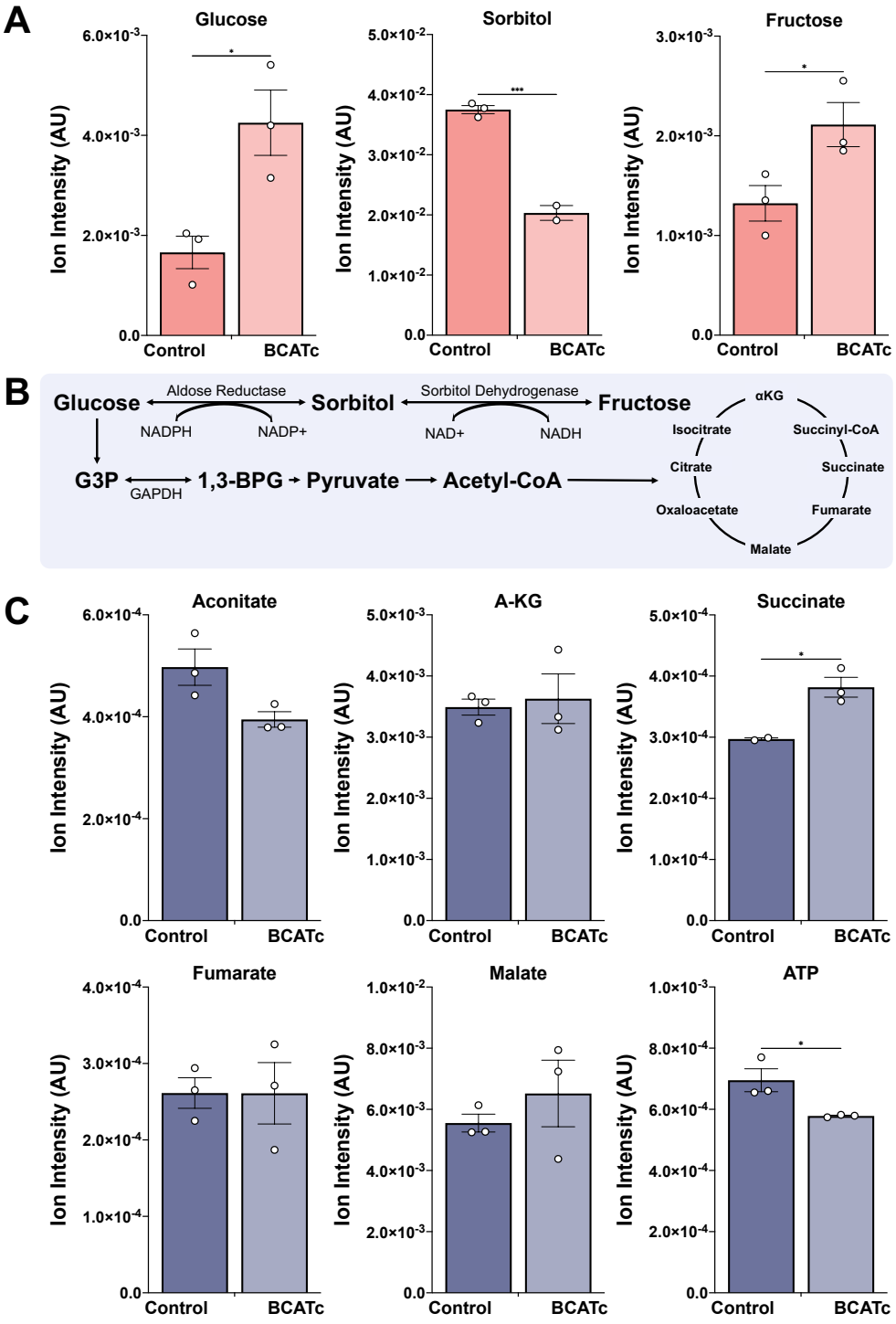

Figure 19. Levels of metabolites in the glycolytic pathway and TCA cycle were significantly affected by BCATc expression. SH-SY5Y cells were transfected with BCATc overexpression vectors and levels of (A) glycolytic and (C) TCA cycle metabolites were assessed untargeted metabolomics using LC-MS/MS. (B) Schematic representation of the association between glycolysis and the TCA cycle. Ion intensity shown as arbitrary units (AU) ± SEM (Unpaired t-test, n = 3) ***p < 0.001, **p < 0.01, *p < 0.05.

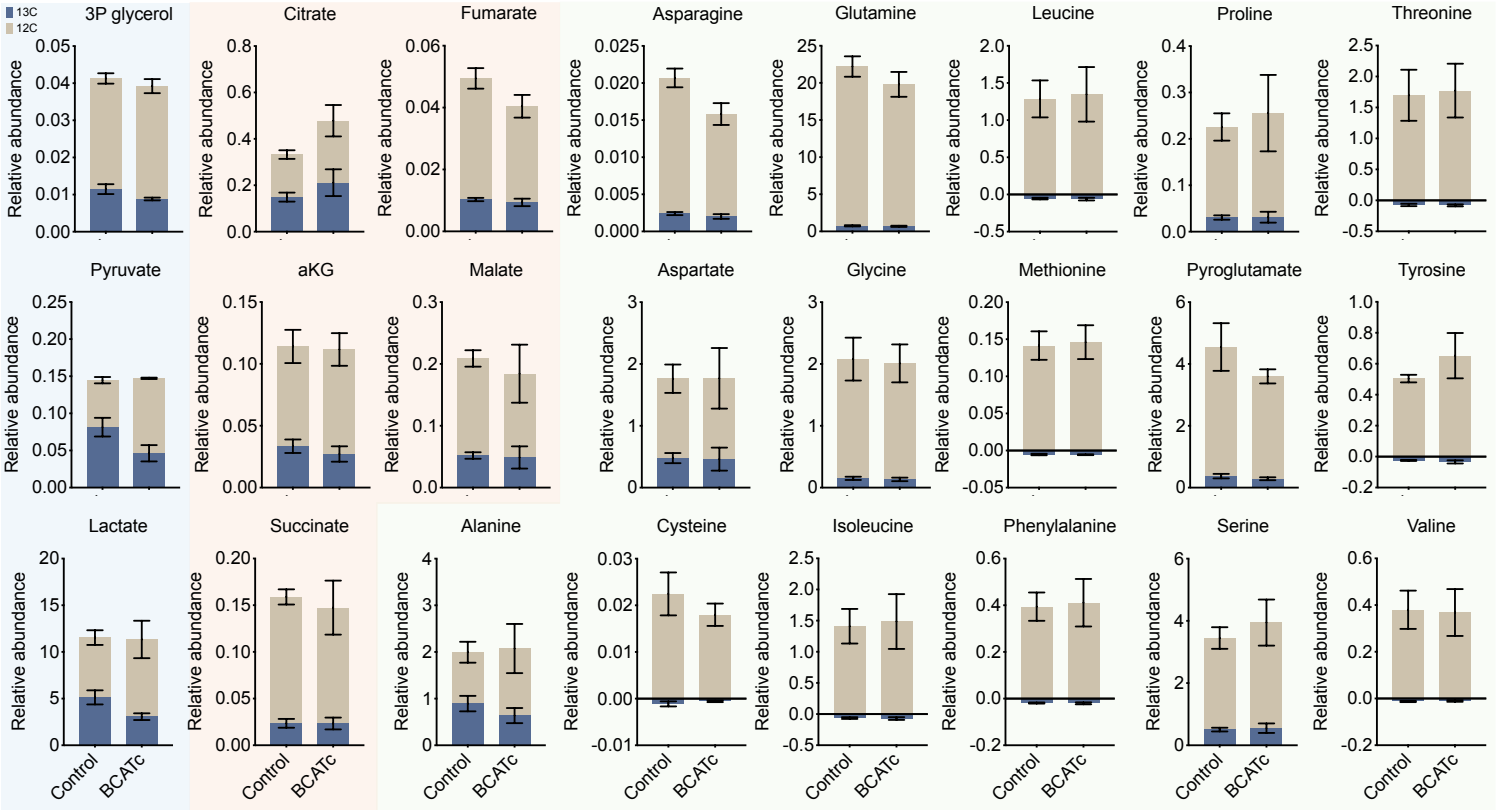

Figure 20. BCATc overexpression affects glycolytic metabolite flux. The BCATc protein was overexpressed in neuronal models and analysed following ^{13}C-glucose flux using scan GC-MS/MS. Flux of ^{13}C-glucose in glycolysis (**blue**), TCA cycle (**beige**) and amino acid metabolism (**green**) were measured in contrast with ^{12}C-glucose. Flux represented as relative abundance ± SEM (Two-way ANOVA, *n* = 3).

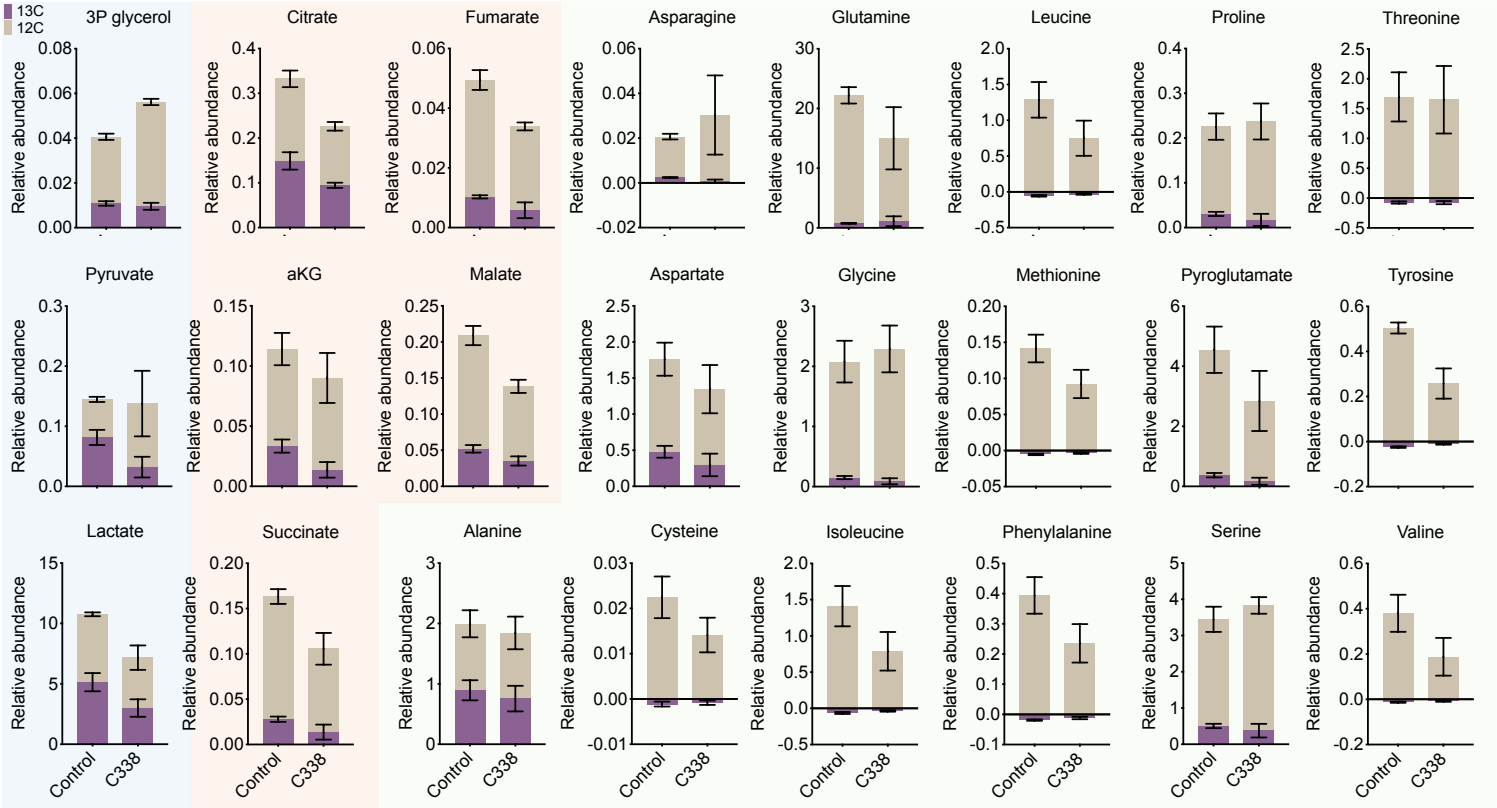

Figure 21. C338S mutant impacts on TCA cycle metabolite flux. BCATc C338S thiol mutant was overexpressed in neuronal cells and analysed using scan mode GC-MS/MS. Flux of ^{13}C-glucose in glycolysis (blue), TCA cycle (orange) and amino acid metabolism (green) was measured in contrast with ^{12}C-glucose. Flux represented as relative abundance ± SEM (Two-way ANOVA, *n* = 3).

4.3.3. Glycolytic and TCA cycle enzymes are differentially regulated by BCATc expression and redox status.

To further evaluate the role of BCATc in regulating glycolysis and the TCA cycle, we next assessed the effect of BCAT on the expression and catalytic activity of key glycolytic enzymes including GAPDH (ab204732), enolase (ab241024), IDH ab102528), MDH (ab183305) and LDH. Overall, overexpression of C338S was significantly more efficient than the overexpression of WT BCATc and C335S, suggesting the thiol at position C335 may play a vital role not only as a redox sensor but also as a glycolytic binding partner (Figure 22). Although there was no significant change in the expression of most proteins in response to overexpression or mutation of the redox active thiol groups apart, TCA cycle proteins MDH and IDH were significantly decreased when cells were overexpressed with C338S ($p=0.0041$ and $p=0.0202$, respectively) (Figure 22).

Overexpression of BCATc resulted in a significant decrease in enzyme activity for GAPDH ($p=0.0449$) and enolase ($p=0.0052$) rather than enzymes particular to the TCA cycle, supporting a role for BCATc in regulating glycolytic flux through its moonlighting role rather than its role as a transaminase (Figure 24). However, LDH was only mildly affected (Figure 23 and 24). Conversely, mutation of the thiol groups of the redox active CXXC motif increased the activity of GAPDH, IDH and MDH, suggesting that the role of BCATc is predominantly to control the flux going through the TCA cycle. While overexpression of C335S significantly increased GAPDH ($p=0.0016$), IDH ($p=0.002$) and MDH ($p=0.0283$), it decreased enolase in a similar manner to wild type overexpression ($p=0.0045$). In contrast, C338S did not affect MDH activity, while GAPDH and IDH were significantly increased ($p=0.0007$ and $p=0.0001$, respectively) and enolase was significantly decreased ($p=0.03$) (Figure 23 and 24).

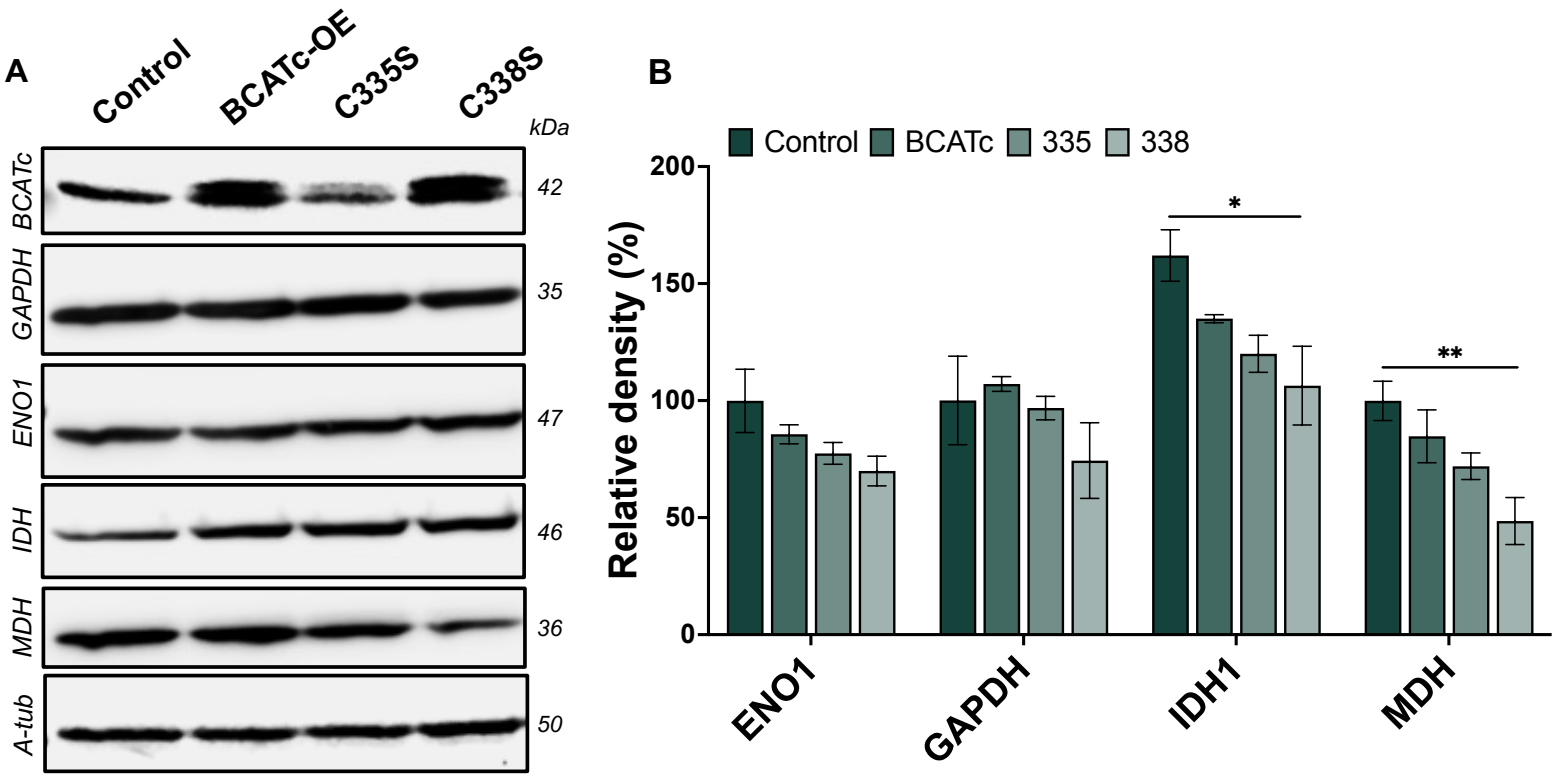

Figure 22. Expression of glycolytic and TCA cycle enzymes were only partially affected by BCATc dysregulation. Following induced changes in BCATc expression in SH-SY5Y cells, expression of GAPDH, enolase, IDH, MDH and LDH was assessed. (A) Western Blot analysis confirming overexpression of the BCATc constructs and the respective protein expression of GAPDH, enolase, IDH and MDH. (B) Respective densitometry analysis shown as percentage relative density ± SEM (Two-way ANOVA, n = 3) *p < 0.05, **p < 0.01.

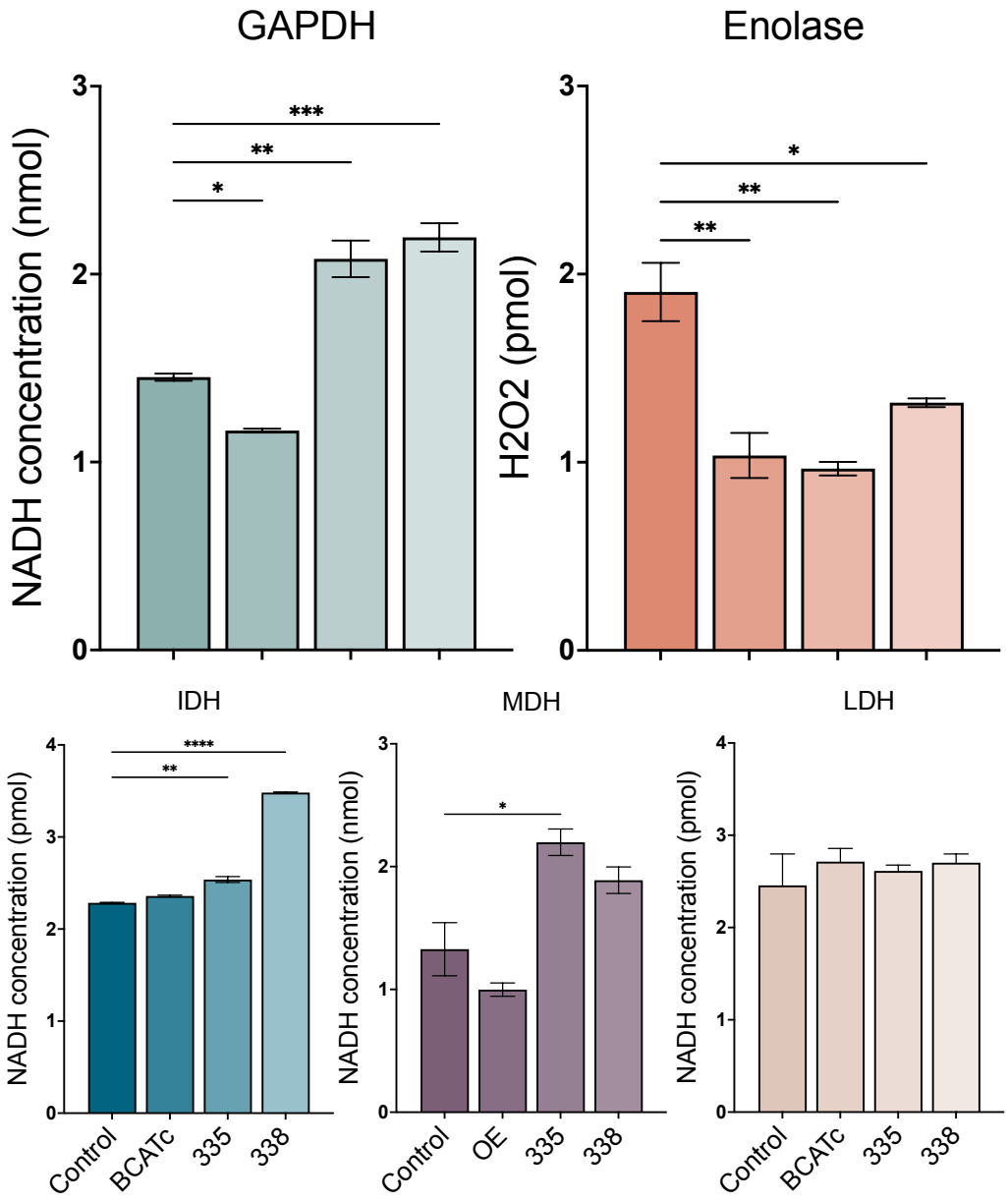

Figure 23. Changes in concentration of enzyme intermediate under BCATc dysregulation. Following overexpression with BCATc and its thiol mutants C335S and C338S, differentiated neuronal models were incubated with substrates for GAPDH, enolase, IDH, MDH and LDH and overall concentration change estimated from absorbance over time. NADH (nmol) or H_2O_2 (pmol) final concentration represented ± SEM (n = 6, One-way ANOVA). *p < 0.05, **p < 0.01, ***p < 0.001., ****p < 0.0001.

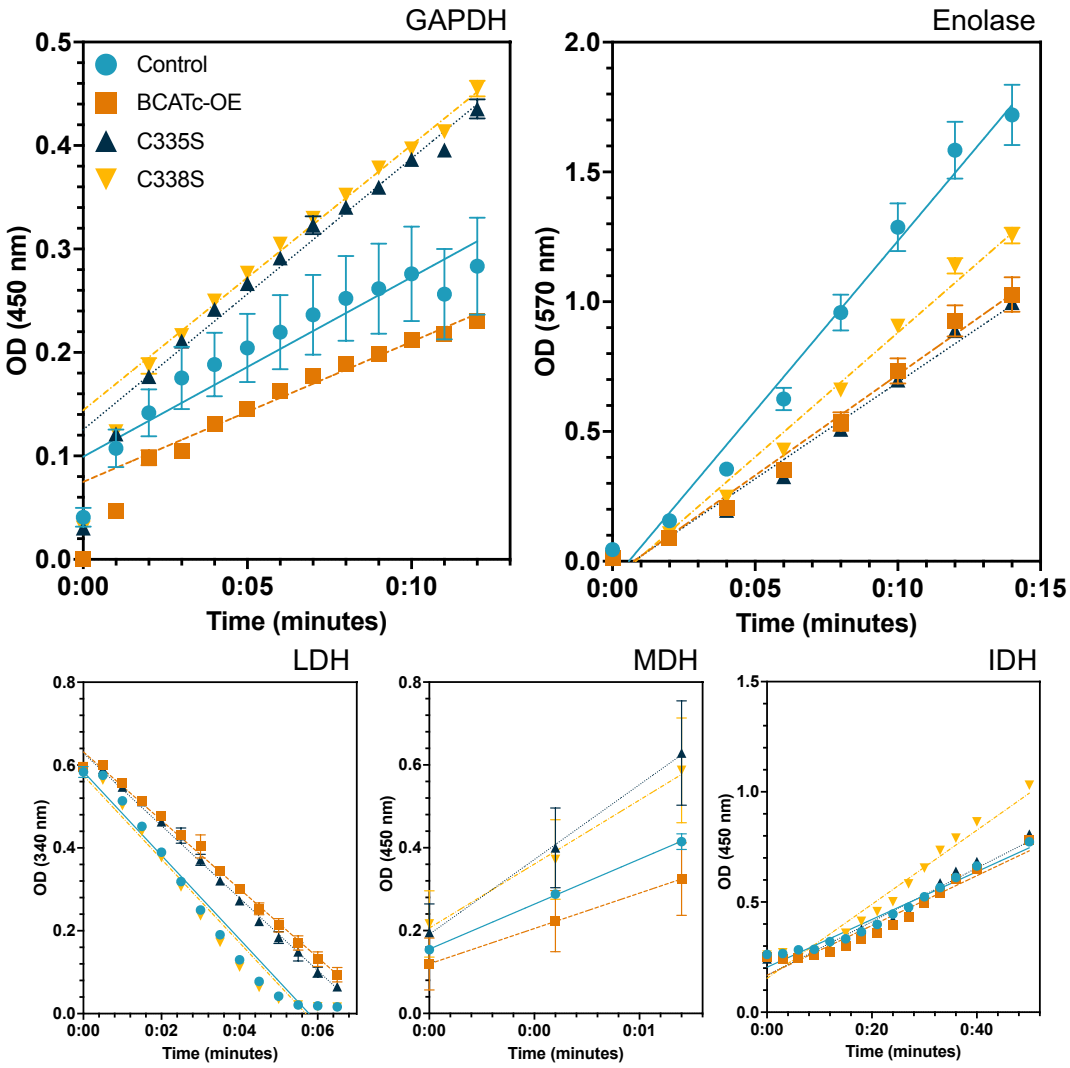

Figure 24. Enzyme activity is significantly impacted by BCATc dysregulation. Following overexpression of BCATc wild type and thiol mutants, the activities of glycolytic enzymes GAPDH, LDH, and enolase, and TCA enzymes MDH and IDH, were assessed over several time points showing the impact of BCATc dysregulation over time. Activity of GAPDH, MDH and IDH was based on the reaction of NADH with a developer measured at λ = 450 nm; LDH activity was measured following NADH oxidation to NAD at decreasing λ = 340 nm; and enolase activity was followed at λ = 570 nm according to stoichiometric reaction of phosphoenolpyruvate intermediate with a chemical probe (n = 4).

4.3.4. Glycolytic rate and oxidative phosphorylation are affected by changes in BCATc expression and redox status

To validate the impact of BCATc overexpression and its thiol mutants on glycolytic capacity, glycolytic rate was assessed using the Seahorse XF analyser. The results showed that there is a significant decrease in overall glycolytic capacity as well as oxidative phosphorylation when differentiated cells were overexpressed with BCATc and its respective thiol mutants, C335S and C338S ($p<0.001$) (Figure 25A). In support of our data that demonstrates that BCATc is a glycolytic regulator, overexpression alone did not affect oxidative phosphorylation, which was evidenced by no changes in the mitochondrial stress test ($p>0.001$) (Figure 25B). In contrast, overexpression of the thiol mutants significantly impaired oxidative phosphorylation ($p<0.0001$), corroborating our metabolite studies (section 4.3.2). Contrary to what had been observed however, glycolytic rate was decreased under all observed conditions ($p<0.0001$).

This shows that although overexpression of BCATc directly contributes to dysregulation in glycolysis, the effects of wild type overexpression have little impact on the TCA cycle and/or oxidative phosphorylation, suggesting the protein's effects are limited to cytosolic pathways. However, upon oxidation and/or loss of either of its thiol groups, the effects are most prominently observed in mitochondrial pathways such as TCA cycle and oxidative phosphorylation, potentially suggesting that the protein can be translocated into mitochondria under oxidised conditions. These results imply that changes in the protein's redox status are vital for its association with glycolytic, TCA and/or oxidative phosphorylation enzymes. This corroborates previously observed data in sections 4.3.1, 4.3.2 and 4.3.3, which showed that binding to glycolytic enzymes occurred under protein overexpression, while enzymes in the TCA cycle were only impaired when its thiol groups were removed.

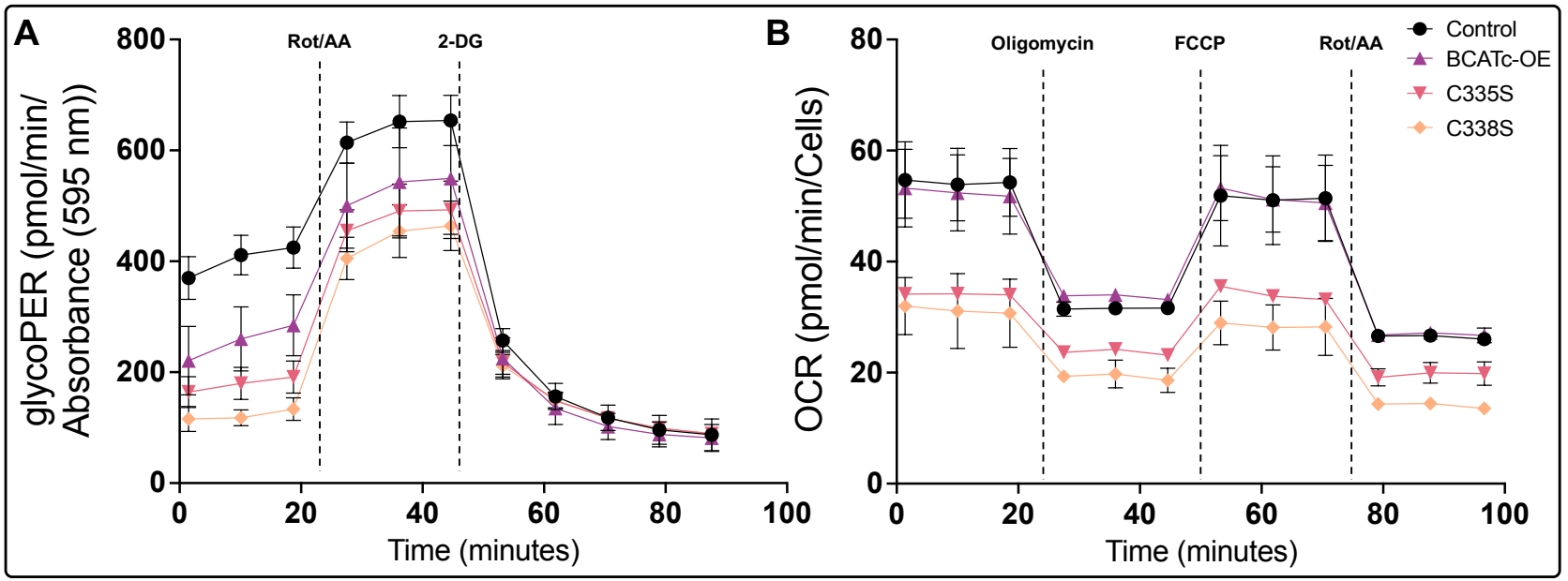

Figure 25. BCATc-OE and thiol mutants negatively impact glycolytic rate and oxidative phosphorylation. Following overexpression of BCATc and its thiol mutants, cells were incubated in balanced, nutrient-rich medium (glucose, glutamine, pyruvate and HEPES), and (A) Glycolytic rate assessed by measuring basal glycolysis prior to inhibition of oxidative phosphorylation with Rot/AA. Compensatory glycolysis was estimated using PER, followed by inhibition of hexokinase and glycolysis by 2-DG. This yielded the PER prior to injection, and thus glycoPER/glycolytic rate using ECAR and OCR. (B) Oxidative phosphorylation was measured using oligomycin (complex V inhibitor), FCCP (membrane potential disruptor) and Rot/AA (complex I and III inhibitors). Dysregulation in energy pathways have a significant impact in the generation of downstream metabolites, either increasing or decreasing overall nutrient availability. Cells were normalized using crystal violet assay. (Glycolytic rate: biological n=12, technical n=3; Mito stress test: biological n=6; technical n=3).

4.4. Discussion

In this chapter, a comprehensive set of biochemical tools were applied to further understand the role of the BCATc protein in metabolic reprogramming, and the downstream implications in the pathology of AD. Untargeted metabolomics identified 5 key cellular energy pathways that are perturbed by BCATc overexpression, namely amino acids and fatty acids metabolisms, glycolysis and the TCA cycle and neurotransmitter synthesis. The activity of enzymes in the glycolytic pathway and the TCA cycle were also dysregulated when BCATc wild type or its thiol mutants were overexpressed, although overexpression of wild type only significantly affected the activity of enzymes in glycolysis but not in the TCA cycle. As a result, while glycolytic rate was significantly decreased in all conditions, oxidative phosphorylation was only affected when the protein's thiols were mutated. This suggests the reactive cysteines are vital in determining BCATc binding partners and shows that the redox environment of the cell is a fundamental contributor.

Collectively, these results support the role of BCATc in metabolic reprogramming and offer further evidence to show that dysregulation in energy pathways, resulting from T2DM or otherwise, are key players in AD pathology. Furthermore, the data discussed in this chapter may further highlight the potential value of discussed metabolites in predicting disease development.

4.4.1. Overexpression of the BCATc protein regulates intracellular metabolite load and may impact on Acetyl-CoA pool

4.4.1.1. Branched chain, aromatics, and general amino acid metabolisms are significantly impaired

The effects on the BCAA pool were a decrease in intracellular concentrations of leucine and isoleucine with a significant increase in valine. This suggests that while leucine and isoleucine metabolism is most likely driven forward as a result of

increased substrate affinity to the BCAT protein, valine catabolism is most likely decreased; out of the three BCAAs, valine has shown the lowest affinity for BCATc, with a k_{cat}/K_m of 51 x 10^3 M^{-1} s^{-1} (in contrast with 220 10^3 M^{-1} s^{-1} for leucine and 223 10^3 M^{-1} s^{-1} for isoleucine) (Davoodi *et al.,* 1998). Dysregulated BCAA metabolism has been previously implicated in both T2DM and AD pathogeneses. McCormack *et al.* (2013) showed that high levels of BCAAs can be predictive markers for the development of T2DM, while Li *et al.* (2018) observed these levels were also increased in the blood of AD patients. The authors also suggested that leucine, but not isoleucine or valine, upregulate tau phosphorylation via mTORC1 activation in mice models, contributing to the AD pathology (Li *et al.,* 2018). However, our data showed that when BCATc is upregulated, similar to what is observed in AD patients (Hull *et al.,* 2015), levels of BCAAs leucine and isoleucine are decreased, while valine is increased. This may suggest that while BCAA levels are initially upregulated as a result of T2DM, these drive overexpression of the BCATc protein in efforts to return the metabolites to physiological levels. Chronic high levels of BCAAs and thus sustained BCAT overexpression (particularly BCATc in neurones), however, ultimately decrease BCAA levels.

This outcome not only impacted the synthesis of respective downstream metabolites but also overall intracellular amino acids availability. For instance, our results showed that levels of alanine, aspartate, glutamine, and histidine were significantly increased when BCATc was upregulated (see section 4.3.1.1, figure 14). In muscles, BCAAS are the main nitrogen source for synthesis of alanine, aspartate, and glutamine (Holecek, 2002). Thus, rapid transamination of and decrease in levels of BCAAs as a result of BCATc upregulation may drive an increase in synthesis of these amino acids. Furthermore, high levels of histidine have been correlated with an increase in the ammonia pool, contributing to the observed increase in alanine and glutamine, and the decrease in BCAAs (Holecek, 2020).

Aromatic amino acids phenylalanine and tyrosine, key compounds in neurotransmitter synthesis, were also significantly decreased (see section 4.3.1.1). BCATc may contribute to an increase in BCAA and decrease in ArAAs uptake as a result of SCLS75/LAT1 transporter competition, affecting neurotransmission substrates (Fernstrom, 2005). This was evidenced by a decrease in levels of phenylalanine and subsequent tyrosine, as well as tryptophan downstream metabolite kynurenine, which can impact on niacin production, NAD and NADP, and regulation of the redox system (Badawy, 2017). This decrease in neurotransmitter precursors has significant impact on neuronal health and may drive loss of long-term potentiation, synaptic plasticity and ultimately lead to cellular death, all of which are key features in the development and progression of AD (Martinez-Serra *et al.*, 2022). Increased glucose levels such as in T2DM and which have been observed in this study (see section 4.3.1.4) may also downregulate SCLS75/LAT1 expression, decreasing amino acid uptake (Yamamoto *et al.,* 2017). In addition, downregulation of SCLS75/LAT1 may also impact on the availability of amino acids for insulin synthesis and secretion in pancreatic β-cells (Kobayashi *et al.,* 2018). These, in conjunction with the potentially high extracellular levels of BCAAs and ArAAs, could lead to transporter overload and further evidence the observed intracellular decrease in those amino acids (Scalise *et al.,* 2018).

4.4.1.2. Dysregulation of fatty acid metabolism by BCATc overexpression decreases cytosol-mitochondria lipid transport and β-oxidation

The increase observed in levels of valine, likely as a result of its impaired catabolism as previously discussed, also correlates with the decreasing levels of carnitine and acylcarnitines, particularly isobutyrylcarnitine. This in turn lead to an increase in levels of long-chain fatty acids palmitic and stearic acid, as well as fatty acid amides oleamide, palmitamide and stearamide (see section 4.3.1.2.) Oxidative

metabolism is vital for brain health and dysregulated fatty acid metabolism can have serious consequences, such as impaired β-oxidation and ultimately Acetyl-CoA turnover (Jones *et al.,* 2010). Long-chain fatty acid metabolism is dependent on the transport of long-chain fatty acids into mitochondria for β-oxidation, transport which is mediated by acylcarnitines such as isobutyrylcarnitine (McCoin *et al.,* 2015). However, acylcarnitines are not only involved in lipid metabolism but also in protein regulation and cholinergic transmission (Jones *et al.,* 2010; Cristofano *et al.,* 2016), and have been previously shown significantly decreased in individuals with AD relative to healthy controls (Huo *et al.,* 2020). This is important as the expression of BCATc has been shown significantly increased in the brains of individuals with AD (Hull *et al.,* 2015), and we have shown that in BCATc overexpression models of AD the levels of these metabolites are likewise significantly decreased (see section 4.3.1.2). In addition, fatty acid amides have also been associated with increased Aβ burden in the CSF of AD patients (Kim *et al.,* 2019), further substantiating the effects of BCATc overexpression in the pathogenesis of the disease.

4.4.1.3. Dysregulated metabolites in neurotransmitter synthesis may impact on neuronal death.

In addition to β-oxidation, there are several mechanisms which dysregulation contributes to the decline in Acetyl-CoA and ultimately impact on energy pathways, lipid synthesis and protein acetylation (Moffett *et al.,* 2013). For instance, we have shown that levels of N-acetylaspartate (NAA), one of the most abundant acetylated metabolites in the human brain, were significantly decreased following BCATc overexpression (see section 4.3.1.3). Downregulation of NAA has not only been implicated in decreased synthesis of brain-specific dipeptide N-acetyl-aspartyl-glutamate (NAAG) but is also suggested to impair Acetyl-CoA synthesis (as acetate

donor) (Moffett *et al.,* 2007). NAAG has been implicated in glutamatergic modulation via activation of presynaptic metabotropic glutamate receptor 3 (mGluR3), thus decreasing glutamate release. The metabolite may also associate, although with lesser affinity, with ionotropic NMDA receptors (Morland and Nordengen, 2022). Fluctuations in NAA and subsequent NAAG may therefore increase glutamate release and contribute to glutamate toxicity, driving neuronal loss and the progression of AD. Furthermore, we have also showed that while choline-containing phospholipid choline alphoscerate (α-GPC) is significantly increased, choline and acetylcholine (ACh), the main neurotransmitter in the PNS and most implicated in the development and progression of AD (Ferreira-Vieira *et al.,* 2016), were significantly decreased. Early studies showed that ACh release is increased as a result of α-GPC in rat hippocampal slices, facilitating learning and memory and decreasing structural changes as a result of ageing (Lopez *et al.,* 1991; Schettini *et al.,* 1992). Moreover, in models where cholinergic transmission is dysregulated, the metabolite was shown to exhibit neuroprotective properties and increase choline acetyltransferase (ChAT) activity (Yu *et al.,* 2020). However, the availability of both substrates, choline and Acetyl-CoA, as well as the activity ChAT, are critical for the synthesis of acetylcholine, which has been shown decreased in individuals with AD (Hampel *et al.,* 2018). Our results suggest that decreasing Acetyl-CoA availability, as a result of previously discussed dysregulation in β-oxidation, glycolysis and BCAA catabolism, also decreases synthesis of acetylcholine from choline, while α-GPC is driven to increase as a compensatory mechanism. This may be further supported by the increased levels of fatty acids, the main components of α-GPC (Traini *et al.,* 2013).

4.4.2. BCATc dysregulation impairs glycolysis and the TCA cycle

4.4.2.1. Glucose and fructose, but not sorbitol, are significantly increased as a result of BCATc overexpression

We have observed that while levels of glucose and fructose are significantly increased, sorbitol levels are decreased in neuronal models overexpressing the BCATc protein (see section 4.3.1.4). Hyperglycaemia is a primary feature in T2DM pathology, and alongside mitochondrial dysfunction and impaired BCAA catabolism has been extensively implicated in the development of AD (Polis and Samson, 2020). In addition, high glucose levels have been shown to induce a shift from glycolysis to the polyol pathway, generating the non-metabolising sugar sorbitol and increasing osmotic pressure and oxidative damage (Chung *et al.,* 2003). The brain, as an energy-demanding tissue, requires efficient ATP turnover from glycolysis, the TCA cycle and oxidative phosphorylation. However, excessive concentrations of metabolites such as glucose and fructose, as well as sorbitol, lead to severe complications (ranging from AGEs and ROS generation to neuronal death) and may in fact suggest a dysregulation in these energy pathways (Butterfield and Halliwell, 2019).

As previously discussed, BCATc overexpression is likely to impact Acetyl-CoA generation via regulation of multiple pathways such as BCAA metabolism and fatty acid β-oxidation. Ultimately, however, Acetyl-CoA is mainly replenished via glycolysis, showing that its downregulation will contribute towards Acetyl-CoA decrease and disruption of additional energy demanding mechanisms (Shi and Tu, 2015). Excessive levels of glucose lead to saturation of hexokinase, enzyme which is feedback-inhibited by its by-product glucose-6-phosphate (G6P), thus preventing glucose from being further metabolised and further contributing to hyperglycaemia (Berg, 2002). Eventually, this imbalance may also drive a decrease in fructose phosphorylation by hexokinase and thus an increase in its availability. This is

important as fructose downstream metabolites fructose-3-phosphate and 3-deoxyglucosone are significantly more prone to glycation, leading to accumulation of AGEs and thus ROS generation which are features of metabolic syndrome and contribute to neurodegeneration (Tang *et al.,* 2012). Curiously, however, levels of sorbitol were decreased when BCATc was overexpressed despite the significantly high levels of glucose. This may suggest that 1. the activity of the enzyme responsible for sorbitol synthesis, aldose reductase, is decreased, while sorbitol dehydrogenase, which converts sorbitol into fructose, is either stable or increased; or 2. Aldose reductase activity is stable and sorbitol dehydrogenase is increased. This also offers an additional pathway through which fructose levels are significantly increased. Furthermore, it is likely that the increase in glycolytic metabolites is also associated with the role of BCATc in regulating the enzymatic activity in both glycolysis and the polyol pathways. These results are discussed in further detail in section 4.4.2.2.

4.4.2.2. Impaired enzyme activity may drive glycolysis disruption.

Overexpression of BCATc wild type significantly decreased the activity of glycolytic enzymes GAPDH and enolase, while mutations of either cysteine residues C335 or C338 had the opposite effect by increasing GAPDH activity; enolase, however, remained decreased. Assessment of glycolytic rate offered further evidence to place BCATc in the centre of glycolytic regulation, with those findings showing that its overexpression significantly decreases glycolysis. However, although the thiol mutants increased the activity of GAPDH, glycolytic rate was similarly decreased, suggesting enolase activity is a crucial step in regulation of the pathway. In support of this, data from C^{13}-glucose tracing studies showed that both BCATc and C338S overexpression decreased levels of pyruvate and lactate, metabolites which are highly dependent on enolase activity. Previously, unpublished data by our research group have shown that, in its reduced state, the BCAT protein associates with

GAPDH, hexokinase and enolase via its unique redox-regulated CXXC motif. Conversely, oxidation decreased these associations and led to alternative binding to polyol enzymes aldose reductase and sorbitol dehydrogenase, for instance (Conway *et al.,* unpublished observations). This shows that binding of BCATc to GAPDH is dependent on its intact CXXC motif and that mutations to either of the cysteine residues disrupt this association. In addition, data presented in the following chapter shows that overexpression of BCATc increases hydrogen peroxide levels, contributing to a highly oxidising environment and thus the activity of several redox-sensitive enzymes including GAPDH (Hyslop and Chaney, 2022)

Decreasing activity of GAPDH, as with BCATc overexpression and which has been observed during hyperglycaemia, can activate the apoptotic, protein kinase C, and hexosamine pathways. This is important as dysregulation in these cellular pathways can lead to changes in processes such as endothelial permeability and blood flow, contributing to the development of diabetic retinopathy (Kanwar and Kowluru, 2009; Madsen-Bouterse *et al.,* 2010) and subsequently increasing the risk of mild cognitive impairment (MCI) (Yu *et al.,* 2020). Contrary to what we have observed, the study by Yu *et al.* (2022) found that high serum levels of neuron-specific enolase (alongside HbA1c) also increased the risk of MCI in patients with diabetic retinopathy. Although our data showed that enolase was significantly decreased when BCATc and its thiol mutants were overexpressed, this may suggest that its activity is impaired as a result of its binding to the BCATc protein irrespective of glucose levels. These results, in addition to the glycolytic rate and C^{13}-glucose tracing analysis, provide evidence to show that glycolysis is significantly impaired when BCATc is dysregulated in neuronal models which may translate to individuals with AD. Together with additional findings in this chapter, these highlight the importance of BCATc in metabolic reprogramming, and suggest that under pathological conditions such as T2DM the impact of BCATc in metabolic

reprogramming is considerably more accentuated which may contribute to the development of AD. However, further studies are required to assess the activity of additional glycolytic as well as polyol enzymes such as aldose reductase and sorbitol dehydrogenase.

4.4.2.3. Regulation of TCA cycle and oxidative phosphorylation by BCATc are redox-dependent

As discussed in the section above (4.2.2.2), overexpression of BCATc wild type and mutants significantly disrupt the glycolytic pathway possibly by binding to and changing enzymatic activity. This contributes to a decrease in metabolites such as pyruvate and lactate and overall glycolytic rate, culminating for a decrease in Acetyl-CoA. Product of glycolysis, β-oxidation and BCAA catabolism, Acetyl-CoA is the primary metabolite in maintaining the TCA cycle and oxidative phosphorylation (Shi and Tu, 2015), pathways which we have shown dysregulated when BCATc expression was altered. Our results showed that while overexpression of BCATc wild type did not affect TCA cycle enzymes nor did it impair oxidative phosphorylation, overexpression of the thiol mutants C335S and C338S had a significant impact on both pathways by significantly increasing IDH and MDH while decreasing overall oxidative phosphorylation.

There are several hypotheses we propose for the role of cytosolic BCAT in regulating mitochondrial enzymes in this model. Recent work by our research group has shown that BCATc could translocate into mitochondria when overexpressed and under starved conditions (Edward *et al.,* unpublished observations). In support of that, Hyslop and Chaney showed that oxidation of GAPDH can encourage its binding to chaperone proteins and transport of bound complex into the nucleus and mitochondria, where it is suggested to initiate survival mechanisms such as autophagy and apoptosis (Hyslop and Chaney, 2022). With the proposed

moonlighting role of BCAT as a redox chaperone protein and its role in autophagy (El Hindy *et al.,* 2014; Harris *et al.,* 2020), this could be a possible route through which BCATc travels into mitochondria. However, upon dissociation from the GAPDH complex, BCATc may be oxidised and/or lose its thiols function, which may facilitate its binding to TCA cycle enzymes. In addition, decreasing BCAA metabolism as a result of mutations to the BCATc thiols can lead to a further decrease in Acetyl-CoA, decreasing citrate availability as observed by C^{13}-glucose tracing studies (section 4.3.2). This may induce reverse reductive carboxylation of α-KG to citrate via mitochondrial IDH (IDH2), increasing enzyme activity and subsequently decreasing α-KG levels (section 4.3.2). This impacts on downstream substrates fuelling oxidative phosphorylation and increasing MDH activity in order to generate oxaloacetate and reinitiate the cycle. This upregulation of IDH has been previously observed from a cancer metabolism perspective by Wang *et al.* (2020), who suggested that an increase in citrate synthetase increases carbon sources entering the TCA cycle and drives an overwhelming increase in IDH flux, all the while increasing citrate and decreasing α-KG (Kang *et al.,* 2021). In addition, Zeng *et al.* (2022) showed that by downregulating the mitochondrial isoform IDH2 in acute myeloid leukaemia models there was an increase in levels of α-KG and reduced lipid synthesis, complementing the observations by Wang *et al.* (Zeng *et al.,* 2022).

Although further studies are necessary to assess pathway dysregulation and the mechanisms which can contribute to mutations to the CXXC motif of BCATc and whether those are physiologically relevant, the evidence thus far shows that BCATc does act as a metabolic reprogramming protein. The functional impact of BCATc dysregulation have been explored and are discussed in Chapter 6, where the link between the pathologies of T2DM and AD is further clarified.

Chapter 5

Metabolic reprogramming contributes to oxidative stress, autophagy dysregulation and accumulation of toxic peptides

5.1. Introduction

Accumulation of toxic Aβ peptides and NFTs from tau hyperphosphorylation are hallmarks in the pathogenesis of AD, with Aβ previously showing association with inhibition of LTP thus leading to learning and memory deficits (Chen *et al.,* 2000). Similarly, amyloid aggregates have been implicated in the damage observed in pancreatic β cells, contributing to T2DM pathology and later the development of AD (Ponce-Lopez *et al.,* 2018).

Clearance of misfolding and aggregating proteins is closely regulated under normal physiological conditions, however under pathological conditions the disruption of cellular clearance can lead to damaging downstream effects such as increase in oxidative stress and cellular death (Barbero-Camps *et al.,* 2018). In particular, dysregulation of the autophagy mechanism can contribute to the progression of T2DM, where inhibition may suppress lipid oxidation (Saito *et al.,* 2019) while hyperactivation may decrease insulin secretion (Yamamoto *et al.,* 2018). It is also suggested that dysregulated autophagy may contribute to both degradation and production of Aβ, once APP can be cleaved within autophagosomes during processing of APP-rich organelles (Nixon, 2007; Nilsson and Saido, 2014).

Harris *et al.* (2020) showed that overexpression of cytosolic BCAT in neuronal models increases trafficking of proteins of the mTOR pathway to the cell membrane especially under insulin stimulation, disrupting physiological initiation of the autophagy pathway. However, although an increase in the expression of autophagy marker LC3 initially suggests autophagy initiation despite the effects of mTOR signalling, LC3 appeared to accumulate in the cell membrane, demonstrating that autophagic flux is dysregulated (Harris *et al.,* 2020). This in turn may enhance oxidative stress, leading to an increase in the inflammatory process and contributing to further aggregation of toxic peptides from Aβ and tau hyperphosphorylation. Strict

regulation of the autophagy pathway is therefore vital for maintaining cellular homeostasis, and overexpression of BCAT as observed in AD patients (Hull *et al.*, 2014) significantly disrupts this process. In addition, the autophagy mechanism is primarily regulated by nutrient availability, making it a prime candidate for dysregulation in metabolic diseases such as T2DM where marked fluctuations in insulin, glucose and BCAAs levels are observed.

This chapter focused on demonstrating the impact of changes in BCATc expression, as a result of metabolic dysregulation or otherwise, in the redox environment of the cell as well as the accumulation of AD-related proteins (i.e., Aβ and tau hyperphosphorylation) and autophagy regulation, preventing clearance of accumulated proteins and likely contributing to the progression of the AD pathology. It follows directly from the metabolic reprogramming discussed in the previous chapter.

5.2. Hypothesis and Aims

5.2.1. Hypothesis

Dysregulation in energy pathways as a result of either sustained overexpression of BCATc or impairing of its redox function, results in increased oxidative stress and impaired autophagy, leading to the accumulation of toxic peptides such as Aβ and hyperphosphorylated tau, key markers of AD pathology.

5.2.2. Specific aims

Specific aim 1: To demonstrate that changes in BCATc expression and redox status increase cellular oxidative stress.

Specific aim 2: To evaluate how autophagy is affected in response to increased levels of the BCATc protein.

Specific aim 3: To analyse whether the effects of oxidative stress and impaired autophagy, underpinned by BCATc and metabolic dysregulation, lead to protein aggregation and cellular death.

5.3. Results

5.3.1. Wild-type and thiol mutant BCAT overexpression independently increase oxidative stress in the cytosol and mitochondria

Oxidative damage is one of the most damaging outcomes from uncontrolled T2DM, affecting major energy pathways in the cell and resulting in neuronal cell death. To assess the impact of BCATc dysregulation on ROS generation in individual cell compartments, particularly cytosol and mitochondria, H_2O_2 was measured following BCATc knockdown and/or overexpression of wild type and thiol mutants C335S and C338S, using confocal microscopy, immunofluorescence and ratiometric fluorescence techniques. These experiments were performed using the genetically encoded fluorescent probe HyPer7, which senses the increase in H_2O_2 intracellularly and can be observed under 420/500 nm excitation and 510 nm emission. Measuring the decrease in 420 nm to the increase in 500 nm (ratiometric) gives the proportion of H_2O_2 produced under stress conditions.

To confirm the effectiveness of the molecular probes, cells were analysed using immunofluorescence 48 h after co-transfection with the BCATc mutants and pCS2+HyPer7-NES (cytosolic) or pCS2+MLS-HyPer7 (inner mitochondrial membrane). Images were obtained from individual wells showing successful Hyper7 transfection and increasing ROS levels (Figure 26C and D). Results for fluorescence ratiometric analysis confirmed the increase in ROS production and additionally showed that, although BCATc is localised to the cytoplasm, the effects that both OE and KD have in both cellular compartments are substantial and impact on ROS production in the mitochondrial matrix, suggesting the products of or the protein itself can directly impact on metabolic pathways within different cellular compartments most likely by altering metabolite levels. In the cytosol, BCATc-KD ($p=0.016$), BCATc-OE ($p=0.0003$) and thiol mutants ($p=0.0026$, C335S; $p=0.0023$, C338S) significantly increased ROS production, with wild type OE showing the highest impact overall

(Figure 26A). These were compared with HyperProbe-only transfected cells to demonstrate the effects are as a result of BCAT dysregulation as opposed to cellular transfection alone. In contrast, BCATc-KD only mildly, although not significantly, decreased mitochondrial matrix ROS production ($p=0.1271$), with overexpression increasing similar to what was observed in the cytosol ($p=0.0008$, BCATc-OE; $p= 0.0300$, C335S; $p= 0.0267$, C338S) (Figure 26B).

SH-SY5Y were also observed using live confocal imaging, further corroborating ratiometric and immunofluorescence studies by allowing visualisation of cells while actively generating H_2O_2, whether in the cytosol or mitochondria. Confocal analysis showed a substantial visual increase in cytosolic ROS production across most analysed parameters (BCATc-OE, C335S, C338S), with no observable differences under BCATc-KD (Figure 27). Furthermore, although mitochondrial ROS was also observed using pCS2+MLS-HyPer7 (Figure 28).

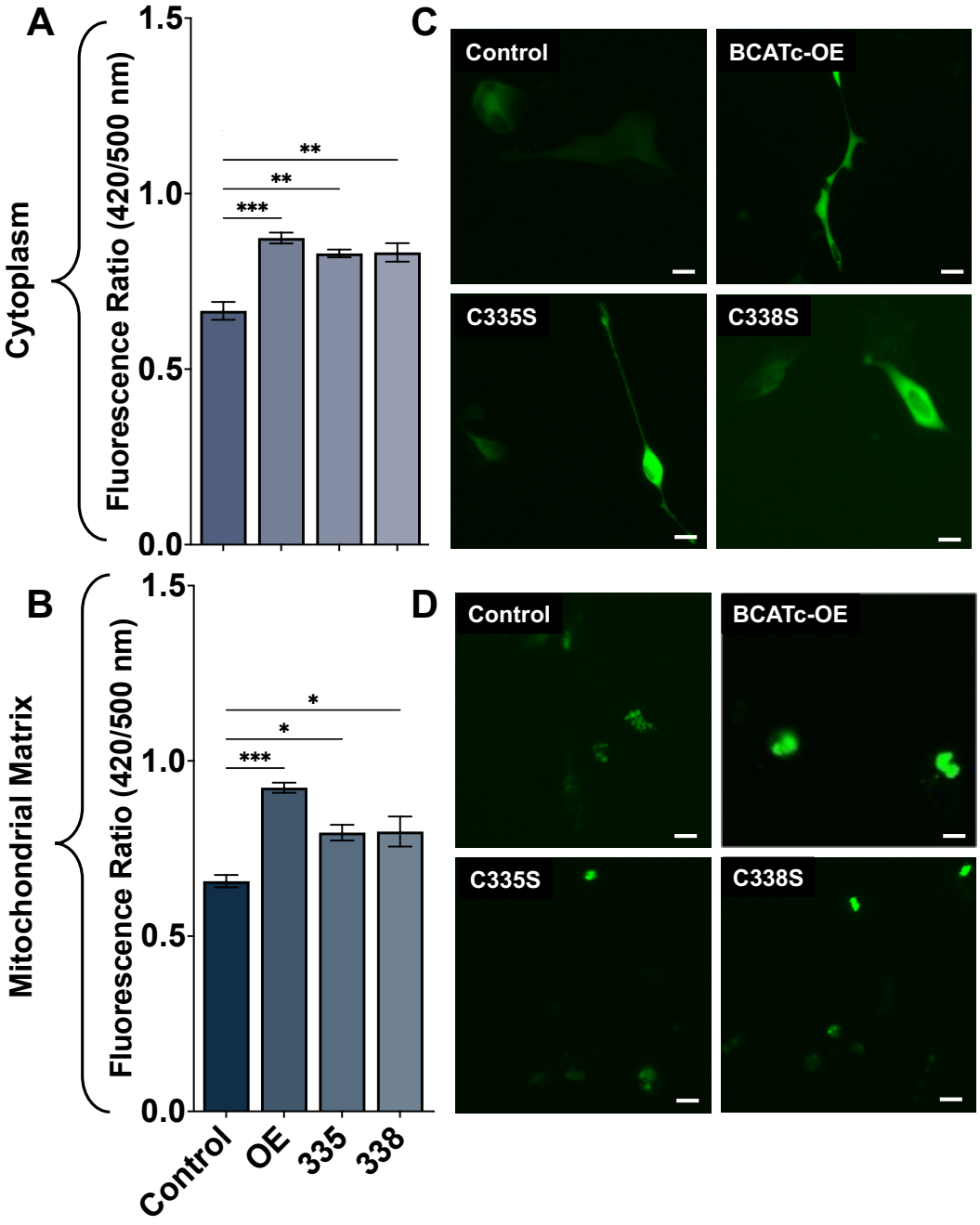

Figure 26. Dysregulation in BCATc expression and redox status increase ROS production in cytoplasm and mitochondria. Fluorescence ratiometric analysis showed a significant increase in (A) cytosolic and (B) mitochondrial matrix ROS when cells were overexpressed with wild type and thiol mutants of the BCATc protein. Immunofluorescence data further evidenced the increase in H_2O_2 production for overexpression in (C) the cytoplasm and (D) mitochondria. Scale bar: 10 μM. Fluorescence ratio shown as ± SEM (One-way ANOVA, n = 4) *p < 0.05, **p < 0.01, ***p < 0.001.

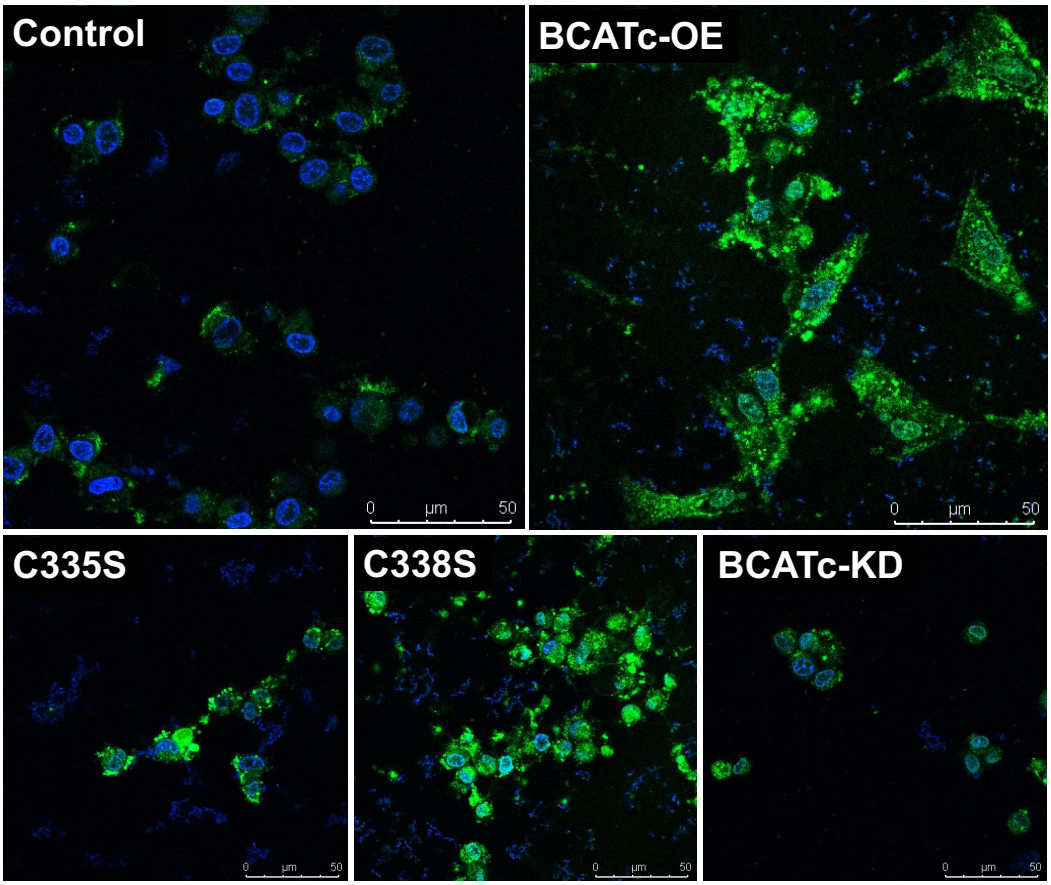

Figure 27. Cytosolic ROS production is increased depending on BCATc expression and redox status. Differentiated cells were transfected with BCATc constructs and cytosolic HyperProbe pCS2+HyPer7-NES for 48 h prior to confocal imaging, or with knock down constructs for 96 h. Fluorescence illustrates H_2O_2 generation relative to control cells. Magnification: 60x. Scale bar: 50 µM (n = 2).

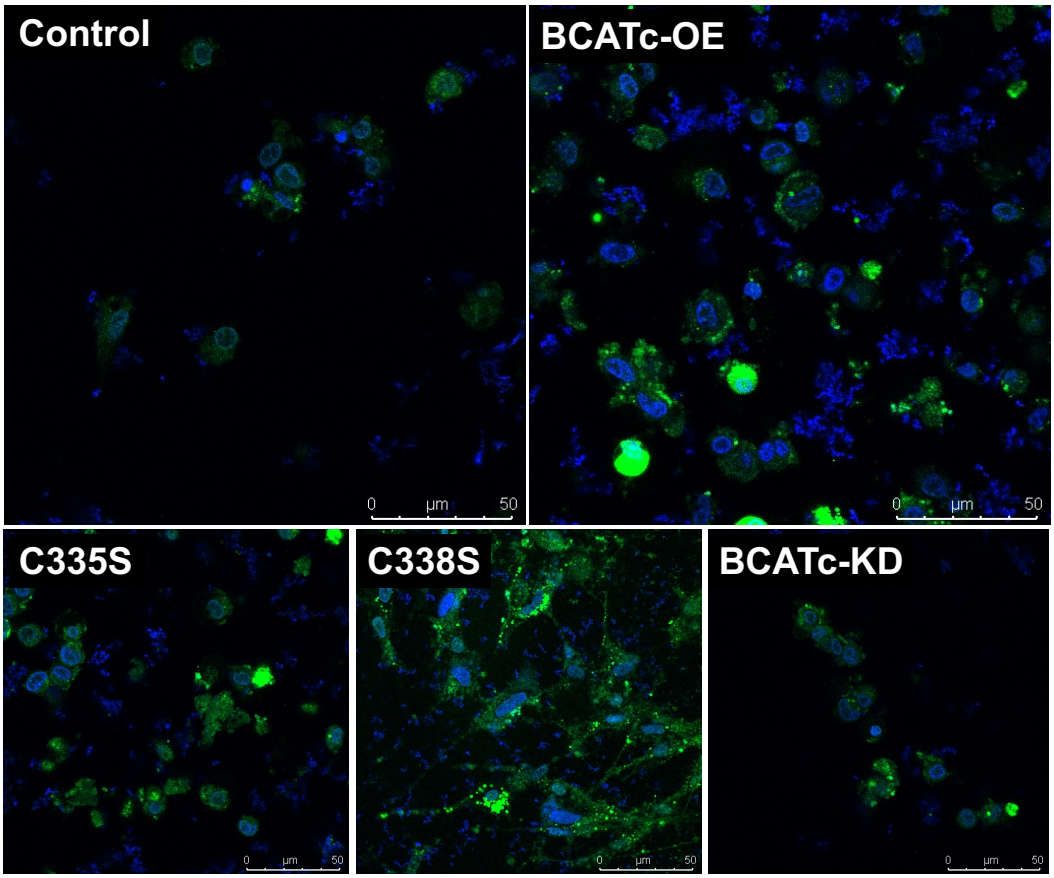

Figure 28. Increased mitochondrial ROS production was also changed depending on BCATc expression and redox status. Mitochondrial HyperProbe pCS2+MLS-HyPer7 was chosen alongside overexpression and knockdown of the BCATc protein and its thiol mutants to show the increase in mitochondria derived H_2O_2. Fluorescence illustrates H_2O_2 generation relative to control cells. Magnification: 60x. Scale bar: 50 µM (n = 2).

To investigate if markers of oxidative stresswere also altered in response to BCAT expression, Western Blot analysis was performed to assess the effects on the expression on antioxidant proteins thioredoxin (TRX), glutaredoxin (GLRX), peroxiredoxin (PRX1) and superoxide dismutase (SOD2) (BCATc-OE and -KD models). The results showed that when BCATc was overexpressed the expression of redox proteins decreased (Figure 29), which may affect the overall antioxidant capacity of the cell and drive further increase in ROS. Although these changes were not significant in this instance, the expression of peroxiredoxin showed the opposite trend with a gradual increase following BCATc-OE. BCATc knockdown however led to a decrease in PRX whilst increasing TRX, GLRX and SOD2 (Figure 29). Although upregulation of SOD2 can increase the production of H_2O_2, downregulation of the protein likely disrupts dismutase capacity of superoxide, a highly reactive and damaging form of the oxygen ion. This shows the importance of redox balancing in the cell, as dysregulation on either side can lead to severe impact on cellular health and potential irreversible changes in its machinery.

5.3.2. Autophagy is affected by changes in the expression of the BCAT protein

Regulation of autophagy, cellular pathway for removal of intracellular debris, is vital for prevention of unprogrammed cell death, increased oxidative stress and regulation of osmotic pressure, maintaining the homeostatic state. Autophagy plays a key role in cell survival under conditions such as nutrient deprivation, providing an energy source for the surrounding tissue and as a result it requires strict control to prevent its malfunction.

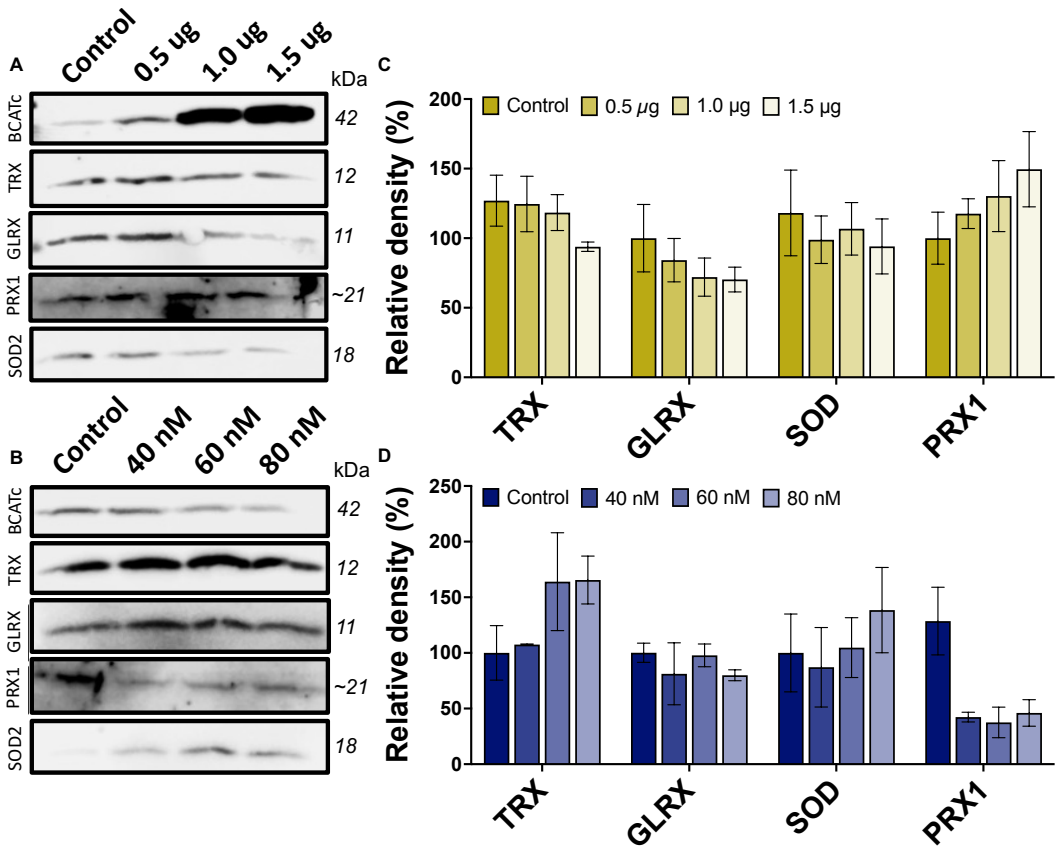

Figure 29. BCATc overexpression and knock down differentially regulate the expression of antioxidant proteins. Differentiated SH-SY5Y cells were (A) overexpressed or (B) knocked down with several concentrations of the BCATc protein, and the expressions of antioxidant proteins such as thioredoxin (TRX), glutaredoxin (GLRX), peroxiredoxin (PRX) and superoxide dismutase (SOD) were assessed. (C) Respective densitometry analysis shown as percentage relative density ± SEM (Two-way ANOVA (ns), n = 3).

Here, Western Blot analysis was used to assess autophagic flux under induced activation or inhibition of the pathway using several treatments. Nutrient deprivation and rapamycin were chosen as autophagy activators primarily via inhibition of the mTOR pathway; and bafilomycin and chloroquine as autophagy inhibitors via disruption of V-ATPase, which prevents lysosome acidification and thus degradation of autophagosome inner membrane and engulfed organelles (Hooper *et al.,* 2022). Glucose and BCAAs were also used as nutrient rich conditions to prevent autophagy and promote protein synthesis.

Neuronal models were initially nutrient deprived in saline (EBSS) for 2 hours and further 1 hour as a nutrient deprived control. Rapamycin (mTOR inhibitor) acted as an autophagy activator at 100 nM concentration, whereas 100 nM bafilomycin and 50 μM chloroquine (V-ATPase inhibitors blocking autophagosome fusion and acidification) were used for inhibition. Western Blot analysis of the autophagy marker LC3 showed a decrease in the ratio of LC3I/LC3II when cells were treated with 100 nM rapamycin or nutrient deprived, whereas 100 nM bafilomycin and 50 μM chloroquine significantly increased LC3II accumulation ($p<0.001$, $p<0.0001$, respectively), suggesting autophagosome degradation was indeed impaired (Figure 30A). Increasing expression of the BCATc protein significantly increased autophagosome formation and potential turnover, as observed with the ratio of the autophagy marker LC3I/II (Figure 30B). BCATc-KD however showed inhibition in autophagosome formation and overall autophagy, the opposite effect to overexpression (Figure 30B).

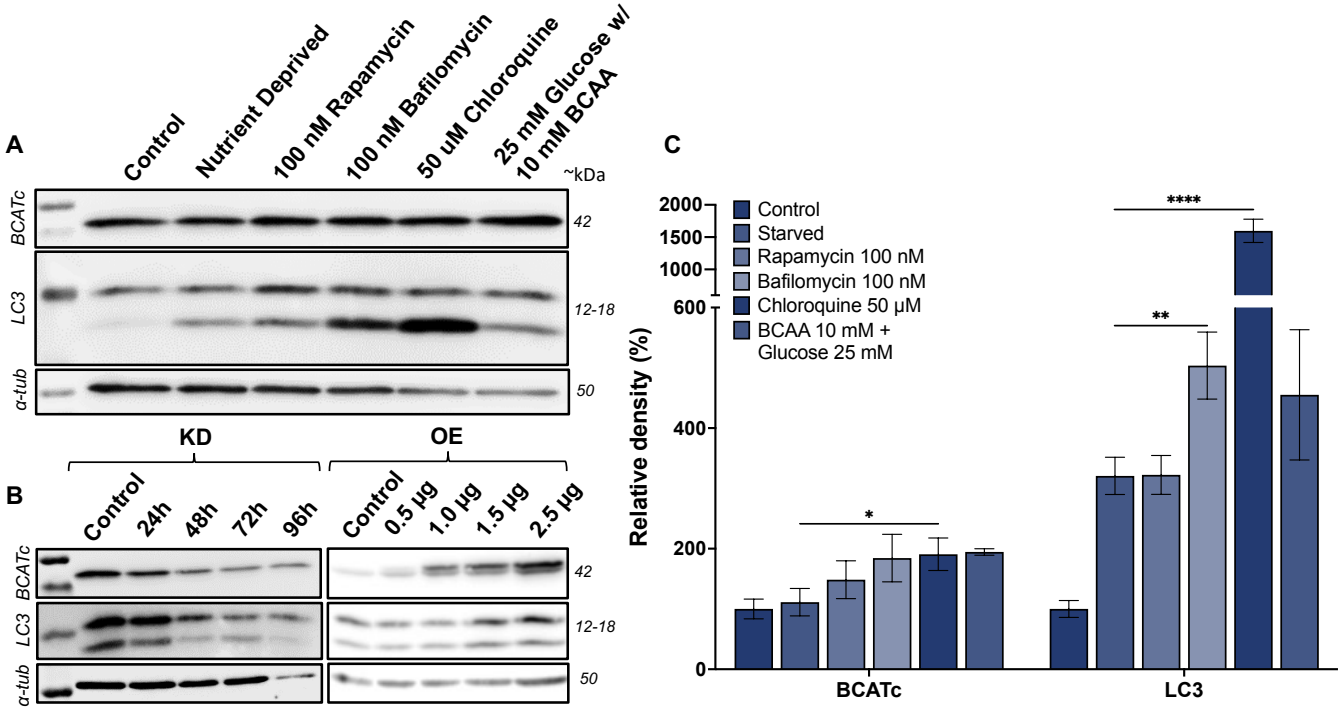

Figure 30.Overexpression and knockdown of BCATc impair autophagy by increasing or decreasing autophagosome formation. (A) Cells were treated with autophagy inducer rapamycin, and inhibitors bafilomycin, chloroquine, and nutrients BCAAs and glucose. The ratio of autophagy marker LC3I/LC3II, reflecting autophagosome turnover, was subsequently assessed and analysed against starved control. (B) BCATc overexpression and knock down showing regulation of autophagy marker LC3. (C) Densitometry analysis shown percentage relative density ± SEM (Two-way ANOVA, n = 3) *p < 0.05, **p < 0.01. (Figure partially extracted from Harris *et al.*, 2020).

5.3.3. Dysregulation of BCATc and autophagy impaired Aβ processing and tau phosphorylation, and led to protein accumulation

Aggregation of toxic peptides such as $A\beta_{42}$, and NFTs accumulation following hyperphosphorylation of MAP-tau are key features in AD progression. Under changes in BCATc expression, APP, Aβ and hyperphosphorylated tau levels were assessed and showed significant increase in a concentration dependent manner, with levels of total tau decreasing accordingly (Figure 31). Mutations in the thiol groups led to a similar increase in those proteins, although the levels were significantly less pronounced than those observed for WT OE (Figure 32).

Immunocytochemistry analysis corroborated these findings by showing both Aβ and hyperphosphorylated tau are increased when the BCATc protein is overexpressed or had its thiols mutated, with Aβ accumulating in a punctate manner across the cell body (Figure 31A and 31B, respectively). These vesicle-like structures, such as autophagosomes, are likely involved in either sequestering Aβ/hyperphosphorylated tau for degradation and preventing further accumulation or contributing towards the aggregation via dysregulation in the autophagy mechanism, a process which has been previously explored and discussed in depth in Harris *et al.* (2020). This suggests that although the autophagy mechanism can be initiated in an attempt to remove toxic aggregation, the BCAT protein likely prevents autophagosome maturation and removal, increasing its levels within the cell and exacerbating the accumulation that should have been prevented otherwise.

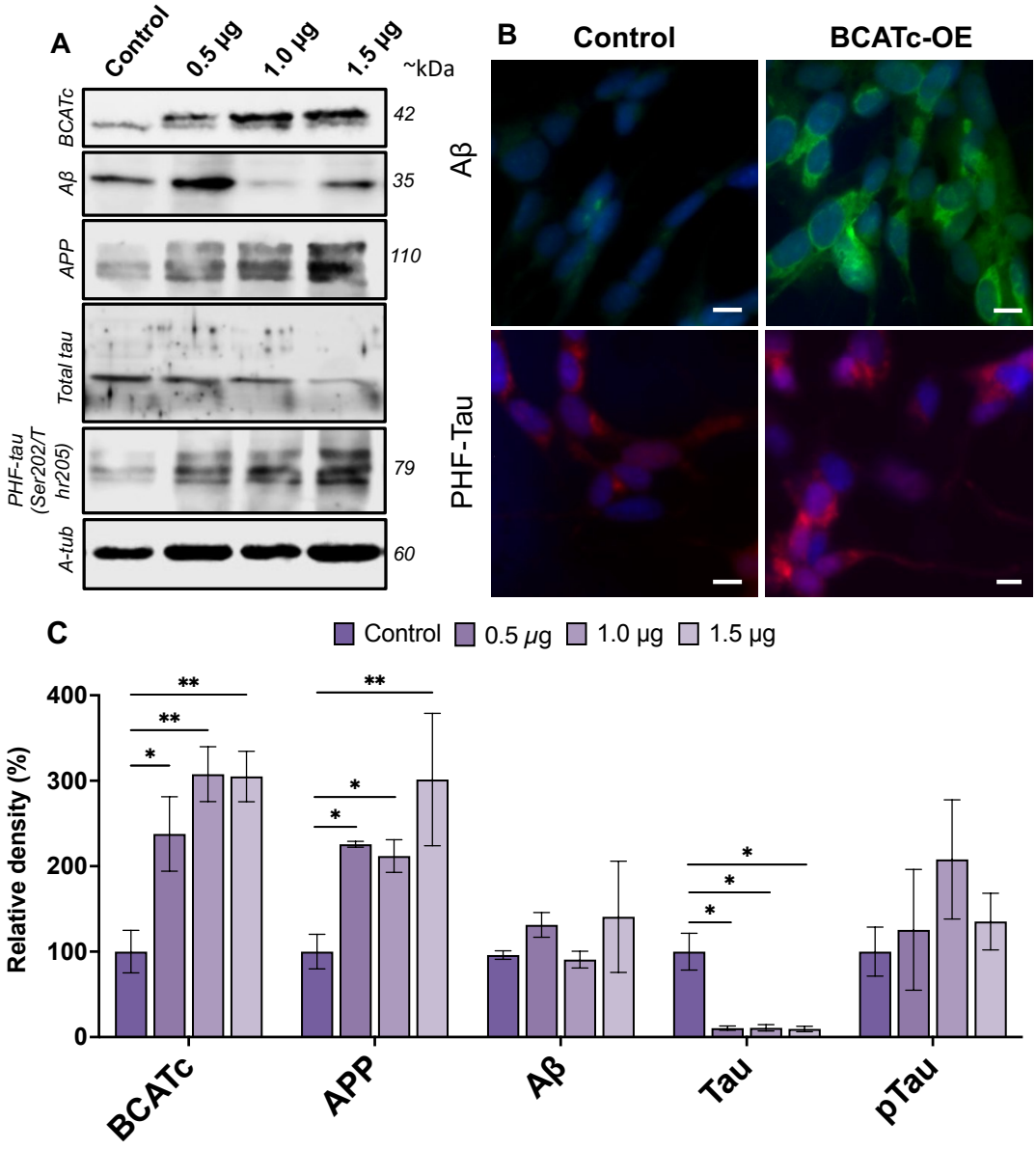

Figure 31. Gradual increase in BCATc expression increases APP, Aβ and hyperphosphorylated tau expression. Differentiated cells were transfected with increasing concentrations of BCATc and expression of several proteins associated with AD pathology were assessed. (A) Western Blot analysis of $A\beta_{1-42}$, APP, Tau and pTau following BCATc overexpression. (B) Fluorescence microscopy illustrating the increase in Aβ and hyperphosphorylated tau (n = 4; Scale bar: 10 µM). (C) Densitometry analysis shown percentage relative density ± SEM (Two-way ANOVA, n = 3) *p < 0.05, **p < 0.01.

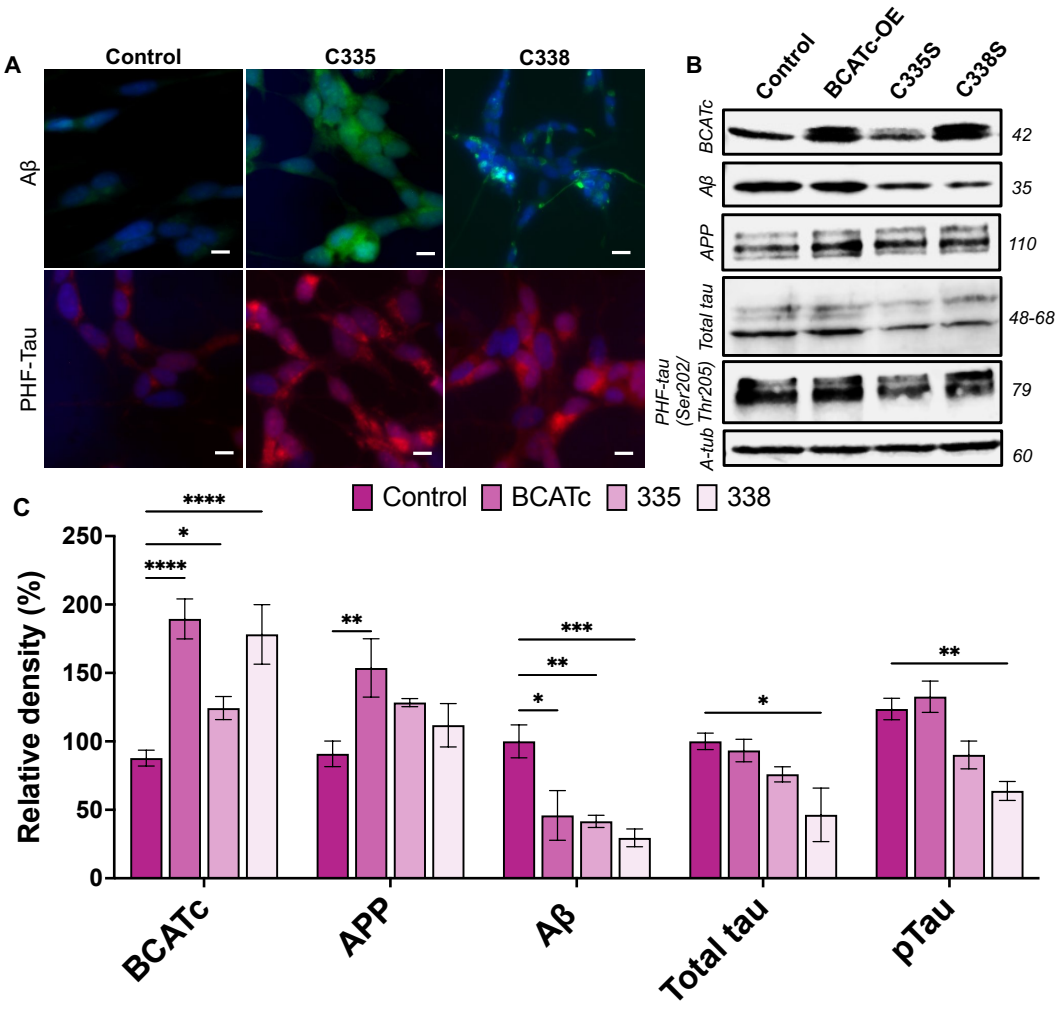

Figure 32. BCATc thiol mutants C335 and C338 dysregulate expression of AD-related proteins. Differentiated cells were transfected with BCATc thiol mutants C335 and C338 and expression AD-related proteins was assessed. (A) Fluorescence microscopy illustrating the increase in Aβ and hyperphosphorylated tau (n = 3; Scale bar: 10 μM). (B) Western Blot analysis of $Aβ_{1-42}$, APP, Tau and pTau following overexpression of BCATc and its thiol mutants. (C) Respective densitometry analysis shown percentage relative density ± SEM (Two-way ANOVA, n = 3) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

In addition, there is contradicting evidence to suggest APP may be cleaved in autophagosomes producing the more toxic, aggregation prone $A\beta_{42}$ peptide (Nilsson and Saido, 2014).

5.3.4. BCATc overexpression and redox status affect cellular viability with and without BCAA/glucose supplementation

Using differentiated, dopaminergic-like neuronal cells (>3 days), viability studies showed a significant decrease in neuronal viability following overexpression of BCATc and its thiol mutants C335 and C338S ($p<0.0001$) (Figure 33). Supplementation with 10 mM BCAAs improved viability particularly when cells were overexpressed with BCATc and C338, but not C335, whereas 25 mM glucose further exacerbated neuronal death in healthy cells with modest improvement observed when BCATc was overexpressed. This suggests that increasing BCAA levels may drive protein synthesis particularly under BCATc overexpression, hence preventing excess BCATc from interacting with binding partners in the glycolytic pathway and disrupting the pathway (as observed in chapter 4). However, hyperglycaemia is a known driver of ROS production and cell death, and binding and downregulation of glycolytic enzymes (i.e., GAPDH and enolase) by BCATc prevents metabolism of excess glucose further increasing cellular damage.

Mutations in the redox sensor C338 also affected neuronal viability in a similar fashion to BCATc overexpression ($p<0.0001$) (Figure 33), although its effects were less pronounced than those observed for BCATc-OE. However, while glucose supplementation seemed to have a similar detrimental effect on both healthy controls and C335S, supplementation with BCAAs significantly impaired viability when C335S was overexpressed ($p<0.01$), in contrast with the improvement observed for BCATc-OE (ns). Overexpression of mutant cysteine 338 (C338S) did not show a significant impact or change in cellular viability in contrast with BCATc-OE and BCAA/glucose supplementation, although it curiously behaved similarly to BCATc-OE with C335S

showing the most observable changes. This could offer more understanding on the roles of the BCAT thiols and its interactions with different pathways involved in cell survival, particularly in respect to the protein's role as a redox sensor and its inability to perform its regular function without the presence of its cysteine residues.

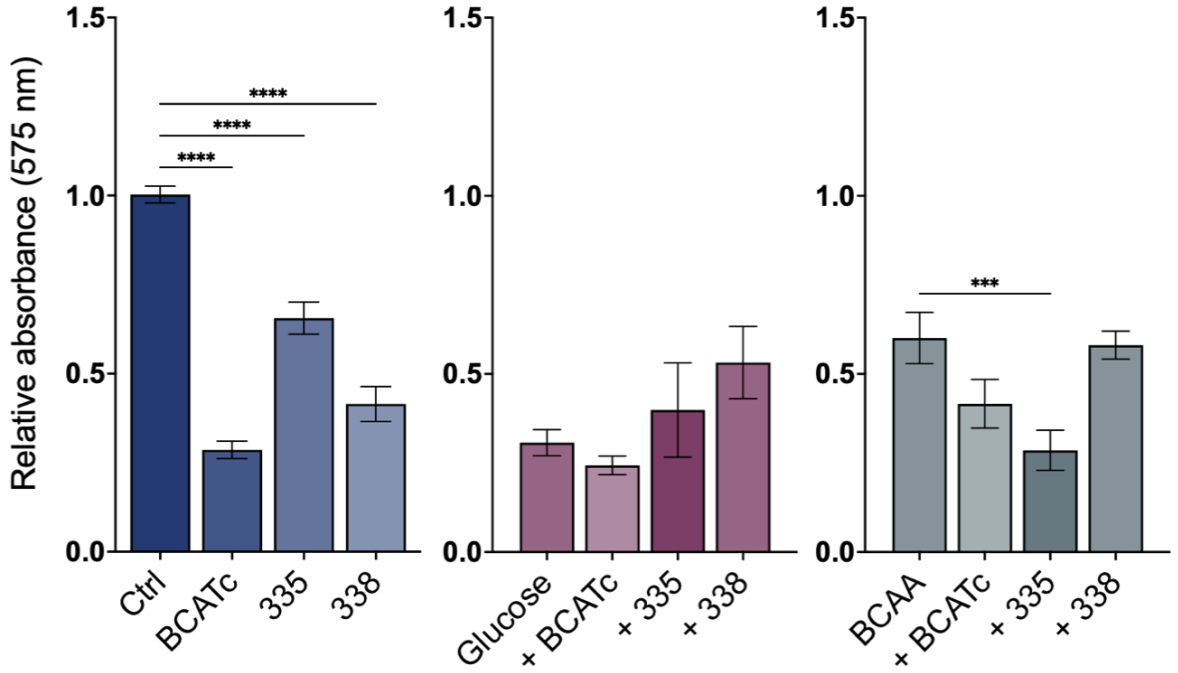

Figure* 33. *Overexpression of BCAT and thiol mutants impacts on neuronal viability with and without 10 mM BCAA and 25 mM glucose supplementation. BCATc and its thiol mutants were overexpressed in SH-SY5Y cell viability using crystal violet was assessed. (A) Cell viability following overexpression of the constructs alone showed an overall decrease. (B) Following 25 mM glucose treatments there was a further decrease in viability for C335S and increase for C338S. (C) Viability including 10 mM BCAA provided modest viability improvement for WT BCATc and C338S, and a further decrease for C335S. Relative absorbance shown ± SEM (One-way ANOVA, n = 4). ***p < 0.001, ****p < 0.0001.

5.4. Discussion

This chapter has explored molecular, biochemical, and imaging techniques to investigate the functional impact of dysregulated BCATc expression in neuronal models, following from the discussed role of the protein in metabolic reprogramming (Chapter 4). Analysis of ROS generation using molecular probes and confocal imaging showed that overexpression of BCATc wild type and thiol mutants had a significant impact in cytosolic and mitochondrial H_2O_2 turnover, and that wild-type overexpression decreased the expression of key antioxidant proteins. However, downregulation of BCATc expression had little to no impact on ROS while increasing antioxidant proteins' expression, suggesting the protein is a regulator of the redox system of the cell. The autophagy pathway was also impacted as a result of changes in BCATc expression (see Harris *et al.,* 2020 for further details), with overexpression increasing and knockdown decreasing autophagosome turnover and thus acting as an autophagy regulator. Finally, Western Blot and imaging techniques showed that BCATc overexpression increases expression and potential accumulation of AD markers such as APP, Aβ and hyperphosphorylated tau, although overexpression of the thiol mutants had a lesser impact. These disturbances ultimately led to a significant decrease in overall neuronal viability, and treatments with BCAAs partially rescued BCATc wild-type viability while exacerbating cell death in C335S mutants. In addition to data showing the impact of BCATc dysregulation in cellular metabolism, these sets of data provide further evidence to suggest the protein's involvement in the biomarkers of pathologies such as T2DM and AD.

5.4.1. Overexpression of BCATc wild type and thiol mutants increase, while knockdown decreases, H_2O_2 turnover in neuronal cell models

Overexpression of the BCATc protein and its thiol mutants increased, albeit to different extents, ROS/H_2O_2 production in both the cytosol and the mitochondria;

in contrast, knockdown of wild type had no impact on ROS generation. However, although fluorometric analysis showed that BCATc exerted the most prominent impact in ROS production in both cellular compartments, confocal analysis suggested that its thiol mutants C335S and C338S had the most substantial increase in mitochondrial ROS relative to cytosolic. These results corroborate findings discussed in Chapter 4, where overexpression of the BCATc thiol mutants had a significant impact in TCA cycle enzymes and significantly impaired oxidative phosphorylation.

Under high levels of glucose, such as what is observed in T2DM and which has been seen in this project, an increase in pyruvate oxidation via the TCA cycle increases electron donors into the electron transport chain, driving downstream generation of superoxide (O_2^-) and subsequent catalysis into H_2O_2 by antioxidant protein superoxide dismutase (Giacco and Brownlee, 2010). We have previously observed that pyruvate levels are decreased as a result of mutations in C338, and that both thiol mutants increase GAPDH activity although decreasing overall glycolytic rate (see Chapter 4 for results and discussion). This could suggest that the pyruvate generated, although at a slower rate, is rapidly converted into Acetyl-CoA and oxidised in the TCA cycle, which would also increase the activity of IDH as previously observed. Because the availability of Acetyl-CoA in the cell is decreased, its generation would ultimately favour the TCA cycle to sustain energy demands for neuronal survival (Jankowska-Kulawy *et al.,* 2022). However, the overwhelming activation of IDH would decrease α-KG and subsequently succinate, which could drive complex II/coenzyme Q to recycle fumarate into succinate, producing H_2O_2 as a result (Wong *et al.,* 2017).

Data from this project assessing the role of overexpressed BCATc wild type showed that its impact was particular to enzymes of the glycolysis pathway such as GAPDH and enolase, whereas its thiol mutants impaired the activity in the TCA cycle

and oxidative phosphorylation. The results in this chapter therefore highlight that overexpressed BCATc wild type has its primary effects in the redox status of the cytosol, where the protein is likely contributing to oxidative damage by dysregulating glucose and fructose catabolism. This can be further evidenced by the lack of ROS generation as a result of BCATc knockdown, suggesting the exacerbated expression of the protein is responsible for cellular imbalances. However, as a result of stress conditions, BCATc may bind to and likely act as a chaperone for oxidised proteins such as GAPDH, transporting it into the mitochondrial inner membrane (Hyslop and Chaney, 2022). There, BCATc would release GAPDH, however its CXXC motif would be susceptible to modifications such as S-nitrosation, S-glutathionylation and S-thiolation (Coles *et al.,* 2009; Nakashima *et al.,* 2018; Gao *et al.,* 2022). In addition, H_2O_2 levels may lead to oxidation of BCATc thiols (SH) to sulfenic acid (SOH), changing its activity (Mailloux and Treberg, 2016). These modifications would thus lead to differential binding of enzymes such as IDH and MDH and subsequent TCA cycle/oxidative phosphorylation imbalance, offering supporting evidence for the increased generation of ROS/H_2O_2 in the mitochondria as a result of BCATc mutations.

5.4.2. Antioxidant proteins are imbalanced when BCATc is dysregulated

The antioxidant system is key in protecting mitochondria from the effects of ROS production and regulating oxidative damage, particularly under pathological conditions such as T2DM and AD (Rabilloud *et al.,* 2001). This is particularly relevant to our neuronal models, where overexpression of wild type BCATc (>150%) correlated with a decrease in the expression of antioxidant proteins thioredoxin, glutaredoxin, peroxiredoxin and superoxide dismutase. Knockdown of the BCATc protein (<50%), however, had the opposite effect by increasing these proteins while decreasing H_2O_2. It has been previously established that BCATc may be subjected to

modifications such as S-glutathionylation, S-nitrosation, S-thiolation and levels of H_2O_2, enabling post-translational modifications such as phosphorylation, acetylation, and disulfide bond formation; decreasing the protein's activity; or rendering it inactive (Coles *et al.,* 2009; Mailloux and Treberg, 2016; Nakashima *et al.,* 2018; Conway *et al.,* 2021; Gao *et al.,* 2022).

With the previously discussed increase in H_2O_2 following BCATc overexpression, it is likely that the increase in ROS as a result of dysregulation in energy pathways such as glycolysis (see Chapter 4) does in fact upregulate antioxidant proteins' synthesis, however BCATc can interact with the thiol groups of these proteins in response to H_2O_2 and prevent its physiological role in redox regulation. This can be further corroborated by the results observed when BCATc is knocked down, where not only H_2O_2 levels remained similar to controls, but TRX, GLRX, PRX and SOD showed a small increase in expression rather than a decrease as observed with overexpression. Moreover, although it is a general consensus that an increase in ROS may influence synthesis and/or translation of antioxidants (Grant, 2011; Picazo and Molin, 2021), a recent study has shown that H_2O_2 may downregulate ribosome biogenesis at the nucleolus level, in turn downregulating protein synthesis (Sapio *et al.,* 2021). Additional studies are required to assess the activity of these enzymes under the conditions studied and alongside overexpression of BCATc thiol mutants. However, these results offer further understanding of the impacts of disrupted energy metabolism in the redox environment of the cell as a result of BCATc dysregulation.

5.4.3. BCATc overexpression and knockdown disrupt autophagic flux

Cellular death as a result of nutrient starvation has been well established and is widely assessed in health and disease, especially in the context of autophagy (Young

et al., 2009). Prolonged nutrient overload, however, may be equally as damaging, although more efforts are required to understand the bioenergetic pathways involved (Qiu and Shlegel, 2018). Thus, autophagy is believed to act as a double-edged sword particularly under pathological conditions (Yang *et al.,* 2017). Here we showed that overexpression of BCATc significantly increased autophagosome generation but in a dysregulated manner, preventing lysosome fusion and thus clearance. Knockdown of the protein had the opposite effect, by decreasing overall autophagosome turnover and thus autophagy activation. Incubation with bafilomycin (inhibitor of V-ATPase-dependent autophagosome acidification) or chloroquine (autophagosome-lysosome fusion inhibitor) (Mauvezin and Neufeld, 2015; Mauthe *et al.,* 2018) revealed an increase in the expression of the lapidated form of the autophagy marker LC3, LC3II, and BCATc, therefore suggesting that BCATc is involved in the accumulation of immature autophagosomes.

Considering its role in BCAA metabolism and in proposed metabolic reprogramming, and its overexpression in the AD brain (Hull *et al.,* 2015), it is not unlikely BCATc plays an integral role in additional nutrient-dependent pathways such as autophagy. For instance, the BCAA leucine is a potent activator of mTORC1, a negative regulator of the autophagy mechanism. Leucine impacts on autophagosome biogenesis via its downstream metabolite Acetyl-CoA, which acetylates mTORC1 component raptor and activates the complex, thus inhibiting autophagy (Son *et al.,* 2020). However, we have previously shown that leucine levels are decreased, and proposed that Acetyl-CoA generation is likely significantly decreased when BCATc is overexpressed, which could suggest a decrease in raptor acetylation and mTORC1 inhibition, driving autophagy activation although in a dysregulated manner.

Leucine, and to some extent isoleucine and valine, have also been shown to contribute to the development of insulin resistance, an early sign of T2DM (Newgard,

2012). Harris *et al.* (2020) showed that, in response to insulin signalling or as a result of changes in cellular redox status, BCATc translocates to the membrane where it binds via S-palmitoylation, while phosphorylation by the α isoform of protein kinase C (PKC) appears to release this association (Harris *et al.,* 2020). The involvement of PKC is particularly interesting in light of a study by Lorden *et al.* (2020). The group showed that APP-overexpressing AD mice models carrying mutations that increase catalytic activity of PKCα by up to 30% (M489V) exhibited severe cognitive decline. These mice had differential phosphorylation of multiple PKC substrates, particularly those involved in synaptic depression, with the phosphorylation of one particular protein also shown to be increased in the brains of AD patients (Lorden *et al.,* 2020). With APP processing shown to be enhanced in autophagic vacuoles (Tung *et al.,* 2012) and BCATc contributing to an increase in APP expression (see section 5.4.4.), we suggest these dysregulated pathways may contribute to PKCα overactivation and the AD pathology. Moreover, differential phosphorylation of BCATc as a result of PKCα mutations may contribute to its abnormal release from the cell membrane.

Together, these studies show that the implications for BCATc overexpression extend beyond its role in metabolic reprogramming to include tightly regulated cell death pathways such as autophagy, further substantiating its role as a moonlighting protein (Conway, 2021). This is important particularly in pathologies where nutrient dysregulation and insulin resistance dictate the rate of disease progression, such as T2DM and AD. For instance, it has previously been shown that autophagy-deficient mice present with reduced insulin secretion, consequently increasing β-cell degeneration and impairing glucose tolerance (Fujitani *et al.,* 2009). In addition, while aggregated proteins such as Aβ may be cleared via autophagy, dysregulation in autophagosome clearance may contribute to the generation of intracellular Aβ, once the machinery required for the generation of the peptide (such as APP components)

are all enhanced within the vacuoles (Tung *et al.,* 2012). Thus, it is vital to explore the changes in expression of AD-related proteins in the context of autophagy and BCATc dysregulation to understand the mechanisms underpinning the development of AD, as a result of T2DM or otherwise.

5.4.4. Increased expression of AD-related toxic peptides as a result of BCATc overexpression

Aβ and hyperphosphorylated tau have long been associated with the development and progression of AD (Serrano-Pozo *et al.,* 2011). Similarly, amyloidosis has been linked with T2DM, where deposits of amyloid fibrils were identified in the pancreatic islets of Langerhans leading to loss in insulin secreting β-cells (Ponce-Lopéz *et al.,* 2019). Whether accumulation of these toxic peptides is the cause or the outcome of the pathology of these diseases, however, remains debatable (Cooper and Zhang, 2010; Amtul, 2016). A previous study by Hull *et al.* (2015) showed that BCATc is significantly overexpressed in the brains of AD individuals, with a subsequent study by Harris *et al.* (2020) showing BCATc overexpression and thus autophagy dysregulation impacts on the expression of proteins and peptides such as APP, Aβ and hyperphosphorylated tau, likely contributing to disease pathology. In this project we showed that BCATc overexpression increases not only Aβ but also its precursor APP, as well as contributing to hyperphosphorylation of microtubule-associated protein tau particularly in phosphorylation sites Ser202/Thr205. In contrast, overexpression of the thiol mutants C335S and C338S led to a decrease in the expression of these proteins, including total tau.

The mechanisms dysregulated by changes in BCATc expression which may lead to protein aggregation and accumulation are multiple. Synthesis and aggregation

of intracellular Aβ for instance has been previously associated with impaired autophagy, where accumulation of autophagosomes overexpressing APP processing components has been observed (Tung *et al.,* 2012). Polis *et al.* (2019) showed that brain amyloidosis is significantly decreased when APP expression is downregulated, correlating with these findings (Polis *et al.,* 2019). Reduction of glycolysis intermediates such as dihydroxyacetone phosphate and phosphoenolpyruvate were also suggested to have positive correlation with $A\beta_{42}$ and $A\beta_{42/40}$ (Bergau *et al.,* 2019), complementing our results showing decreased glycolytic rate and dysregulated Aβ expression as a result of BCATc dysregulation. Moreover, Le Douce *et al.* (2020) demonstrated that glycolysis-derived L-serine was significantly decreased in 3xTg-AD mice (harbouring APP Swedish, MAPT P301L, and PSEN1 M146V transgenes), which reflected on reduced glycolytic flux and subsequent impairment of synaptic plasticity and memory (Le Douce *et al.,* 2020).

Aβ has also been indirectly implicated in tau hyperphosphorylation and subsequent development of neurofibrillary tangles (Blurton-Jones and LaFerla, 2006). Accumulation of extracellular Aβ plaques can contribute to ROS generation and signal synthesis and release of pro-inflammatory cytokines (IL1β, for instance) by surrounding microglia, which in turn upregulate kinases such as cyclin-dependent kinase 5 (CDK-5) and GSK-3β increasing tau phosphorylation, microtubule destabilisation and NFT formation (Barron *et al.,* 2017). Furthermore, Du *et al.* (2022) showed that increasing intracellular ROS levels correlate with formation of tau oligomers in the hippocampi of individuals with AD as well as transgenic mice, and that clearance of mitochondrial ROS significantly decrease tau oligomers *in vitro* (Du *et al.,* 2022). However, whether Aβ increase and tau hyperphosphorylation occur as a direct result of BCATc overexpression, or indirectly via mechanisms which are disrupted by the BCATc protein, requires further elucidation.

5.4.5. BCATc overexpression significantly decreases neuronal viability although BCAAs may aid cell death rescue

Neuronal viability as a result of overexpression of BCATc wild type and thiol mutants, supplementation with high levels of glucose or BCAAs, was significantly decreased. While neuronal death is an important process to remove abnormal and potentially harmful cells in order to maintain normal brain function and homeostasis, uncontrolled neuronal loss in specific parts of the brain, such as the hippocampus and frontotemporal lobe, leads to brain atrophy and is a key feature in neurodegenerative diseases such as AD (Moujalled *et al.*, 2021). This neuronal loss observed in AD pathology is often associated with the accumulation of extracellular senile plaques of Aβ, which can lead to cytotoxicity involving increased intracellular Ca^{2+}, mitochondrial impairment, ROS generation and oxidative damage, and activation of multiple pathways involved in cell death (Niikura *et al.,* 2006). However, recent work suggests that Aβ oligomers as opposed to fibrils are the most neurotoxic, contributing to an increase in extra synaptic glutamate levels and NMDAR excitotoxicity, ultimately inhibiting LTP particularly in the hippocampal area and inducing mechanisms involved in LTD (Aleksis *et al.,* 2017). Similarly, hyperphosphorylated tau has also been linked to exacerbated neurodegeneration by polymerising into paired helical filaments and NFTs, which increase neurotoxicity thus enabling neuronal death (Gong and Iqbal, 2008). We have previously shown that BCATc overexpression significantly increases generation of ROS and the expression of APP, Aβ and hyperphosphorylated tau, which could suggest multiple mechanism through which neuronal loss is triggered.

In addition to BCATc, our results showed that glucose and BCAAs alone significantly decrease neuronal viability, although BCAA to a much lesser extent. Chronic exposure to high levels of glucose has been associated with neuronal

damage and is one of the primary concerns in diabetic neuropathy, which presents in approximately 60% of patients with T2DM (Zhou *et al.,* 2019). Similarly, high levels of BCAAs have been shown to contribute to ROS generation, a key mechanism in inducing cell death (Zhenyukh *et al.,* 2017). We have observed that a combination of glucose and BCATc overexpression further decreased viability. Considering the high levels of glucose our data has previously shown when BCATc was overexpressed (see Chapter 4), it is not surprising that the further addition of glucose would exacerbate neuronal death. In contrast, BCAA supplementation decreased viability to a much lesser extent, and in the case of BCATc and C338S it did ameliorate the cell death observed. This has been previously observed in mice models of retinal degeneration and glaucoma, where Hasegawa *et al.* (2018) showed that treatments with BCAAs, but not glucose, increased ATP levels and attenuated neurodegeneration (Hasegawa *et al.,* 2018). This suggests that BCAA supplementation may counteract the effects of BCATc overexpression likely by stimulating its transamination role and ultimately increasing ATP, while hyperglycaemia exacerbates neuronal death as a result of the dysregulation in the glycolytic pathway following BCATc overexpression.

To conclude, the results in this chapter showed that BCATc dysregulation impacts on ROS turnover, the autophagy mechanism and subsequent aggregation of toxic peptides, which ultimately lead to neuronal death. This provides further evidence to understand the moonlighting roles of the BCATc protein, suggesting these might be much greater than currently supposed (Conway *et al.,* 2021).

Chapter 6

Summary of findings and future work

6.1. Summary of findings

Chronic hyperglycaemia, high levels of fatty acids and dysregulated BCAA metabolism are key features in the progression of T2DM (Boden, 2003; Karmi *et al.,* 2010; Campos, 2012). The transaminase in BCAA metabolism, BCAT, has been shown to be overexpressed in AD individuals (Hull *et al.,* 2015), with more recent studies establishing an association between increased levels of the aforementioned metabolites and its neurotoxicity-inducing properties which impact in the development of AD (Schönfeld and Reiser, 2017; Tournissac *et al.,* 2018; Siddik *et al.,* 2019; Zhang, *et al.,* 2021). Although the exact cause of BCATc overexpression in AD remains unclear, we suggest that it is a consequence of chronically increased concentrations of BCAAs such as what is observed in T2DM, with subsequent overexpression disrupting energy metabolism and further exacerbating the already damaging oxidative stress.

In the proposed model, the neuronal cells studied would reflect the sustained damage as a result of chronic intake and/or exposure to BCAAs (Figure 34). Under pathological conditions such as T2DM, increased concentrations of circulating BCAAs would contribute to the dysregulation of several cellular processes, all of which may contribute to oxidative stress and cellular death. These include, but are not limited to, decreasing levels of Acetyl-CoA, product of BCAA catabolism, β-oxidation of fatty acids and glycolysis, and substrate for the TCA cycle and fatty acid synthesis; decrease in neurotransmitter synthesis, release and uptake; impairment in the uptake of ArAAs, histidine and methionine, for instance as a result of SCLS75/LAT1 transporter specificity (Scalise *et al.,* 2018); increase in glucose and fructose levels by contributing to insulin resistance, thus increasing hyperglycaemia and subsequently the development of AGEs (Karusheva *et al.,* 2019); and increase in fatty acid synthesis (while decreasing β-oxidation) (Crown *et al.,* 2015).

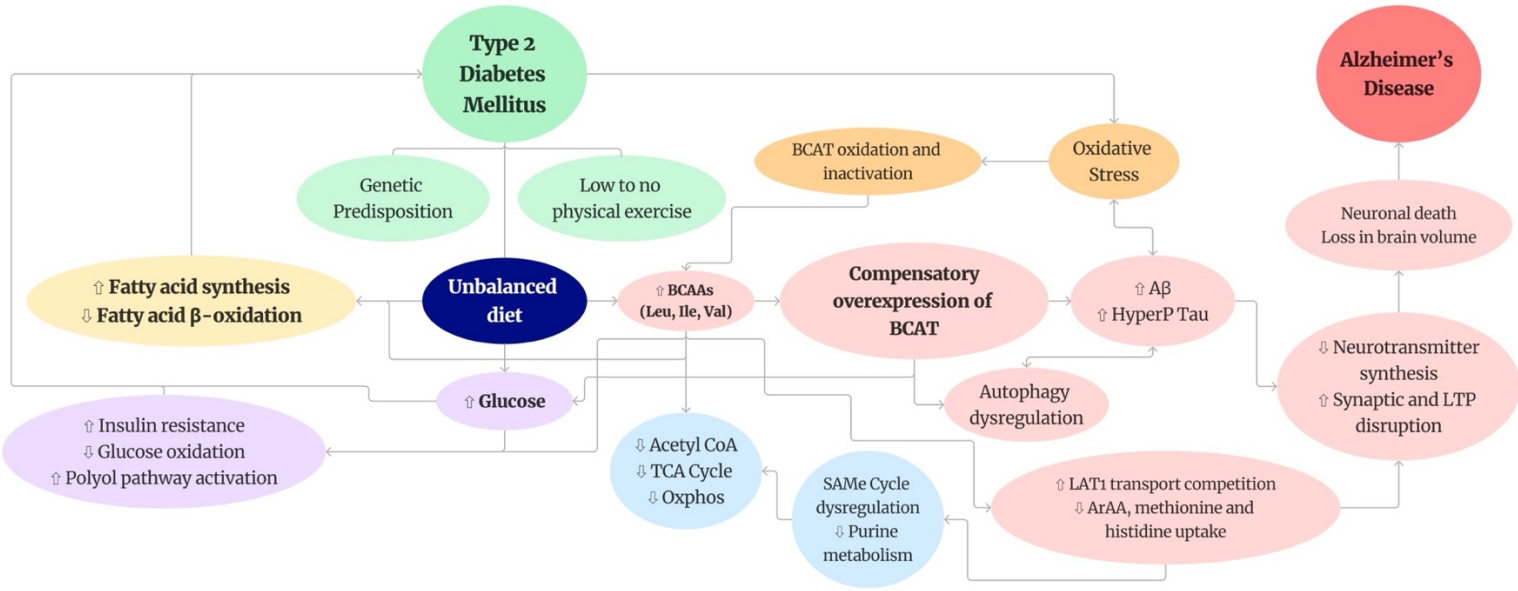

Figure 34. Diet as a proposed central point for metabolic dysregulation, T2DM and the development of AD. Significant increases in carbohydrates, proteins and fats can lead to otherwise vital compounds such as glucose, BCAAs and fatty acids having harmful effects. For instance, the development of T2DM relies significantly on metabolic dysregulation, with factors such as genetic predisposition and low to no physical exercise further contributing to its pathology. Increasing levels of BCAAs can also lead to the overexpression of its transaminase, the BCAT protein, which this work has shown links to glycolysis, autophagy regulation, and upregulation of toxic peptides Aβ and hyperphosphorylated tau. Aggregation of these peptides can contribute to oxidative stress, which in turn can oxidise and inactivate the BCAT protein and further increase BCAA availability. Dysregulation in BCAA metabolism can increase insulin resistance and fatty acid synthesis while decreasing acetyl CoA, the TCA cycle and oxidative phosphorylation, as well as the aromatic amino acid pool in the brain and thus neurotransmitter synthesis. These, combined with aggregation of toxic peptides, can disrupt neurotransmitter synthesis and LTP formation, contributing to neuronal loss and the development of AD.

BCAAs would stimulate BCATc synthesis in order to sustain its increased catabolic demand, and its overexpression under a highly oxidised environment would lead to protein oxidation and thus inactivation, impacting on downstream energy pathways by either decreasing the products of BCAA catabolism (i.e., Acetyl-CoA) or directly impacting on the activity of enzymes in individual pathways, as observed in chapter 4. Wang *et al.* (2019) observed that accumulation of BCKAs, by-product of BCAA catabolism, significantly impact on TCA cycle and oxidative phosphorylation in hepatocyte mitochondria, further supporting the role of BCAT in regulating those pathways (Wang *et al.*, 2019).

Saturation of the glycolytic pathway and further accumulation of glucose in the first instance have numerous consequences within the cell, from increase in advanced glycation end products (AGEs), to generation of reactive oxygen species (i.e., H_2O_2 and O_2^-) and thus oxidative damage, particularly in the pathogenesis of T2DM and AD (Murphy and LeVine, 2010). BCATc overexpression was found to significantly increase the levels of glucose, most likely via disruption of the glycolytic pathway by binding key enzymes such as GAPDH and enolase. Changes in the redox status of BCATc however, such as removal of either one of its reactive cysteines, while still impacting on the glycolytic rate (but rather increasing GAPDH activity) affect additional pathways such as the TCA cycle and oxidative phosphorylation. This shows that the protein's thiols are vital for protein-protein interaction and influence its binding partners, similar to what has been suggested by Conway *et al.* (unpublished observations).

Moreover, regulation of the autophagy mechanism, which if impaired has been shown to contribute to the aggregation of misfolded proteins and lead to an increase in oxidative stress (Di Meco *et al.,* 2020), is highly dependent upon dietary control - an inconstant in individuals with T2DM (Gao *et al.,* 2015). The results in

Chapter 5, alongside data shown by Harris *et al.* (2020), highlighted the potential outcomes of dysregulation in autophagy turnover as a result of BCATc overexpression, such as accumulation of toxic APP, Aβ and hyperphosphorylated tau aggregates, hallmarks in the pathogenesis of AD. In addition, BCATc overexpression also increased generation of H_2O_2 in the cytosol and mitochondria, and decreased expression of antioxidant proteins such as TRX, GRX, SOD and PRX. Knock down models of the protein showed the opposite with a decrease in ROS and increase in antioxidant proteins, validating the experimental results. This increase in oxidative stress and decrease in the antioxidant system, alongside AGEs formation, may contribute to the development of intra- and extra-cellular protein aggregates by disruption of microtubule assembly (tau), induction of an inflammatory response and driving clearance via the autophagy pathway (Aβ) (Barron *et al.,* 2017; Du *et al.,* 2022).

Collectively, these results offer extensive evidence to suggest that the redox-dependent BCATc protein plays a key role in metabolic reprogramming in neuronal models, disrupting energy pathways such as glycolysis and the TCA cycle by binding to and altering the activity of its enzymes and thus the availability of its metabolites. This culminates in redox imbalance, accumulation of toxic peptides and ultimately neuronal death. The data collected and discussed therefore support the central hypothesis in this project, having met the aims set to be investigated. It evidences that from lifestyle and dietary choices culminating to the development of metabolic conditions such as T2DM, the likelihood of progression to neurodegenerative conditions such as Alzheimer's disease can be overwhelming. The people involved in this project hope to be able to contribute further to the understanding of the molecular and biochemical mechanisms involved in the pathologies of T2DM and AD, potentially offering insights into measures necessary to prevent their development altogether.

6.1. Limitations

- Untargeted metabolomics was performed in undifferentiated SH-SY5Y neuronal models, which express both the mitochondrial and cytosolic isoforms of the BCAT protein. We suggest BCATm may be compensating for BCATc particularly in knock down models, although it would contribute to exacerbate overall BCAT overexpression in these models. In future experiments, cells will be differentiated into dopaminergic neuronal models and metabolite load analysed under BCATc dysregulation, including knockdown and thiol mutants.
- The activity of the BCAT thiol mutants has been extensively assessed in earlier *in vitro* protein studies by our research group (Conway *et al.,* 2002; Conway *et al.,* 2004; Conway *et al.,* 2008). However, whether the activity of these mutants remains unchanged in biological system such as neuronal models has not been established. Future experiments will include activity assessment using concentrations of pure protein and H_2O_2 as oxidation substrate.
- The development of the BCATm thiol mutants in CMV vector could not be performed although a similar protocol to BCATc was used. This will be addressed in future work in order to fully develop and validate the thiol mutants of both isoforms of the BCAT protein, to achieve successful transfection and characterisation in cell lines.

6.2. Future work

- With the association of BCAA metabolism proteins and metabolites, such as BCKD and its substrates BCKAs, with insulin resistance, the abundance of these compounds will be assessed in further metabolomics and biochemical studies following insulin treatments in neuronal models. BCKD expression and BCKA levels will also be analysed under BCATc dysregulation using biochemical and proteomics analyses.
- Additional enzymes of the glycolytic pathway such as hexokinase and phosphofructokinase will be assessed, alongside polyol pathway enzymes aldose reductase and sorbitol dehydrogenase. This may provide further evidence of disruption not only in glycolysis but also the polyol pathway, which if impaired is likewise harmful impacting on osmotic pressure, and ROS and AGEs generation.
- Overall protein aggregation and AGEs will also be assessed following overexpression and knockdown of the BCATc will type and thiol mutants in our neuronal models. These will include metabolomics and biochemical analyses, with potential for molecular studies.
- We will explore TRX overexpression plasmids to assess whether this would rescue ROS following BCATc overexpression. In addition, as PRX levels may increase levels of H_2O_2, PRX expression and dimerisation will be analysed under normal physiological and BCATc-dysregulated conditions. Analysis of ROS using the Hyper molecular probe will be performed.

6.3. Appendix

6.3.1. BCATc overexpression vector, from section 2.2.3.

According to Harris (2016), Chapter 3, title 'Overexpression of hBCAT', subheading 'Creating the hBCATc expression vector', the OE vectors utilised in the experiments outlined in this thesis were developed as follows: '*The recombinant hBCATc/m gene was synthesised into the pENTR221 Gateway® entry vector and transformed into competent E.coli by GeneArt®. This plasmid was recombined into a Gateway® pDEST™26 (containing an N-terminal 6X His tag) for human cell expression using the LR Clonase® II enzyme mix. Blue/white colonies were visualised after 24 hours for the pUC19 plates, where white colonies were positive transformants containing the pDEST™26 vector along with the hBCATc/m gene. The transformation efficiency was calculated to be 6.72 x 107 CFU/µg. Validation of the hBCATc/m expression vector was tested using the PacI and AscI restriction enzymes and separation on a 0.8% agarose gel […]. Restriction digest of the expression vector was used to confirm that the vector contained the hBCATc gene along with the specific restriction sites*.'

6.4. References

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