Chapter 5

Is there a role for hBCATc in neuronal cell signalling?

5.1 – Introduction:

The broad expression of hBCATc in the brain reflects an important role in the regulation of neuronal glutamate levels as well as a potential to modulate the effects of leucine on signalling pathways (Sweatt *et al*., 2004). In terms of cell signalling, hBCATc is expressed in the neurons of the arcuate nucleus, the location of the central melanocortin signalling system (Cone, 2005). The arcuate neurons of the hypothalamus, where glutamate and GABA underlie the majority of fast synaptic activity, are involved in homeostatic regulation of energy metabolism via effects on the feeding system (van den Pol, 2003). The signalling pathway for this regulation involves the protein kinase mammalian target of rapamycin (mTOR) shown to be controlled by amino acid availability, of which leucine, a branched-chain amino acid, is the primary indicator (Hara *et al*., 1998). Harris and Lawrence (2003) found that in both animals and various cultured cell types, abundant leucine leads to an increase in mTOR activity, as it stimulated the phosphorylation of mTOR downstream targets (Lynch *et al*., 2000). The presence of hBCATc in the arcuate neurons implies a mechanism for controlling a leucine-mTOR signal in the hypothalamus. Particularly as the availability of leucine for signalling through the neuronal mTOR pathway may be controlled, at least in part, by hBCATc activity. This mechanism involving hBCATc in mTOR signalling is supported by the findings of this study, whereby association of hBCATc with the membrane means that it is suitably located to function as both a receptor and a mediator of the signalling pathway.

From research examining the redox associations between hBCAT and neuronal cellular proteins under oxidative stress, it has been shown that hBCATc binds specifically to several proteins that either have known reactive cysteines or CXXC motifs. Furthermore, these proteins have phosphorylation sites and/or are directly involved or controlled by G-protein signalling, known to be modulated by

peroxide. Because these neuronal cell signalling proteins are linked through redox interactions with hBCATc, which has several phosphorylation sites, these proteins may also be regulated by phosphorylation (Conway *et al*., 2008). This implies that hBCATc may play a pivotal role in regulating and/or mediating the degree of S-thiolation with specific neuronal proteins involved in G-protein cell signalling, thus supporting the notion that hBCATc associating with the membrane may facilitate this interaction.

The localisation of hBCATc is known and is indicative of a role in neuronal cell signalling pathways; however, the interactions and the mechanisms involved are currently unknown. The hBCATc sequence has a number of potential phosphorylation sites for both PKC and Casein kinase II (Figure 5.1.1), although their use *in vitro* is yet to be confirmed. However, previous work has shown that PKCα phosphorylates isolated hBCAT in both a time and concentration dependent manner (El Hindy, Unpublished data). This work aims to investigate the nature of hBCATc membrane localisation in relation to treatment with known cell signalling molecules and substrates for hBCATc. Furthermore, the mechanisms involved in facilitating this association will be analysed, particularly regarding the impact of phosphorylation and the redox conditions of the cell.

MKDCSNGCSAECTGEGGSKEVVGTFKADLIVTPAIVTP
ATILKEKPDPNNLVFGTVFTDHMLTVEWSSEFGWEKPH
IKPLQNLSLHPGSSALHYAVELFEGLKAFRGVDNKIRLF
QPNLNNMDRMYRSSAVRATLPVFDKEELLECIQQLVKL
DQEWVPYSTSASLYIRPTFIGTEPSLGVKKPTKALLFVL
LSPVGPYFSSGTFNPVSLWANPKYVRAWKGGTGDCK
MGGNYGSSLFAQCEAVDNGCQQVLWLYGEDHQITEV
GTMNLFLYWINEDGEEELATPPLDGIILPGVTRRCILDLA
HQWGEFKVSERYLTMDDLTTALEGNRVREMFGSGTA
CVVCPVSDILYKGETIHIPTMENGPKLASRILSKLTDIQY
GREERDWTIVLS

Figure 5.1.1 - hBCATc amino acid sequence. The reactive cysteines and the conserved CXXC motif are indicated in red. Phosphorylation sites for PKC are shown in green, tyrosine kinase sites in blue, cAMP in purple, and Casein kinase II in orange.

5.2 – Specific aims:

- **Specific aim 1:** Evaluate if hBCATc can associate with the cell membrane.
- **Specific aim 2:** Investigate the effects of known signalling molecules on hBCATc membrane localisation, including key substrates for hBCAT such as leucine and glutamate.
- **Specific aim 3:** To demonstrate whether hBCAT can be phosphorylated in SH-SY5Y cells using co-immunoprecipitation and western blot analysis.
- **Specific aim 4:** Assess the effect of phosphorylation of hBCATc on membrane localisation and identify the mechanisms responsible for regulating it.

5.3 – Results:

The cytosolic hBCAT protein has been previously observed, using immunocytochemistry with confocal microscopy, to migrate to the cell membrane under conditions of oxidative stress caused by exposure to hydrogen peroxide. These findings suggest a potential role for hBCATc in neuronal cell signalling, whereby it could function as part of a receptor-mediated signalling pathway or translocate to the membrane to metabolise or transport substrates. Key signalling molecules, such as glutamate and leucine, are both substrates for hBCAT and thus may influence hBCAT localisation. To confirm that the proposed association is at the membrane and to investigate the extent and control of this membrane migration, subcellular fractionations of neuronal cells treated with various stimuli were analysed by Western blot analysis.

5.3.1 – Western blot analysis of IMR-32 and SH-SY5Y subcellular fractions.

To validate the suitability of this method to investigate hBCATc membrane association, a crude subcellular fraction was produced from IMR-32 cells to produce a nuclear, cytosolic and membrane fraction (Figure 5.3.1). Referring to the method stated in Chapter 2.19, the last steps normally used to separate the lipid raft fraction of the membrane were not used as this was an exploratory experiment. Western blot analysis confirmed that hBCATc was associated with the membrane, where expression was evident in the cytosolic and membrane fractions. The non-fractionated IMR-32 cell lysate validates the western blot analysis and shows that hBCATc was present in the cell line. There was no band present in the nuclear fraction suggesting that hBCATc expression was confined to the cytosol and the cell membrane. Interestingly, under these conditions the membrane fraction showed a band for hBCATc at a higher molecular weight, approximately 65 kDa, compared to the expected 43 KDa. This poses a possibility that in this cell line, post-translational

modifications such as glycosylation or lipid addition to the protein restrict its migration through the gel.

Using proteins that are specific to the membrane, cytosol or nucleus, as positive controls, we validated that expression of hBCATc was specifically localised to these areas whilst also demonstrating that the fractions produced by this method contain only proteins specific to that location. For these experiments antibodies to Na, K-ATPase, a membrane-specific protein, and Hsp90, a cytosol-specific protein, were used to verify the fractionation. A second neuroblastoma cell line was also used (SH-SY5Y) to test if the membrane localisation of hBCATc was not just specific to IMR-32 cells. hBCATc was present in all of the collected fractions for both cell lines, but was highest in the cytosol and non-raft membrane fractions (Figure 5.3.2). In contrast to the previous result (Figure 5.3.1), membrane hBCATc was expressed at its expected molecular weight of 43 KDa (Figure 5.3.2). This could be a result of the lipid raft isolation step disrupting the potential lipid modification, as this step was not carried out previously. As can be seen from the Na, K-ATPase and Hsp90 blots, the fractionation procedure was successful as these fraction-specific proteins show expected bands in their corresponding lane. The expression of hBCATc in other than the cytosolic fraction is a novel finding and with the membrane fraction it will be interesting to investigate the nature of this localisation, whether it is transient dependent on stimulus and what stimuli effect this trafficking.

5.3.2 – Western blot analysis of stimuli effecting hBCATc membrane localisation.

As leucine is a key nutrient signal, it may play an important role in recruiting hBCATc to the membrane. Leucine was chosen as it is the BCAA that most readily crosses the blood-brain barrier and also has roles in numerous cell signalling pathways including mTOR. After starving SH-SY5Y cells for one hour, they were

treated with 5 mM, 10 mM or 20 mM leucine for three hours respectively, and then analysed for subcellular expression (Figure 5.3.3). As can be seen from comparing the bands for hBCATc, its expression does not change in the cytosolic fraction, however in the membrane fractions; treatment with leucine significantly reduced the amount of hBCATc membrane localisation in the 5 mM (p = 0.0052) and 10 mM (p = 0.0005) samples.

Glutamate is also a signalling molecule, where binding to mGluR1 initiates varied signalling responses including activation of coupled Ca^{2+}, K^{+}, and Na^{+} channels, and G protein signalling cascades involving MAPK, PKB and Src kinase. These processes involve recruitment of other receptors and protein complexes to the membrane to facilitate successful downstream signalling. There may therefore be an impact on hBCATc membrane localisation under exposure to varying glutamate concentrations. Cells were treated with 5 mM, 10 mM or 20 mM glutamate for 30 minutes, respectively. This resulted in a decrease in membrane hBCATc, observed to drop initially at 5 mM and 10 mM glutamate, but then more at the 20 mM glutamate concentration (Figure 5.3.4).

Previous studies have indicated that insulin enhances leucine mediated regulation of mTOR. To study if the combined treatment effects hBCATc membrane localisation, cells were treated with 5 mM leucine, 100 nM insulin, 5 mM leucine and 100 nM insulin together, for 3 hours following a 1 hour starvation in EBSS. The treatments were all prepared in EBSS to exclude the effects of nutrients present in the cell media. Each sample is compared to the starvation control (EBSS only), which has reduced hBCATc membrane association in comparison to the media control sample. In contrast, the cytosolic fractions show an increase in hBCATc expression in the starved sample. As previously demonstrated, treating the cells with leucine (p = 0.0996) significantly reduces membrane association of hBCATc, but interestingly, when treated with insulin (p = 0.0237) there was a significant

increase in membrane localisation. Treating with insulin and leucine simultaneously appears to attenuate this association but it is still greater than that observed in the leucine only treatment (Figure 5.3.5). The increase in membrane localisation of hBCATc when treated with 100 nM insulin had the greatest overall difference and was significantly different (p = 0.0237).

5.3.3 – Western blot analysis of co-immunoprecipitated SH-SY5Y cells examining phosphorylation.

The observed migration of hBCATc to the external cell membrane suggests a role for hBCATc in cell signalling. One of the key mechanisms for maintaining signalling pathways is through phosphorylation of substrates. Previous work by Dr El Hindy (Unpublished, 2014) has demonstrated that the isolated reduced hBCAT protein can be phosphorylated by PKCα in a both time and concentration dependent manner. It is not known however, whether or not hBCAT in a cellular environment is also phosphorylated by PKCα. To this end, prior to extraction, SH-SY5Y cells were incubated in phosphorylation buffer for 2 hours to encourage cellular phosphorylation. The cell lysates were incubated for 1 hour with protein G mag sepharose beads which were cross-linked to 1 µg of either antibodies for hBCAT (hBCATc and hBCATm, or the commercial antibodies for hBCAT; hBCAT1 and hBCAT2) or IgG isotype control. The samples were then eluted and separated on a 12% SDS-PAGE gel and analysed by western blot analysis. When the samples were probed with anti-Phospho-(Tyr), a number of bands were observed suggesting that numerous proteins that have been precipitated out of the cell lysate have been phosphorylated at a tyrosine residue (Figure 5.3.6). Importantly, for all of the lanes using hBCAT antibodies there is a band at approximately 43 KDa, showing that hBCAT has been successfully precipitated out from the cell lysates and that it is also phosphorylated at a tyrosine residue (Figure 5.3.6).

As a control for this experiment, an antibody for control IgG was used to demonstrate that the hBCAT band shown in the other lanes was not a result of non-specific binding. There was no band at the weight of hBCAT in this IgG control lane, thus illustrating the validity of the experiment. There are, however, numerous bands at higher molecular weights than hBCAT in all of the lanes, suggesting that they have been precipitated out via non-specific IgG interactions. The same membrane was then probed for hBCATc and then hBCATm to check that the band observed at 43 KDa was hBCAT. As demonstrated in Figure 5.3.7, for both hBCATc and hBCATm there are bands at 43 KDa indicating a successful immunoprecipitation, with an absence of a band in the IgG control lane. The hBCATc blot shows bands in all four hBCAT lanes, not just the hBCATc and hBCAT1 (Commercial antibody for hBCATc) lanes due to cross-reactivity between the two isoforms. There is a clear band at 50 KDa observed in all of the lanes, which is the heavy chain IgG fragment of the primary antibody, which has been eluted alongside the protein samples that were analysed by western blot analysis. From this experiment, and together with the previous work carried out by Dr El Hindy (Unpublished, 2014), it was established that the hBCAT protein can be phosphorylated by PKCα. Taking this work forward, it was of interest to investigate if phosphorylation impacts the localisation of hBCATc to the membrane.

5.3.4 – Western blot analysis of subcellular fractions subject to phosphorylation by PKCα.

As shown previously, treatment with insulin caused the greatest increase in membrane localisation. Using specific inhibitors (Go6976 and staurosporine) and activators (phospho buffer and PMA) of PKCα, the effect of hBCATc phosphorylation on membrane localisation was examined. After a 2 hour incubation with the respective buffer (EBSS or phospho) containing either the activator or inhibitors with and without insulin, SH-SY5Y cells were fractionated and analysed by

western blot analysis. The results indicate that phosphorylation of hBCATc inhibits localisation to the membrane, potentially interfering with the signalling mechanism (Figure 5.3.8). When comparing the inhibited sample to the EBSS control, it suggests that inhibiting hBCAT phosphorylation actually results in an increase in membrane localisation (Figure 5.3.8). Interestingly, when a sample was treated with insulin and 5 mM glucose simultaneously, the membrane localisation increased even further (Figure 5.3.8).

Together these findings suggest that hBCATc, in its un-phosphorylated form, can migrate to the cell membrane when cells are stimulated by insulin. However, when phosphorylated by PKCα this prevents the trafficking effect induced by insulin. Further work by Dr El Hindy (unpublished, 2014) showed that the mechanism by which hBCAT is phosphorylated can be redox regulated, specifically through the hBCAT's conserved CXXC motif. Using this knowledge, oxidising and reducing agents were used together with the treatments for activating and inhibiting hBCATc phosphorylation. When the cells are oxidised, previous work has demonstrated that hBCAT cannot be phosphorylated, whereas under reduced conditions it can be phosphorylated. Upon fractionation and western blot analysis, cells that have been reduced have a clear reduction in membrane hBCATc (Figure 5.3.9). When comparing the EBSS with the PKCα activator between reduced and oxidised samples, it is clear that the decrease in membrane hBCATc caused by phosphorylation is diminished in the oxidised sample, perhaps due to the oxidised CXXC motif of hBCATc preventing successful phosphorylation. The insulin sample provides further support for the previous experiments showing the increase in membrane localisation, whereby EBSS alone provides the control level, the oxidised sample has a slight increase in membrane hBCATc and then the oxidised with insulin has the highest level of membrane hBCATc.

5.4 – Discussion:

Through subcellular fractionation with Western blot analysis, it has been demonstrated that hBCATc can associate with the cell membrane. Furthermore, under insulin treatment this association was significantly increased. After establishing that hBCAT can be phosphorylated by PKCα *in vitro*, using immunoprecipitation with western blot analysis, use of PKCα activators and inhibitors showed that upon hBCATc phosphorylation, the membrane fraction of hBCATc was decreased. Furthermore, the redox state appeared to regulate this phosphorylation, whereby reduced conditions permitted phosphorylation and therefore decreased hBCATc membrane localisation, whereas oxidation prevented hBCATc phosphorylation and thus increased the hBCATc membrane fraction.

5.4.1 - Autophagy links with the cell membrane.

As demonstrated in the previous chapter, the hBCAT proteins play a role in the mTOR and autophagy pathways, with evidence of co-localisation with the autophagic vacuole marker, LC3. Elements of the autophagy pathway have been linked to the cell membrane, perhaps demonstrating a role for hBCATc in translocating to the membrane. Research has suggested that the plasma membrane contributes to the early autophagosome precursor structures. This is dependent on the association of Atg16L1-positive vesicles with the plasma membrane through Atg16L1-AP2/clathrin heavy-chain interactions (Ravikumar *et al.,* 2010). This could be an even earlier stage in the autophagy assembly, where these autophagosome precursors are membrane structures preceding phagophores (Ravikumar *et al.,* 2010). The large surface area of the plasma membrane would thus serve as a large membrane reservoir, facilitating periods of autophagosome synthesis at levels many-fold higher than under basal conditions, without compromising other cellular processes. As hBCATc is co-localised with autophagic vacuoles, it would be interesting to investigate at what stage this association with

autophagy occurs, with evidence of membrane association potentially suggesting the initiation stages.

Another link between autophagy and the membrane is demonstrated by the lysosome-associated membrane proteins (LAMPs). These are integral transmembrane proteins that are expressed on lysosomes and can be shuttled to the plasma membrane during cell activation (Holcombe *et al.,* 1993). In chaperone mediated autophagy (CMA), proteins selected for degradation bind to the lysosomal membrane via the cytosolic tail of LAMP-2A (Cuervo and Dice, 1996). For a protein to be selected for CMA it must contain the CMA-targeting motif, a pentapeptide sequence of KFERQ (Dice *et al.,* 1986). Recognition of this sequence is specifically by heat shock cognate protein of 70 kDa (hsc70), a multifunctional chaperone involved in numerous cellular processes such as protein refolding and protein targeting to other organelles (Chiang *et al.,* 1989).

Under cellular stress, such as starvation and oxidative stress, the percentage of CMA-active lysosomes (containing both hsc70 and CMA substrates) can increase from 20% to 80% of the total cellular lysosomes (Cuervo *et al.,* 1997). In nearly all cell types, nutrient shortage is responsible for maximally activating CMA (Kaushik and Cuervo, 2012). CMA is in fact up-regulated under conditions that encourage protein damage such as oxidative stress and protein denaturing compounds, particularly for neuronal survival (Finn and Dice, 2005; Kiffin *et al.,* 2004; Cuervo *et al.,* 1999; Dohi *et al.,* 2012). There is also evidence demonstrating a role in removing key proteins associated with neurodegenerative diseases, including APP and tau in AD (Wong and Cuervo, 2010). Thus, the up-regulation of hBCATc observed in AD will likely impact on CMA, through its role in nutrient metabolism and refolding of misfolded proteins (Hull *et al.,* 2015; El Hindy *et al.,* 2014). Furthermore, the findings from both chapter 4 and 5 demonstrate that hBCAT is up-regulated under starvation conditions, and also membrane expression

is increased when the cells are subject to oxidative stress. Both of these are key initiators of CMA. However, activation of CMA is initiated after approximately 10 hours of starvation, occurring after up-regulation of autophagy, which typically occurs after 30 minutes of starvation but does not last for more than 8-10 hours (Aniento *et al.,* 1993). This time frame is not captured in the studies carried out here, as all cellular treatments were for no longer than 4 hours. Consequently, CMA should not be observed as a response in these experiments. Future studies should look at the effect of longer treatment periods on hBCATc localisation and activity.

5.4.2 - Insulin signalling and the possible role for hBCATc.

Treating SH-SY5Y cells with insulin was shown to significantly increase the membrane association of hBCATc, suggesting a role in the insulin signalling pathway. Insulin's signalling effects are mediated through activation of PI3K, a lipid kinase responsible for phosphorylating phosphoinositides. Through stimulation by various growth factors and cytokines, PI3K is recruited to the membrane, resulting in enzyme activation and generation of PtdIns(3,4,5)P_3 from its lipid substrate, phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) (Katso *et al.,* 2001; Cantley, 2002). Dephosphorylation of PtdIns(3,4,5)P_3 at the D-3 position by the tumour suppressor PTEN reverses the action of PI3K (Katso *et al.,* 2001). PtdIns(3,4,5)P_3 generated in the membrane by PI3K recruits members of numerous intracellular signalling pathways, coupling PI3K signals to downstream effector molecules (Katso *et al.,* 2001). It is proposed here that hBCATc could be considered one of these downstream effectors, demonstrated by the significant increase in membrane expression of hBCATc upon insulin treatment.

One of the downstream effectors activated through PI3K is protein kinase B (PKB, also known as Akt), which is essential for both mediating the effects of insulin on glucose homeostasis and regulating the effects of insulin and IGFs on mTOR signalling and cell growth. PKB phosphorylates and inactivates an extensive range

of substrates involved in diverse cellular processes. The effects of insulin on cell growth are directed through phosphorylation and inactivation of tuberin sclerosis complex 2 (TSC2), an inhibitor of the GTPase Rheb (Inoki *et al.,* 2002; Tee *et al.,* 2003). Rheb activation through insulin stimulation leads to activation of the mTOR pathway, including S6 kinase (Garami *et al.,* 2003). Work in Chapter 4 has already demonstrated that hBCATc overexpression resulted in increased S6 kinase activation, clear evidence for a role in the insulin-mediated mTOR pathway, particularly due to the increase in membrane expression of hBCATc after insulin treatment.

Similarly to the Ras and mitogen-activated protein kinase (MAPK) pathways, physiological control of PI3K signalling is likely to involve negative feedback mechanisms to monitor strength and duration of PI3K activation (Harrington *et al.,* 2005). Negative regulation of PI3K, as demonstrated by prolonged exposure to insulin, results in a decrease in both the electrophoretic mobility and the level of insulin receptor substrate (IRS)-1 protein (Haruta *et al.,* 2000). Activation of PKB, although initially stimulated by insulin exposure, decreases after prolonged insulin treatment in a similar manner to the decrease in IRS-1 levels. This suggests attenuation of PI3K activity, functioning as a negative feedback pathway (Haruta *et al.,* 2000). Interestingly, a common mechanism for IRS-1 reduction is through an increase in proteasomal degradation, demonstrated by the use of proteasome inhibitors whereby IRS-1 reduction after prolonged insulin stimulation is prevented (Haruta *et al.,* 2000; Greene *et al.,* 2003). Additionally, blocking ubiquitination through use of a cell line with a mutation in the ubiquitin-activating enzyme E1 prevented IRS-1 degradation (Zhande *et al.,* 2002). This suggests potential involvement of autophagy in reducing over-stimulation of IRS-1, a process hBCATc has been clearly linked to in Chapter 4 (Figure 5.4.1). It would therefore be interesting to monitor membrane hBCATc, alongside PI3K activation, over a period

of time after insulin treatment. This would demonstrate whether or not hBCATc plays a role in the negative feedback mechanism for PI3K.

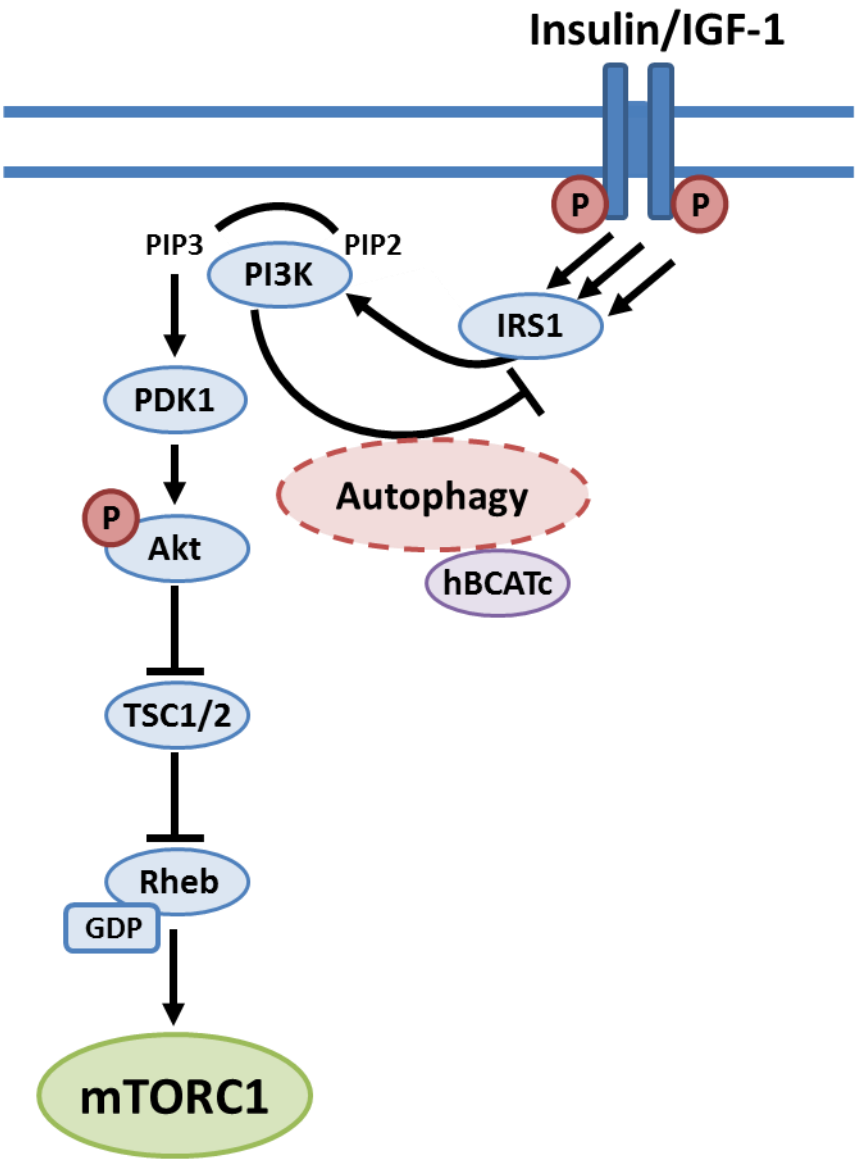

Figure 5.4.1 - Negative feedback mechanism of the Insulin/PI3K pathway. Overstimulation of the insulin/PI3K pathway results in decreased electrophoretic mobility and level of IRS-1. PI3K activity is attenuated through inhibiting IRS-1 by means of proteasomal degradation after prolonged insulin exposure.

5.4.3 - The effect of leucine treatment alongside insulin.

When the cells were treated with insulin and leucine together, the increase in membrane expression of hBCATc, observed with insulin treatment, was attenuated. The presence of amino acids has been shown to decrease activation of PI3K after insulin treatment, associated with both an increase in IRS-1 inhibitory phosphorylation and a reduction in IRS-1 stability and association with PI3K (Tremblay and Marette, 2001; Patti *et al.,* 1998). This effect also correlates with S6-K, whereby a temporary increase due to potentiation of mTOR signalling then leads to a decrease in S6 K, demonstrating the negative feedback control of PI3K activity by this kinase (Tremblay and Marette, 2001).

Support for the reduction in IRS-1 gene transcription by S6 K has been given through suppression of S6 K by RNA interference (RNAi) restoring the abundance of IRS-1 mRNA in TSC2-deficient cells (Harrington *et al.,* 2004). Furthermore, mammalian cells overexpressing Rheb, which induces S6 K, result in the decrease of both IRS-1 protein levels and PKB activation in response to insulin. Co-transfection with a kinase-deficient S6 K or kinase-dead mTOR variant prevented this decrease (Shah *et al.,* 2004). Thus, there is extensive evidence for amino acids, such as leucine, stimulating mTOR and inhibiting insulin signalling via IRS-1.

If hBCATc is recruited to the membrane upon insulin stimulation of IRS-1, the additional presence of leucine will inhibit IRS-1 activation, consequently reducing hBCATc recruitment to the membrane. As hBCATc appears to increase S6 K activation, demonstrated in Chapter 4, it could function as part of the negative feedback mechanism in place to prevent over-stimulation of IRS-1 whereby downstream activation of S6 K and autophagy associations both work to reduce IRS-1 activation.

5.4.4 - Glutamate signalling causing a decrease in membrane hBCATc.

The membrane expression of hBCATc was significantly reduced when cells were treated with glutamate, most notably at the highest concentration of 20 mM. Glutamate is a widely distributed excitatory neurotransmitter which, through its action on glutamate receptors, modulates a number of neuronal functions including synaptic plasticity and organisation, long-term potentiation, and excitotoxicity (Willard and Koochekpour, 2013). However, neuronal cell death can occur through its excitotoxic action, resulting in increased calcium load, activation of proteases, and generation of ROS and nitric oxide (Dong *et al.,* 2009).

Stimulation of neuronal glutamate receptors results in an increase in intracellular Ca^{2+} via varying signalling mechanisms. iGluRs directly open an ion channel upon glutamate binding, whereas mGluRs result in Ca^{2+} influx via indirect ion channel opening and/or by second messenger signalling systems. Binding of Homer1 to the Homer binding motif, a proline-rich extended C-terminus of mGluRs, recruits the scaffold protein Shank, facilitating direct interaction between mGluRs and NMDARs and causing Ca^{2+} influx (Tu *et al.,* 1999). Activation of PLCs by Gα11 causes cleavage of PIP2 into IP3, released into the cytosol, and DAG, which remains in the plasma membrane. These products initiate numerous signalling cascades, however, when working cooperatively also result in further protein activation through stimulating the MAPK, ERK1/2 and mTOR pathways (Figure 5.4.2) (Menard and Quirion, 2012). Subsequently, PKC is recruited to the membrane through cooperatively binding DAG and Ca^{2+}.

PKC is maintained in the membrane in an active state by membrane-bound receptor for activated protein kinase C proteins such as RACKs (receptor for activated C kinases) (Ramakers *et al.,* 1997). Furthermore, additional Ca^{2+} release is activated from the ER Ca^{2+} stores due to IP3 receptor activation by IP3. PKC activation plays a role in receptor desensitisation by phosphorylating mGluR1a, a

critical mechanism for returning resting membrane potential after action potential propagation (Figure 5.4.2) (Dhami and Ferguson, 2006). Therefore, recruitment of PKC to the cell membrane due to glutamate stimulation may result in phosphorylation of localised hBCATc; which from this chapter's findings results in a reduction in hBCATc membrane expression. In addition to this, hBCATc could also be required for reducing the cytosolic levels of glutamate through its transamination reaction of BCAAs. Further experiments could be carried out in future to confirm this by treating the cells with glutamate before immunoprecipitating hBCAT and probing for phosphorylation.

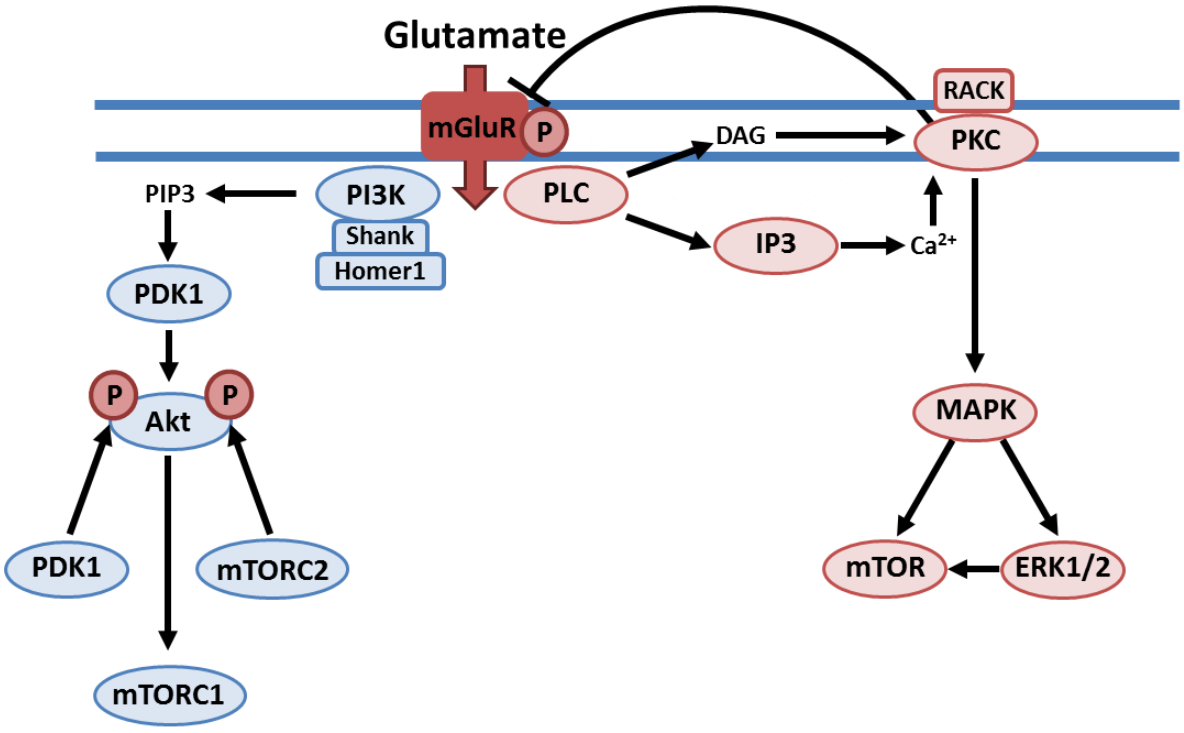

Figure 5.4.2 – Glutamate signalling through mGluR. Activation of PLC results in the production of IP3 and DAG, which cooperatively recruit PKC to the membrane where its activation stimulates mTOR via MAPK and ERK1/2. PKC is also responsible for phosphorylating and desensitising mGluR as a negative feedback mechanism. mGluR activation also recruits PI3K, producing PIP3 and stimulating mTOR via Akt.

Glutamate signalling has been linked to the mTOR pathway, where homer1 proteins are also responsible for coupling of mGluR1 to PI3K; activation of PI3K

consequently leads to accumulation of PIP3 and recruitment of pleckstrin homology (PH) domain-containing proteins such as Akt1. Recruitment of Akt1 to the membrane via its PH domain causes phosphorylation by mTORC2 and PDK1 (Figure 5.4.2) (Cybulski and Hall, 2009). Activated Akt1 has been shown to be both neuroprotective and pro-proliferation in numerous neuronal cell types (Rong *et al.,* 2003; Chong *et al.,* 2003; Allen *et al.,* 2000). Furthermore, Akt1 also activates mTORC1 members including TSC2, resulting in increased translation of proteins required for cell growth (Alsina *et al.,* 2012). This highlights the complexities of the neuronal signalling pathways, where glutamate, insulin and leucine all impact on mTOR signalling at various stages. Therefore hBCATc involvement, through altered expression and localisation, is both dynamic and varied.

Glutamate cytotoxicity has been previously observed in SH-SY5Y cells, however a recent study demonstrated that pre-treatment with insulin prevented glutamate-induced cell death (Nampoothiri *et al.,* 2014). With the maximal cell viability observed at 100 nM, insulin treatment also reduced the production of ROS and prevented apoptosis. This neuroprotective action of insulin is intriguing, particularly due to the stark contrast in membrane hBCATc expression; where it is increased upon insulin treatment but decreased with glutamate. Work by Skeberdis *et al.* (2001) found that insulin potentiates NMDA receptors through delivering new channel molecules to the cell surface via regulated exocytosis. Furthermore, they also showed that the insertion of new receptors was through a SNAP-25 mediated form of SNAP-receptor-dependent exocytosis.

Activation of PKC also induces recruitment of NMDA receptors to the cell surface in cultured hippocampal neurons (Lin *et al.,* 2001). PKC involvement appears to be a requirement, as insulin potentiation of NMDA receptor activity was inhibited by calphostin C, a selective PKC inhibitor (Liao and Leonard, 1999). A model was suggested where insulin regulates membrane fusion events of NMDA

receptor-containing vesicles by PKC-mediated phosphorylation of SNAP-25 (Skeberdis *et al.,* 2001). Not only does this demonstrate the mutual relationship between insulin and glutamate, but also shows the crucial involvement of PKC. Potentially, this involvement of PKC in mediating new NMDA receptors could prevent phosphorylation of hBCATc, permitting membrane localisation. Or alternatively, hBCATc could be functioning alongside SNARE-dependent exocytosis to increase NMDA receptors due to insulin stimulation. Future work could look to analyse binding partners of hBCATc that are specific to the membrane fraction and also observe the cellular location of PKC in relation to hBCATc under glutamate treatment with and without insulin.

5.4.5 - PKC mediated phosphorylation of hBCATc.

There are a number of potential phosphorylation sites present on the hBCATc structure which have been previously shown to be phosphorylated by various neuronal PKC isoforms, most prominently with PKCα (El Hindy *et al.,* Unpublished). Furthermore, PKC phosphorylation was mediated through changes in the redox sensitive reactive thiols of hBCAT (El Hindy *et al.,* Unpublished). These studies, however, were carried out on the isolated recombinant protein, and so whether this phosphorylation occurred *in vitro* was not known. Phosphorylation of other proteins involved in BCAA metabolism has already been observed, in particular the BCKD complex. Phosphorylation by BDK, and consequently inhibition of BCKD, is triggered by low levels of α-ketoisocaproate, a BCAA metabolite (Paxton *et al.,* 1984). PKCα belongs to the conventional group of isoforms, including α, β and γ, which have been well characterised for their role in the mammalian brain, unlike the novel and atypical isoforms ζ and -ı/λ which aren't activated by calcium, DAG or PMA (Ono *et al.,* 1989; Geraldes and King, 2010). PKCα functions in numerous cell signalling pathways and by linking PKCα to the regulation of hBCAT suggests a role for hBCAT as a neuronal signalling molecule. In these experiments, hBCAT was phosphorylated under conditions that encourage

phosphorylation through PKCα. Furthermore, when these conditions were applied to cells before membrane analysis, phosphorylation of hBCATc reduced membrane expression.

The optimal conditions for hBCAT phosphorylation have been shown to require the presence of DAG and calcium (El Hindy *et al.,* Unpublished). PKCα has a greater affinity for DAG when calcium is bound, which are both required to bind in order to produce a conformational change, releasing the pseudosubstrate domain and thus activating PKCα (Newton *et al.,* 2001; Hancock, 2010). The phosphorylation sites in the hBCAT sequence have either arginine (K), histidine (H), or lysine (R) surrounding the serine or threonine sites, these are basic residues which are known to bind negatively charged phospholipids consequently enhancing interactions with PKCα (Madison *et al.,* 1986). Phosphorylation by PKCα is therefore possible for hBCATc, however confirmation through mass spectrometry analysis after encouraging phosphorylation is required to establish the specific site.

Similar to other redox proteins, such as neurogranin, reduced conditions were previously shown to be optimal for hBCATc phosphorylation (Sheu *et al.,* 1996; El Hindy *et al.,* Unpublished). The cysteine rich motifs, unique to PKCα, are readily oxidised and have been shown to stimulate PKC activity of the regulatory domain upon oxidative modification (Gopalakrishna *et al.,* 2000). Thus, available thiol groups of reduced hBCAT may potentially be required to successfully interact with PKC. This has been supported by the findings here, whereby treatment with hydrogen peroxide alongside PMA (a PKC activator) results in higher membrane expression of hBCATc compared to treatment with DTT and PMA. The expression of membrane hBCATc when treated with hydrogen peroxide and insulin is only slightly higher than the sample treated with hydrogen peroxide and PMA. This small difference could be due to the fact that ROS interact with insulin receptor tyrosine phosphorylation, disrupting insulin signalling and affecting PI3K activation and IRS

phosphorylation (Tirosh *et al.,* 1999; Evans *et al.,* 2003). Thus, the effect insulin has on membrane hBCATc expression may be reduced under oxidising conditions.

Decreased levels of PKC have been observed in the brains of AD patients, perhaps due to high levels of Ca^{2+} favouring phosphatase activation (Alkon *et al.,* 2007; LaFerla *et al.,* 2002; Foster *et al.,* 1997). Overall, the balance of PKC to phosphatase levels is significantly altered in the neurodegeration process, which will have an impact on the extent of hBCAT phosphorylation (Norris *et al.,* 1998). It is not yet known how hBCAT phosphorylation affects its transaminase activity, however, there is potential for adverse effects on BCAA and glutamate levels in AD environment. However, PKC activators have been shown to help reduce levels of Aβ40 and Aβ42 and also improve cognitive impairment (Etcheberrigaray *et al.,* 2004). Perhaps phosphorylation of hBCAT impacts its role in autophagy and mTOR signalling, resulting in increased clearance of Aβ through autophagy. This work has shown that membrane expression of hBCATc is reduced by phosphorylation under reduced conditions and is increased when treated with insulin and prevented from being phosphorylated with inhibitors and hydrogen peroxide. Future studies are needed to confirm if insulin affects the phosphorylation state of hBCATc in order to determine the sequence of events. Furthermore, the effect of phosphorylation on hBCAT activity and its subsequent impact on mTOR and autophagy remains to be elucidated.

5.4.6 - Potential mechanisms for membrane translocation of hBCATc.

The signals that instigate hBCATc membrane localisation have been demonstrated, however the mechanism involved for trafficking the protein to the membrane is unknown. A potential mechanism by which this could occur would be through protein modifications such as n-myristoylations and palmitoylations which result in changes of activity, regarding protein binding and regulation. N-myristoylation involves the irreversible covalent binding of myristate to the N-

terminal glycine (Farazi *et al*., 2001). This protein modification usually occurs co-translationally after removal of the interior methionine residue via cellular methionylaminopeptidases (Wolven *et al*., 1997). However, N-myristoylation is also present post-translationally as demonstrated in the pro-apoptotic protein BID, where proteolytic cleavage reveals a myristoylation motif (Zha *et al*., 2000). Myristate binding promotes reversible weak interactions between the protein and the membrane and is often in sync with other mechanisms, such as palmitoylation, to regulate protein targeting and function. N-myristoylation is known to effect proteins involved in signal transduction cascades, and can produce diverse functions such as transmembrane signalling through binding to G proteins, and changes in intracellular destinations (Farazi *et al*., 2001). Upon entering the sequence for hBCATc into CSS-Palm 2.0, a number of lipid modification sites are predicted (Table 5.1).

In addition to the N-myristoylation site, there are 3 S-palmitoylation predicted sites. Palmitoylation is a lipid modification involving the attachment of a 16-carbon saturated fatty acid, palmitate, which has pleiotropic effects on protein function. Diverse aspects of neuronal protein trafficking and function can be regulated through palmitoylation, including influencing the membrane binding and targeting of the modified proteins (Resh, 2006).

Table 5.1 - CSS-Palm 2.0 prediction of hBCATc lipid modification sites.

Position	Peptide	Score	Type
4	****MKD**C**SNGCSAE	2.263	S-Palmitoylation
7	*MKDCSN**G**CSAECTG	13.101	N-Myristoylation
8	MKDCSNG**C**SAECTGE	3.977	S-Palmitoylation
12	SNGCSAE**C**TGEGGSK	2.814	S-Palmitoylation
338	SGTACVV**C**PVSDILY	1.123	S-Palmitoylation

A two-signal model of membrane association theory suggests that either the presence of myristate plus palmitate or two palmitates can direct a protein to the membrane (Resh, 1994). This is clearly demonstrated by the Src family of protein tyrosine kinases, which are localised to subspecialised regions on the plasma membrane through covalent modification by myristate, which is co-translationally attached to a glycine at position 2 of the protein through an amide link. Seven out of the nine Src family proteins are then also modified post-translationally by the reversible attachment of palmitate to cysteines near the N terminus of the protein via a thioester linkage. The palmitoyl acyltransferase enzyme is responsible for the palmitoylation of myristoylated Src family proteins (Berthiaume and Resh, 1995), which is part of a large DHHC (Asp-His-His-Cys) motif family. The DHHC protein family of palmitoylating enzymes generate and maintain the specialised compartmentalisation of substrates in polarised neurons (Fukata and Fukata, 2010). Regarding G proteins, the α_s subunit of the heterotrimeric G proteins have also been shown to be reversibly palmitoylated through regulation with isoproterenol, a β-adrenergic receptor agonist. This leads to a shift from the membrane bound to cytosolic form of the protein (Wedegaertner and Bourne, 1994).

The importance of exposed cysteine residues has been demonstrated by research looking at $P59^{fyn}$ (Fyn), a cytosolic Src protein tyrosine kinase, which has shown that mutating the cysteine-3 residue reduces the extent of palmitoylation and membrane binding of Fyn (Alland *et al*., 1994). Additionally, use of chimeric Fyn proteins has also demonstrated that the palmitoylation of cysteine residues in the N-terminal region of Fyn is required for efficient membrane association. A mutation of the N-terminal glycine to alanine (G2A) of Fyn results in a non-myristoylated protein that does not associate with the membrane, and also abolishes palmitoylation. Myristoylation is not an absolute requirement for palmitoylation or membrane association of Fyn, as it is suggested that the role of myristate is to enhance accessibility of Fyn to a membrane-bound palmitoyl acylttransferase (Wolven *et al*.,

1997). With regards to myristoylation and palmitoylation of hBCATc, the protein sequence provides a number of potential sites for both N-myristoyation and S-palmitoylation, implying a potential mechanism for the membrane association of hBCATc (CSS-Palm, 2016). Additionally, the crystal structure of hBCATc, unlike hBCATm, shows the presence of an additional four solvent accessible cysteine residues as well as its CXXC motif (Goto *et al*., 2005) providing residues for potential palmitoylation and myristoylation. The results from these experiments show that hBCATc associates with the membrane, although further studies need to investigate whether the predicted lipid modification sites are active *in vitro*.

5.4.7 - Summary.

It has been clearly demonstrated that hBCATc can associate with the cell membrane, with this localisation being significantly increased with insulin treatment. Key substrates for hBCATc, however, did not affect membrane localisation to the extent that insulin did, suggesting a role separate to that of BCAA transamination. Furthermore, phosphorylation of hBCAT by PKCα was confirmed *in vitro,* and was shown to reduce hBCATc membrane localisation. This phosphorylation relies on a reduced environment, whereas oxidation and inhibition of PKCα facilitated membrane localisation. This work suggests a role for hBCATc to play in neuronal cell signalling, particularly insulin signalling pathway, with potential implications for insulin modulation of neuronal excitability and impacting the mTOR and autophagy pathways. Future work needs to analyse the effect of membrane localisation on hBCATc enzyme activity and elucidate the translocation mechanism.