Chapter 6

Synopsis, conclusion, and future work

Contents

6.0 – SYNOPSIS AND CONCLUSION.

This study had three main objectives: i) Synthesis of an hBCATm inhibitor, ii) Development of knock-down models for hBCAT, iii) Modelling of brain vascular tissue; and the elements combined with the overall aim of determining the effect of dysregulated hBCATm in cerebral tissues. It was hypothesised in this thesis that expression of hBCATm would affect expression of other metabolic proteins including BCKD and GDH. For the first time we show that BCKDHA and AUH, but not GDH protein levels are correlated with hBCATm expression in neuronal cells (**Chapter 4.3**). Evidence in support of an hBCATm/BCKD/GDH metabolon to control L-glutamate synthesis is described, which has implications for neurotransmission and production of downstream metabolites and redox molecules. As the previously proposed models of the BCAT-glutamate-glutamine cycle (Yudkoff *et al.*, 1996; Hutson *et al.*, 1998) are based around rat models, an amendment was proposed by Hull *et al.* (2012) to include a shuttling of metabolites in humans between endothelial cells, astrocytes, and neurons. Furthermore, it was previously hypothesised that GDH may be vital for the cycle (Rothman *et al.*, 2012), and it was demonstrated for the first time in this thesis that GDH is regulated by L-glutamate concentration in a hBCATm expression dependent manner (**Figure 4.16**).

Using the data from this thesis an update on the cycle is proposed (**Figure 6.1**). It is hypothesised that as hBCATc is upregulated in response to oxidative stress (Harding *et al.*, 2003) that the primary role of the enzyme in human neurons is L-glutamate synthesis directly for glutathione production, potentially in a separate L-glutamate compartment. Indeed, both hBCATc and glutathione synthase have been mapped to the neuronal soma and proximal dendrites, rather than the axon of neuronal cells (Hull et al., 2012; Robinson *et al.*, 2000) suggesting that L-glutamate synthesised by hBCATc is not used for neurotransmission. Neuronal increases in BCATc expression are associated with cell survival (Kholodilov *et al.*, 2000) which may be the result of this increased downstream glutathione synthesis. This hypothesis is dependent on production of L-glutamate in astrocytes originating from KG, however, both

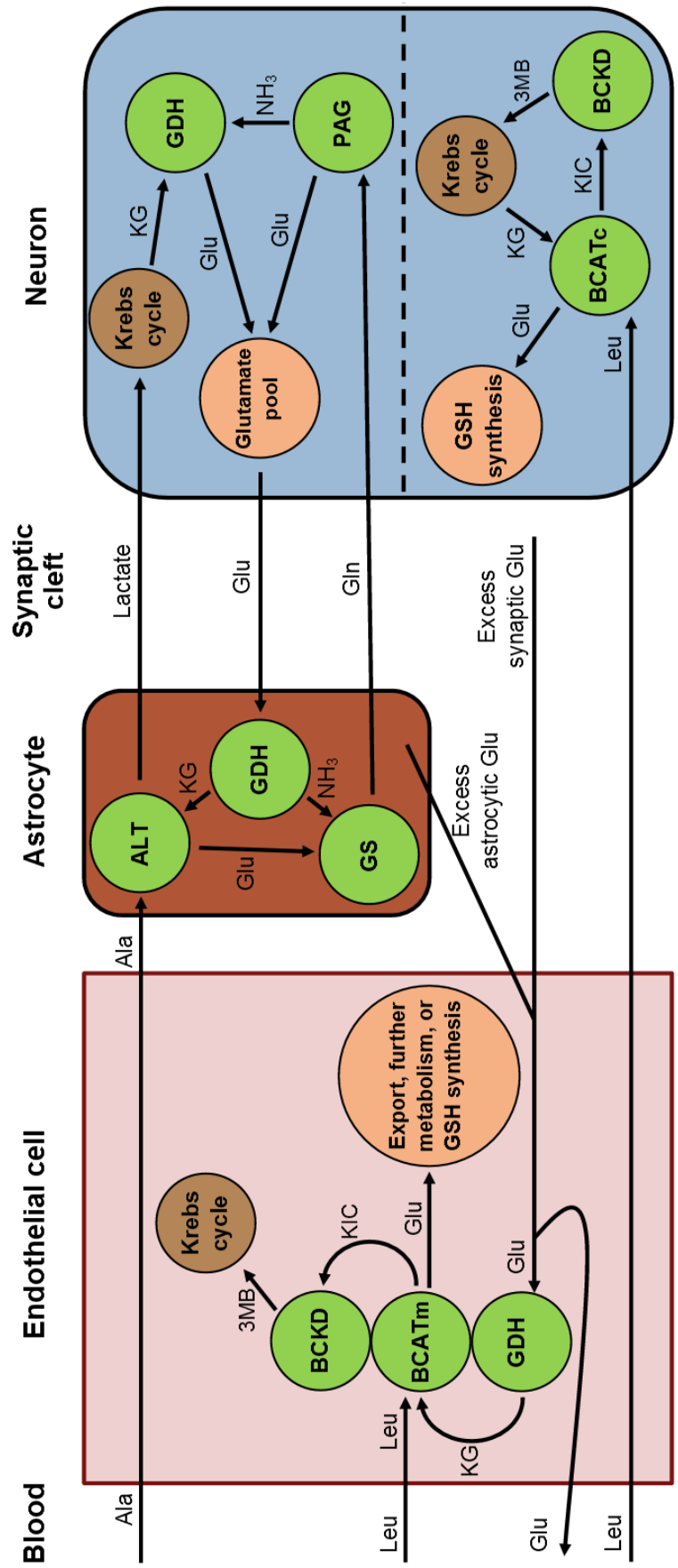

***Figure 6.1* – A new proposed hBCAT-glutamate-glutamine cycle incorporating GDH.** A new cycle is proposed as an expansion to that hypothesised by Hull *et al.* (2012), based on observations within this thesis. The L-glutamate in neurons can be added to a L-glutamate pool and utilised for neurotransmission, as an energy source, or for glutathione synthesis. As hBCATc is upregulated by oxidative stress (Harding *et al.*, 2013) it is proposed that the enzyme serves to provide L-glutamate primarily for glutathione synthesis in a separate compartment. **Abbreviations:** 3MB – 3-methylbutanoyl-CoA. Ala – L-alanine. ALT – Alanine transaminase. BCKD – Branched-chain α-keto acid dehydrogenase. GDH – Glutamate dehydrogenase. Gln – L-glutamine. Glu – L-glutamate. GS – Glutamine synthase. GSH – Glutathione. KG – α-ketoglutarate. KIC – α-ketoisocaproate. Leu – L-leucine. PAG – Phosphate-activated glutaminase.

ALT and AST are well described as contributors to the human glutamate-glutamine cycle in these cells (Rothman *et al.*, 2012; Waagepetersen *et al.*, 2000). Indeed, kinetic data recently demonstrated that the rate of catalysis of the hBCAT, ALT, AST, and GDH enzymes individually is too slow to account for the rapid cerebral nitrogen turnover, and it is likely that several of these enzymes work in tandem (Rothman *et al.*, 2012).

In the endothelial cells, hBCATm is proposed to have several roles as previously discussed (**Chapter 4.4.3**). Briefly, under healthy conditions, BCAA metabolism may contribute to increased energy demands of endothelial cells through ketogenesis, while allowing for L-glutamate to contribute to the glutamate-glutamine cycle or become itself utilised as an energy source (**Figure 4.28**). Alternatively, under oxidative stress, L-glutamate is consumed for glutathione synthesis, while increased BCKA concentrations alter neuronal metabolic pathways in a manner yet undetermined, which has been shown to increase resistance to oxidative stress (Kim *et al.*, 2007). Indeed, synthesis of glutathione is inducible in vascular endothelial cells (Urata *et al.*, 2009). It is proposed that L-glutamate synthesis, as opposed to degradation, is favoured by hBCATm in the endothelial cells for three reasons. Firstly, degradation would require an accumulation of BCKA substrate, however, monocarboxylate transporter 1 (Mct1) favours transport through the BBB and accumulation within neurons (Conn & Steele, 1982; Conn *et al.*, 1983; Mac & Nałecz, 2003; Mac *et al.*, 2000). Secondly, while L-glutamate may be uptaken in the astrocytes and oxidised to KG (McKenna *et al.*, 1996), hBCATm is markedly absent from the astrocytes (Hull *et al.*, 2012). In order for hBCATm to contribute to brain L-glutamate oxidation it must instead be uptaken by the endothelial cells of the blood-brain barrier, however, it is well described that L-glutamate is readily effluxed by endothelial EAATs and that efflux may be increased as an adaptive measure to increased brain interstitial fluid L-glutamate concentrations (Reviewed by Teichberg *et al.*, 2009). In this case, the contribution of hBCATm to L-glutamate oxidation may be minor. Finally, knock-down of hBCATm was demonstrated to decrease glutathione concentration (**Figure 4.19**). The SHSY-5Y cell line is a neuroblastoma cell line, and

oxidative stress is an intrinsic aspect of cancerous cells (Hileman *et al.*, 2003; Kumar *et al.*, 2008). Here, knock-down of hBCATm may have directly decreased glutathione synthesis in oxidatively stressed cells, leading to the observed depletion (**Figure 4.19**). Further evidence is required to accept this hypothesis, primarily through using [^{15}N]BCAA tracer studies in human endothelial cells to measure accumulation of [^{15}N]L-glutamate and subsequently [^{15}N]-radiolabelled glutathione.

It was also found in this study that GRx and TRx were regulated by hBCATm knock-down, suggesting that hBCATm may be involved in activation of the ARE. While similar to AD, PDI only appears to receive a post-translational modification and expression is not impacted (Kim *et al.*, 2000; Uehara *et al.*, 2006). The implication is that TRx and GRx are overexpressed in response to oxidative stress of the endothelial cells, where they may be utilised to repair oxidised endothelial proteins or exported into the cerebrospinal fluid (CSF) along with glutathione to support the whole brain redox environment (Arodin *et al.*, 2014).

In relation to this, a two-hit hypothesis of AD has been proposed by Zlokovic (2011) (**Figure 1.11**) whereby a combination of both cerebrovascular permeability and of neuronal death are required for progression of the disease state. As AD is a complex and multifaceted disease this hypothesis may only be a part of the pathology, however, there is scope to integrate some of the findings on hBCATm expression from this thesis. The two-hit hypothesis proposes that early in the disease, vascular insult causes an influx of peripheral Aβ into the brain which can damage both endothelial cells and neurons, leading to further vascular insult and neuronal death. The vascular insult leads to increased BBB permeability, allowing increased entry of peripheral Aβ into the brain, as well as hypoperfusion and neuronal injury (Reviewed by Zlokovic, 2011).

In this thesis, it is hypothesised that Aβ signals upregulation of hBCATm in the endothelial cells, which acts as a messenger for upregulation of ARE promoted proteins to support cells and to increase glutathione synthesis (**Figure 6.2**). The ARE is already

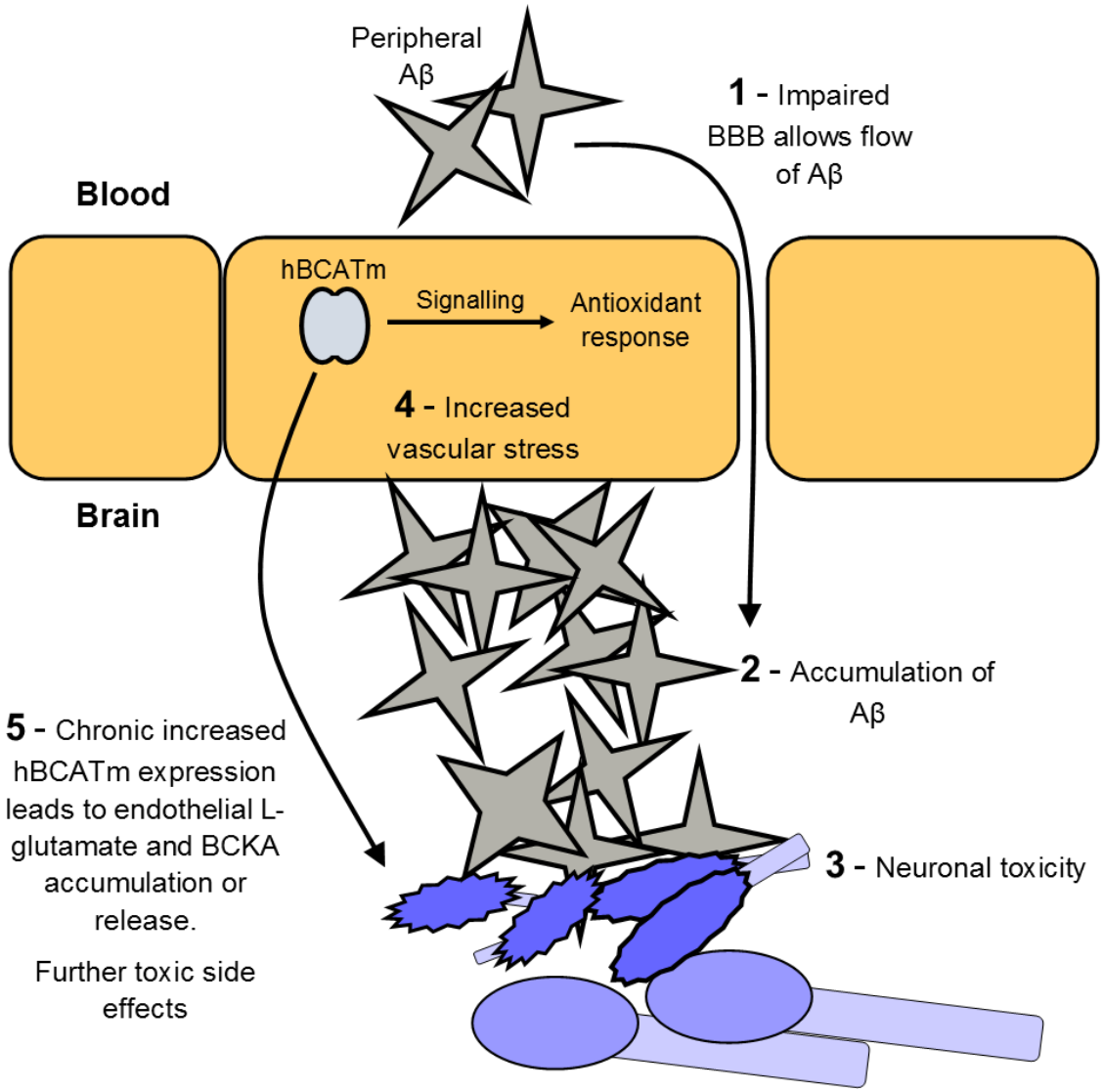

***Figure 6.2* – Vascular stress in AD pathogenesis.** It has been hypothesised that in the early stages of AD, cerebrovascular dysfunction and BBB impairment precedes neuronal pathology (Zlokovic, 2011). Increased BBB premability leads to an influx of Aβ which can damage both neurons, and endothelial cells – compounding the pathology. In this thesis, it was found that hBCATm can regulate expression of antioxidant proteins and molecules, potentially through modification of PDI, while a previous study found that hBCATm is overexpressed in AD in correlation with Braak staging (Hull *et al.*, 2015). It is therefore proposed that overexpression of hBCATm is a response to oxidative stress, and upregulated as a countermeasure. However, chronic stress and hBCATm overexpression may lead to release of excitotoxic concentration of L-glutamate and BCKAs as the system becomes overwhelmed.

well described in this capacity as protective in brain vascular disorder (Wei *et al.*, 2011; Zhao *et al.*, 2007; Wang *et al.*, 2007). However, the implications of chronic upregulation are unknown and it has been hypothesised by Hull *et al.* (2015) that overproduction of L-glutamate could result in the release of excitotoxic concentrations of the neurotransmitter, as well as toxic BCKAs. When this occurs the synthesis of L-glutamate exceeds the synthesis of glutathione, causing an accumulation of L-glutamate. This may further exasperate neuronal stress present from Aβ accumulation, leading to cell death through slow excitotoxicity (**Chapter 1.8.2**). Alternatively, the antioxidant promoting aspects of hBCAT may become overwhelmed and no longer have an impact. Additionally, migration of immune cells to aid clearance of Aβ may result in release of cytokines which can lead to further vascular permeability (Martin *et al.*, 1988; Halle *et al.*, 2008). Recent human trials of antibody for Aβ for AD treatment have failed, which may due to the immune inflammatory response and an impact on BBB permeability (Nicoll *et al.*, 2003; Ferrer *et al.*, 2006).

Finally, this provides a point to integrate the three aims of the study. It is considered here that hBCATm is neuroprotective in the initial stages of the disease, but either becomes overwhelmed or pathological through chronic increased activity. If it were possible to inhibit L-glutamate synthesis by hBCAT, while retaining the ARE signalling aspect then this could prevent the 'second hit' of the two hit hypothesis and thus progression of AD. It is proposed that treatment with an hBCATm specific inhibitor could prevent the pathogenic accumulation of L-glutamate. Alternatively, in this thesis it has been proposed that hBCATm contributes to the antioxidant response by providing substrate for glutathione synthesis and signalling activation of the ARE. Knock-down of hBCATm prevents both direct interaction of hBCATm with other proteins, and also transamination. Specific chemical inhibition of hBCATm transamination would decrease the contribution to glutathione synthesis, while still allowing protein-protein interactions which may signal ARE activation. It may be possible to test the hypothesis that hBCAT contributes to glutathione synthesis by treating endothelial or neuronal cells with either

benzofenac or gabapentin, respectively, and also H_2O_2 to induce an oxidative stress response. If this signalled an increase in ARE activation, but not glutathione synthesis, then it can be accepted that hBCAT is a significant donor of L-glutamate for glutathione synthesis. However, as previously described, adequate L-glutamate concentration is required for neuronal repair from insult (Ikonomidou & Turski, 2002), placing a requirement for precise control of inhibition as treatment of the hBCAT inhibitor could result in pathological L-glutamate depletion.

To conclude, evidence has been provided for hBCATm regulation of anapleurosis, L-glutamate production, glutathione synthesis, and redox chaperone expression. We hypothesise that hBCATm is upregulated in response to cerebrovascular insult, and that hBCATm is chronically overexpressed in AD because of sustained insult (Hull *et al.*, 2015). Using the evidence available it is proposed that the upregulation is a double-edged sword, resulting in upregulation of antioxidant proteins and glutathione, but also excitotoxic concentrations of L-glutamate and α-keto acids over a sustained period. It is believed that treatment with an hBCATm aminotransferase inhibitor could attenuate the pathological aspect, and thus could be a treatment for brain vascular disorder and AD. Although, further evidence for this is required, many of the tools for this further work been developed in this study, providing a strong foundation to build upon.

6.1 – FUTURE WORK.

This thesis provides evidence that hBCATm has a vital role in regulation of the Krebs cycle, L-glutamate synthesis, and redox status by regulating expression of other key proteins and molecules. A new hypothesis has been proposed that hBCATm is an important protein in resistance to oxidative stress through induction of anabolic signals, protein-protein interactions, and glutathione synthesis. To further investigate this, the following experiments and projects could be considered:

- Development of the BOPAA family compounds by further structure-activity relationship studies. Suggested modifications of the compound include:
    - Reintroduction of the 2-hydroxylphenylacetic acid pharmacophore to ring 1.
    - Substitution of the carboxylic acid group of ring 1 to a tetrazole bioisostere.
    - Substitution of the linking ether for an alkyl, ester, amide, or thioester group.
    - Substitution of the 3-position group on ring 1 to larger groups containing a hydrogen bond acceptor.
    - Extension of the 4-methyl group of ring 2 to ethyl or propyl groups.
    - Changing the position of the methyl group of ring 2.
    - Introducing function groups to improve aqueous solubility.

- Using the hBCATm inhibitors to evaluate if there is a different cellular impact between inhibition or knock-down. This would determine if downstream effects are a function of hBCATm transamination activity, or a different protein interaction.

- The impact of hBCAT expressional changes on amino acid and α-keto acid concentrations, both inter and extracellularly. This could be evaluated using a variety of chromatography techniques such as HPLC and LC-MS and by using radiolabelled substrates. In particular:

    - The impact of neuronal hBCATc overexpression, knock-down, or inhibition on L-glutamate concentration.

  - The effect of endothelial hBCATm overexpression, knock-down, or inhibition on L-glutamate concentration.
  - The effect of starvation, L-leucine or L-glutamate treatments on these cell models.
  - Transmission of hBCAT metabolites in a co-culture model, particularly when hBCAT is knocked-down, overexpressed, or inhibited.
  - Concentrations of glutathione in response to inhibition of either hBCAT isoform and oxidative stress.

- Further optimisation of the human brain microvascular co-culture model, particularly of a tri-culture. In addition, evaluation of the impact of hBCAT expression or inhibition on blood-brain barrier integrity.

- Further characterisation of the effect of hBCAT on cellular redox status. In this study hBCAT clearly has an impact on the expression of redox chaperones and redox molecules. Several experiments are proposed:

  - Pull-out of PDI protein to identify post-translational modification in response to hBCATm expression.
  - Mass spectrum analysis of the two bands observed on western blot analysis of PDI.
  - qPCR microarray analysis over a wide range of redox genes to identify other downstream effects.
  - A reporter assay to determine if hBCATm expression enhances transcription from the antioxidant response element.
  - Far western blotting and pull-out studies to establish if hBCATm associates with Nrf2 or PERK as downstream modulators of the ISR pathway.

- Protein mapping studies of ALT in the human brain. This has implications for ketone body metabolism, as well as nitrogen cycling for the glutamate-glutamine cycle.