Chapter 3

Overexpression of hBCAT

3.1 - Introduction:

Due to the increase in hBCAT expression previously shown in the AD brain compared to matched controls, a neuronal cell model using a protein overexpression system would facilitate a means of observing the impact of hBCAT overexpression on cell signalling pathways, in particular mTOR and autophagy (Hull *et al.,* 2015). Overexpression of hBCAT in neuronal cells was developed in order to study gene function and regulation, and also protein function. An ideal transfection method should have high transfection efficiency, low cell toxicity, minimal effects on cell physiology, and be both easy to use and reproducible (Kim and Eberwine, 2010). For these reasons, a chemical method of transfection, using cationic lipids, was chosen. This was the first method successfully used to introduce foreign genes into mammalian cells, and is also the most widely used (Schenborn and Goiffon, 2000; Holmen *et al.,* 1995; Washbourne and McAllister, 2002).

The principle of this method is that the positively charged chemical, jetPRIME, forms a complex with the negatively charged nucleic acids. These positively charged complexes are then attracted to the negatively charged cell membrane where they are incorporated into the cell via endocytosis (Polyplus-transfection, 2015). The transfected DNA is then delivered to the nucleus where it is expressed and results in protein translation. This method is dependent on the nucleic acid/chemical ratio, solution pH and cell membrane conditions. Thus, the transfection efficiency is low compared to other methods such as viral transfection, however there is relatively low cytotoxicity, no mutagenesis or extra-carrying DNA, and there is no size limit on the plasmid (Kim and Eberwine, 2010).

The nucleotide sequence for hBCAT was optimised for mammalian cell expression, i.e. for codon usage, and the cDNA was synthesised and cloned by GeneArt® into a Gateway® entry vector pENTR™221 (Invitrogen). Gateway technology enabled efficient transfer of the hBCAT gene from an entry vector into a

mammalian destination vector pDEST™26, using site-specific 'LR' recombination properties of bacteriophage λ and specific recombination proteins that comprise LR Clonase® II (Landy, 1989). Bacteriaphage λ can grow as a lytic phage in *E.coli*, which can cause the bacteria to lyse and release the viral progeny. However, λ can also integrate site specifically into the *E. coli* genome by lysogenisation, the process allows the viral genome to be transferred to daughter cells until conditions trigger the phage to release from the genome (Ptashne, 1992). The lysogenic phage property is the basis of the Gateway™ cloning system. 'LR' recombination reaction is mediated by LR clonase enzyme mix, which contains intergrase (Int), excisionase (Xis), integration host factor (IHF), the specific recombination (att) sites found on the entry vector and destination vectors that allow the efficient transfer of the gene of interest from the entry vector to the destination vector (Figure 3.1.1).

The hBCAT gene in the Gateway® entry vector is flanked by attL1 and attL2 (100 bp each). The destination vector (pDEST™26) is also flanked by attR1 and attR2 (125 bp each) recombination sites. The orientation of the gene is maintained throughout the recombination reaction (transfer of the gene from the entry to the destination vector) as attL1 reacts only with attR1 and attL2 reacts with attR2. This recombination produces an expression vector, containing the hBCAT gene in the destination vector, which will allow the regulatory elements of human cytomegalovirus (CMV) to promote overexpression of the protein. CMV allows efficient, high-level expression of the recombinant protein, in this case hBCAT, in mammalian cells (Nelson *et al.,* 1987). The expression vector also contains an N-terminal 6X His tag for detection and purification of recombinant proteins, the pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*, the ccdB gene for negative selection after the LR recombination, a T7 promoter for *in vitro* transcription, and ampicillin resistance for gene selection in *E. coli*.

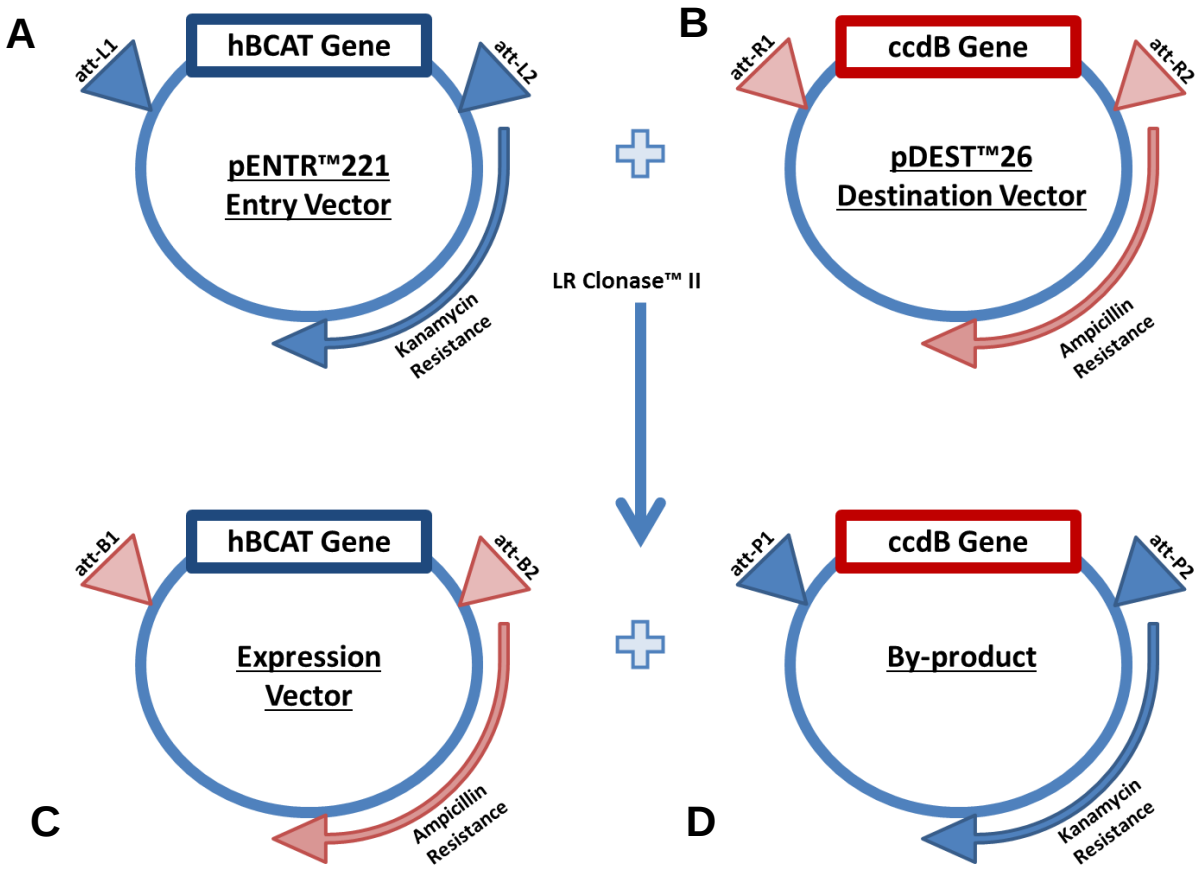

Figure 3.1.1 - LR Recombination reaction between hBCAT entry and destination vector. hBCAT entry vector supplied by GeneArt is flanked by a recombination specific site attL1 and attL2 (A). These sites recombine with attR1 and attR2 sites on the destination vector pDEST28 (B) in the presence of LR Clonase II enzyme mix. This allows efficient transfer of the hBCAT gene from the entry vector into an expression vector donated by the destination vector (C). This reaction produces a non-viable by-product plasmid that does not give any colonies due to the presence of the ccdB toxic gene.

3.2 - Specific aims:

- **Specific aim 1:** Optimise the transfection procedure of the neuroblastoma cell line IMR-32
- **Specific aim 2:** Develop and optimise mammalian overexpression plasmid for hBCATm and hBCATc with a 6X His-tag
- **Specific aim 3:** Optimise transfection of SH-SY5Y cells with both hBCATc and hBCATm overexpression plasmids

3.3 - Results:

hBCAT has been shown to be significantly up-regulated in AD (Hull *et al.,* 2015). Therefore, to evaluate the role of increased hBCAT, the design of a cell model using hBCAT overexpression is required. To enable confirmation of successful transfection of the neuronal cell line, an easily detectable control plasmid was required. eGFP vectors (pcDNA3-EGFP and pHygEGFP) do not contain the hBCAT gene but will enable optimisation of the transfection procedure as they are easily detectable due to fluorescence at 509 nm. After optimisation this vector can be used alongside future hBCAT transfections as a transfection control.

3.3.1 - Transfection with eGFP using the pcDNA3-EGFP vector.

To assess the transfection efficiency of IMR-32 cells using jetPRIME® transfection reagent, pcDNA3-EGFP and pHygEGFP vectors were isolated on LB plates containing 100 µg/mL ampicillin. The eGFP can be seen to fluoresce due to excitation at 490 nm and the emission peak at 509 nm (Figure 3.3.1). The vectors were then purified by mini-prep and separated on a 0.8% agarose gel by electrophoresis (Figure 3.3.2). The lower, brighter band shown in lanes A-D between 2.5 kb and 3 kb represents the supercoiled form of each of the plasmids which is approximately half the size of the open circular form found at approximately 6 kb. The open circular plasmid corresponds to the actual size of the synthesised plasmid; 6.16 kb for pcDNA3-EGFP and 5.8 kb for pHygEGFP. The absence of any bands in lane E demonstrates that the water used for sample preparation is DNA/RNA free and is not responsible for any sample contamination. Due to the extra bands in lane C, which are indicative of nicks in the plasmid or sample contamination, the pHygEGFP vector was not suitable for transfection. The pcDNA3-EGFP plasmid, with no additional bands in either lane A or B, was subsequently used for IMR-32 cell transfection.

The purified pcDNA3-EGFP DNA was transfected into confluent IMR-32 cells using jetPRIME® transfection reagent and incubated at 37°C for various time points. After 24 hours, the transfected cells were expressing the eGFP as demonstrated under the CY2 filter in a gel doc (Figure 3.3.3). In order to quantitatively measure the transfection efficiency, flow cytometry analysis was used whereby approximate cell viability was calculated together with the percentage population that are expressing eGFP, by gating the median fluorescence values (Figure 3.3.4). As shown by comparing R1 value of the control (A,C+E) with the transfected (B,D+F), there was an average decrease of 10% in cell viability of the transfected samples, which does not notably change over the various time points. This was to be expected due to the harsh nature of the transfection procedure. Additionally, by comparing the M2 values, on average 40% of the viable population have been successfully transfected at all 3 times points, with little change over time (B,D+F). For future experiments, transfected cells were incubated for 48 hours to ensure maximum transfection without any significant losses in cell viability.

3.3.2 - Creating the hBCATm expression vector.

The recombinant hBCATm gene was synthesised into the pENTR221 Gateway® entry vector and transformed into competent *E.coli* by GeneArt®. To assess the purity of the samples, 1 µg of the purified DNA was loaded onto a 0.8% agarose gel containing 0.5 µg/mL of ethidium bromide (Figure 3.3.5). In lanes A, E, F and G there are two bands at approximately 4 kb and 2 kb, with the lower band representing the supercoiled form of the 4 kb plasmid. The absence of any other bands, including in the negative control (I), suggests that there is no sample contamination. Restriction digest of the entry vector was used to confirm that the vector contained the hBCATm gene in addition to the specific restriction sites. When incubated with only one of the restriction enzymes, either PacI (B) or AscI (C), the linearized form of the plasmid is shown at approximately 4 kb. When cut

with both restriction enzymes (D+H), the hBCATm gene of 1.2 kb is present together with the rest of the plasmid at 2.8 kb. As the restriction enzymes have not cut all of the available DNA, there is still a band at 4 kb for the complete hBCATm entry vector.

The hBCATm gene from the Gateway® entry vector, pENTR221, 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 hBCATm gene. The transformation efficiency was calculated to be 6.14 x 10^7 CFU/µg. Validation of the hBCATm expression vector was tested using the PacI and AscI restriction enzymes and separation on a 0.8% agarose gel (Figure 3.3.6). In lanes A-D and F there is a band just below 4 kb which is the supercoiled expression vector, approximately half of the actual size of the expression vector which is 7.4 kb. Restriction digest of the expression vector was used to confirm that the vector contained the hBCATm gene along with the specific restriction sites. This is demonstrated in lanes E and G by the lower band at 1.2 kb which is the hBCATm gene, and the remaining linear plasmid at 6.2 kb. The BCATm entry vector used for the LR reaction was loaded (H) with its restriction digest (I), along with the Gus LR positive control (J) to check for any sample contamination. The absence of any additional bands, including in the negative control (K), suggests that there is no sample contamination.

3.3.3 - Western blot analysis of IMR-32 cell lysate transfected with hBCATm-His and bound to His Mag Sepharose Ni.

Although the transfection rate was examined previously using pcDNA3-EGFP (Figure 3.3.4), it was important to check the transfection efficiency with the hBCATm-His expression vector due to a difference in plasmid size. Confluent IMR-

32 cells were transfected with hBCATm-His expression vector DNA using jetPRIME® transfection reagent and incubated at 37°C for 9, 24, 32 and 48 hours. The cell lysates were extracted and analysed using western blot analysis. From the density of the bands observed in Figure 3.3.7 it can be seen that the expression of hBCATm-His increases over the selected time periods (L2-L5), where a time-dependent increase in protein expression is observed. Importantly, in the 24, 32 and 48 hour samples the overexpression of hBCATm-His can be seen by the significant increase in pixel intensity (Figure 3.3.7 B). The overexpression appears to plateau after 32 hours (Figure 3.3.7 B).

A 30 minute pull-down was carried out using 75 µL of His Mag Sepharose Ni and 150 µL of the cell lysate from the 48 hour overexpression of hBCATm-His alongside a control using the 48 hour overexpression of Gus. The eluted precipitation proteins were also analysed using western blot analysis (L8+L9) in Figure 3.3.7 A. In the hBCATm probe there is only a band observed in the hBCATm-His overexpressed pull-down (L8) and not in the Gus overexpression pull-down (L9). This demonstrates that the His tag on the synthesised hBCATm successfully binds to the His Mag Sepharose Ni beads and that cellular hBCATm only specifically binds to the antibody.

3.3.4 - Creating the hBCATc expression vector.

The recombinant hBCATc 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 gene. The transformation efficiency was calculated to be 6.72 x 10^7 CFU/µg. Validation of the hBCATc expression vector was tested using the PacI

and AscI restriction enzymes and separation on a 0.8% agarose gel (Figure 3.3.8). In lanes A and D there is a band just below 4 kb which is the supercoiled expression vector, approximately half of the actual size of the expression vector which is 7.4 kb. Restriction digest of the expression vector was used to confirm that the vector contained the hBCATc gene along with the specific restriction sites. This is demonstrated in lanes C and F by the lower band at 1.2 kb which is the hBCATc gene, and the remaining linear plasmid at 6.2 kb. The GFP control vector (G) was also loaded to check for any sample contamination. The absence of any additional bands, including in the negative control (H), suggests that there is no sample contamination.

3.3.5 - Transfecting SH-SY5Y cells with hBCAT overexpression vectors.

Using the newly created hBCAT overexpression vectors, SH-SY5Y cells were transfected for 48 hours with varying concentrations of 0.5 µg - 2.5 µg. Western blot analysis of the cell lysates for hBCAT demonstrated a successful transfection and overexpression (Figure 3.3.9). This is clear from comparing the increased hBCAT densitometry of the overexpression samples to the media and transfection control lanes. Furthermore, transfection with the hBCAT overexpression plasmids demonstrated a gradual increase in the respective hBCAT isoform expression alongside increasing plasmid concentration. A transfection control was used in both experiments to control for the transfection reagents and process without the hBCAT sequence present. Transfection with the control GFP plasmid appears to result in a decrease of both hBCATc and hBCATm expression.

These results show that the overexpression plasmids for both hBCATc and hBCATm have been successfully created. Through using the optimised transfection procedure using jetPRIME®, these plasmids are successfully integrated into the neuronal cell lines facilitating overexpression of the hBCAT protein. Immuno-detection with antibodies specific for hBCATc and hBCATm at the correct molecular

weight confirms that the protein produced by the plasmid is the respective hBCAT isoform. This method will now be used to study the impact of hBCAT overexpression on neuronal cell signalling pathways, particularly mTOR and autophagy in regards to AD pathogenesis.

3.4 - Discussion:

The expression of the hBCAT proteins has been demonstrated to be significantly upregulated in the brains of patients with AD compared to matched control patients, with hBCATm in particular being upregulated by up to 140% (Hull *et al.,* 2015). Additionally, in rat models examining striatal target injury, mRNA expression of BCATc was increased in the substantia nigra. This correlated with cell death, but the neurons that were immunopositive for BCATc had normal physiology and rarely contained apoptotic chromatin (Kholodilov *et al.,* 2000). Moreover, administration of brain-derived neurotrophic factor (BDNF), which demonstrated neuroprotective effects in newborn rats with lesion of the visual cortex, resulted in up-regulation of BCATc (Madeddu *et al.,* 2004). In transgenic mice overexpressing BDNF, the increase in BCATc expression was in a time and region-dependent manner (Castellano *et al.,* 2006). These findings all provide support for a neuroprotective role of the upregulation of the BCAT proteins in pathogenic conditions.

In order to investigate the effects of this upregulation, there is a need for an hBCAT overexpression model for use in neuronal cell lines. The method used to achieve this involved cloning of the hBCATm and hBCATc nucleotide sequences into a Gateway® entry vector pENTR221 by GeneArt® (Invitrogen). Gateway technology facilitated the transfer of the hBCAT gene from an entry vector into a human destination vector (pDEST™26) using site-specific LR recombination properties of bacteriophage λ and the specific recombination proteins that comprise LR Clonase™ II (Landy, 1989). Once the hBCATm/hBCATc expression vector had been formed from the LR recombination, the DNA was purified and ready for transfection. In order to transfect IMR-32 and SH-SY5Y cells, jetPRIME® transfection reagent was used. jetPRIME® is a cationic polymer-based molecule that forms positively charged complexes with DNA which can then penetrate the cell through endocytosis. Factors that affect the level of

expression include plasmid size, the amount of plasmid used per transfection, the strength of the promoter, the cell type being transfected, the efficiency of the transfection and the toxicity of the transfection reagent (Andrell and Tate, 2013). The pDEST™26 vector contains the human cytomegalovirus promoter (CMV) which allows efficient, high-level expression of the recombinant protein, in this case hBCATm/hBCATc (Andersson *et al.,* 1989). The jetPRIME® transfection reagent has not been previously used for transfection of IMR-32 cells, but has been successfully used with SH-SY5Y cells, another neuroblastoma cell line (Charrier *et al.,* 2012).

Overexpression studies are of crucial importance for understanding the role of particular proteins in disease pathogenesis (Prelich, 2012). In mouse models of AD, overexpression of amyloid β (Aβ) has been successfully used to develop understanding of AD pathogenesis in regards to the amyloid cascade hypothesis (Schwab *et al.,* 2004). From the results presented in this report (Figure 3.3.7 and 3.3.9) it can be concluded that a successful transfection and overexpression system of hBCATm-His and hBCATc-His in both IMR-32 and SH-SY5Y cells has been presented. SH-SY5Y cells were used in addition to IMR-32 cells due to their wide use as a neuronal model, particularly in regards to AD pathogenesis, as a result of their dopaminergic origin and expression of AD-related proteins such as tau (Smith *et al.,* 1995; Chen *et al.,* 2014). This model will enable the interactions and effects of hBCAT to be investigated under various conditions with respect to a potential role in regulating autophagy, under both normal and pathogenic conditions.

As one of the cellular mechanisms that prevent accumulation of misfolded proteins, autophagy degrades large protein complexes in order to prevent toxic aggregates forming (Nixon, 2006). Due to the upregulation of the hBCAT proteins and the accumulation of AVs, both observed in the brains of AD patients, there are potential links between hBCAT and autophagy dysregulation to investigate. In particular, the role hBCAT plays in regulating mTOR signalling through its effects on

leucine metabolism, a key regulator of autophagy (Chan *et al.,* 2009), remains to be revealed. Therefore, through use of an hBCAT overexpression system demonstrated here, the relationship between the upregulation of hBCAT and its effects on autophagy can be examined in a cellular environment.