7 Discussion

Given the proposed role of the hBCAT proteins play in brain glutamate production, it is important to demonstrate whether the distribution observed in animal and cell models is representative of humans. For the first time this work demonstrates key differences in hBCATm localisation between previous distributional studies in cell and animal models, and humans. Similar to previous models, hBCATc was largely neuronal specific. It was then hypothesised that these hBCAT proteins may be altered in diseases where glutamate toxicity plays a role, such as AD. This work then continues to investigate the expression of the hBCAT proteins in AD and demonstrates, for the first time, the overexpression of hBCATm in the vasculature of AD subjects compared to controls. Finally, a cell culture model was used to investigate novel functions of the hBCAT proteins and is discussed in relation to AD pathology and BCAA metabolism.

7.1 Localisation of hBCAT and hPDI in relation to function

Given the key role that BCAT enzymes play in brain glutamate regulation, it is important to establish the distribution of these isoforms in the human brain relative to rat and cell culture models. Consistent with the rat and cell culture models, hBCATc was predominantly observed in neuronal cells. This supports the role of hBCATc in the synthesis of the neurotransmitter glutamate and as the precursor for glutamine synthesis required for GABA production in GABAergic neurons. Distribution of hBCATc expression in hormone secreting cells offers additional roles for hBCATc in hormonal regulation and secretion in the brain. Moreover, unlike the cell culture and rat

models which observed expression of BCATm in astrocytes, there is extensive immuno-positive labelling for hBCATm in the vasculature of the brain with no evidence of labelling within astrocytes. This suggests a different role for hBCATm in glutamate regulation in the human brain from that previously proposed by Hutson *et al.,* (1998). It was further demonstrated in this work that hPDI and hBCAT localised to the same cell type in both neuronal cells and the vasculature. This and other findings are discussed in further detail with respect to published models.

7.1.1 Localisation and proposed function of hBCATm in the brain vasculature

The expression of hBCATm was pronounced throughout the endothelial layer examined in all areas of the human brain. The endothelial layer forms the blood brain barrier (BBB) and acts as a physical barrier through the formation of tight junctions between adjacent cells, separating the luminal (blood-facing) and abluminal (brain-facing) surfaces (reviewed in Abbott *et al.,* 2006). Cerebral endothelial cells have a high density of mitochondria (Oldendorf *et al.,* 1977), giving greater capacity for energy production than other capillary endothelial cells and explaining the punctuate labelling of mitochondrial hBCATm in these cells. Although smaller molecules such as CO_2 and O_2 and lipophilic compounds can permeate this barrier, membrane-specific transporters on both the abluminal and luminal side of the barrier dictate and regulate the passage of nutrients and metabolites. As labelling for hBCATm was consistently located in the endothelial layer it is relevant to

consider the transport mechanisms of the BCAAs and glutamate within these cells.

The BCAAs, particularly leucine can easily traverse the BBB through the L-system facilitative transporters (Oldendorf, 1973; Smith, *et al.,* 1987; Zlokovic, 2008). Conversely, it is widely accepted that the facilitative carrier on the luminal side allows minimal glutamate entry, which is thought to protect the brain from peripheral glutamate plasma changes that could be potentially toxic to brain cells. Light and EM studies observed that the Na+-dependent excitatory amino acid transporters (EAAT) are on the astrocytic feet rather than the abluminal capillary endothelial membrane (Chaudhry, *et al.,* 1995) and evidence for EAAT1, EAAT2 and EAAT3 on this membrane has been reported (O'Kane *et al.,* 1999). One possibility is that these transporters associate with the endothelial membrane upon exposure to excess glutamate; however this also raises the possibility of regular glutamate transport to vascular cells (Beart and O'Shea, 2007). The presence of EAATs indicates that endothelial cells are equipped for abluminal uptake of glutamate, playing a role together with astrocytic cells in controlling glutamate regulation in the brain. This work demonstrates that the endothelial cells themselves have metabolic machinery, the hBCATm protein, to metabolise this glutamate indicating a potential role in neuroprotection.

Previous studies utilising astro-glial primary cultures derived from the brains of new-born Wistar rats demonstrated that BCATm was largely located in astrocyte populations, the basis of which formed the hypothetical BCAA-

BCKA nitrogen shuttle (Figure 1.6) (Yudkoff *et al.,* 1996; Yudkoff, *et al.,* 1997; Hutson *et al.,* 2001). In contrast, no astrocyte labelling was detected in the human brain. The explanation for this discrepancy is not clear. One possibility is that the expression of BCATm is induced under conditions of cell proliferation, as demonstrated in studies by Perez-Villasenor *et al.,* and Ichihara (Ichihara, 1975; Perez-Villasenor *et al.,* 2005). Bixel *et al.,* appreciated that the presence of BCAT in cell culture models may be spurious as the cells are taken out of their usual context, and cellular interactions are not appreciated (Bixel *et al.,* 1997). Alternatively, these studies may highlight the difference in BCAT expression between species that was previously reported by Goodwin *et al.,* (1987) who observed differential BCAT expression in skeletal muscle in sheep compared to mouse models.

Utilising rat models, it was originally proposed by Hutson *et al.,* (1998) that transamination catalysed by BCATm should operate in the direction of glutamate production due to the relatively high concentration of αKG in the mitochondria and the relatively low concentration of glutamate (Figure 1.6) (Hutson *et al.,* 1988). Contrary to this, fasting rat hepatocytes infused with lactate, pyruvate and oleate (conditions expected to result in glutamate accumulation) observed that mitochondrial concentrations of glutamate were 10-20 mM and up to 35 times higher than that in the cytosol (Zuurendonk *et al.,* 1976). Glutamate may well be the more concentrated metabolite, or under pathologically high levels of glutamate, the reverse reaction becomes favourable. Here, in these studies hBCATm was detected in the vasculature,

it is therefore proposed that the role of the hBCATm isoform in endothelial cells is to operate co-operatively with astrocytes and neuronal cells to metabolise glutamate produced upon excitation, (taken from either the ECF or through interaction with the astrocytic feet) with the BCKAs forming αKG and the respective BCAAs. These BCAAs may subsequently be taken up by the brain, to replenish glutamate stores in neurons (by hBCATc) or removed and exchanged into the blood, leaving αKG to enter the TCA cycle (Figure 7.1).

Currently, the proposed mechanism by which the endothelial cells metabolise or remove excess glutamate is by direct uptake of glutamate through Na+-dependent transporters. This creates a rise in the glutamate gradient in the endothelial cells, which has been proposed to subsequently exit into the blood through facilitated transport (O'Kane, *et al.,* 1999). A more complex, yet to be demonstrated pathway, through the glutamate-glutamine cycle, was suggested to involve the initial conversion of glutamate to glutamine in astrocytes, followed by its transfer to endothelial cells where it is recycled to glutamate and delivered to the blood. Neither model included the potential for further metabolism of glutamate within endothelial cells. The novel finding of the hBCATm in these cells therefore raises the possibility of an alternative mechanism of glutamate regulation in the brain. However in cell models, Hutson *et al.,* demonstrated that in astrocytes containing BCATm, BCKAs but not BCAAs stimulates transamination of glutamate to αKG and decarboxylation to pyruvate and lactate (i.e. the oxidative breakdown of glutamate) (Hutson *et al.,* 1998). This relates to the hBCATm expression in

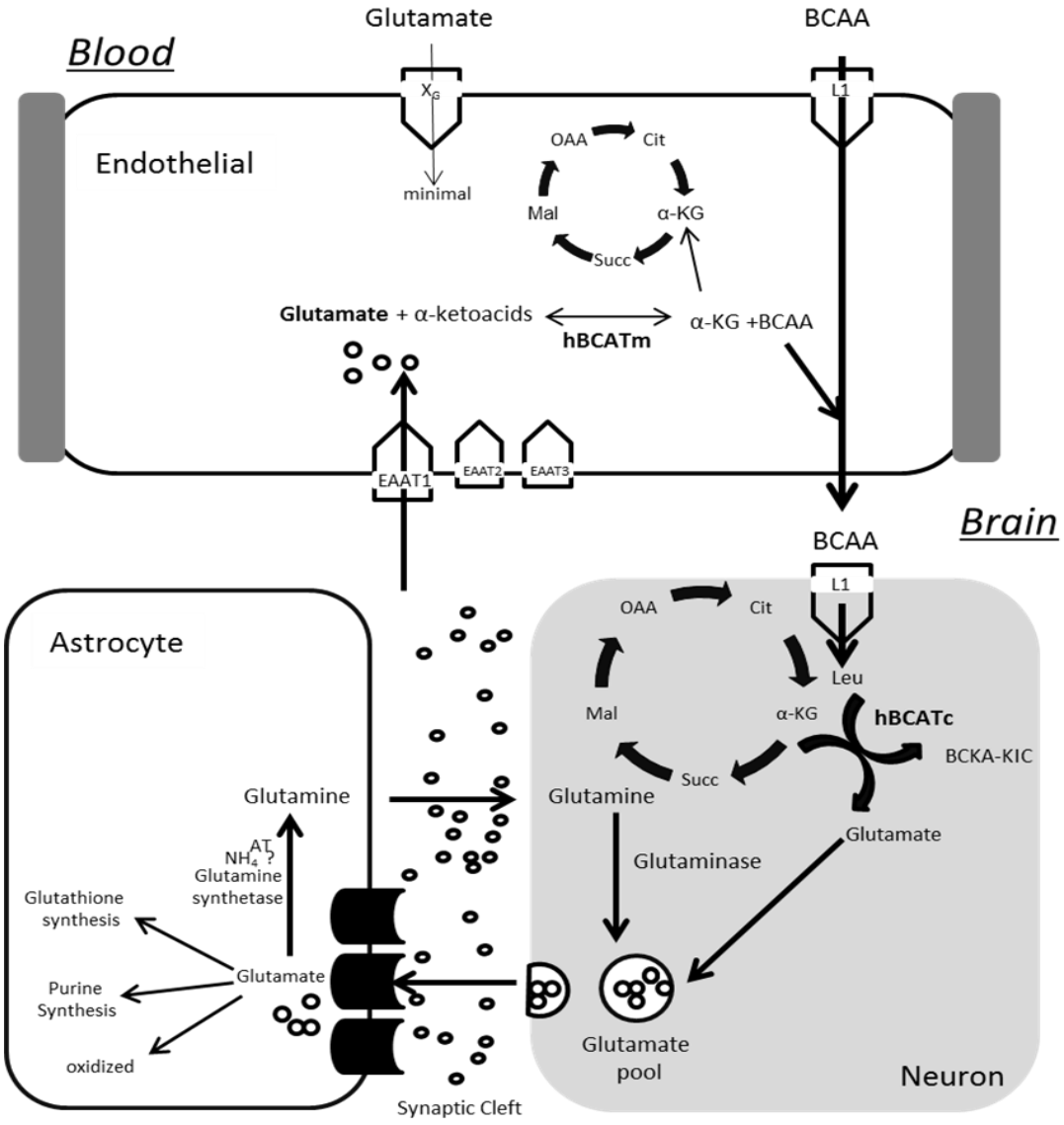

Figure 7.1 Model proposed for hBCAT signalling in the human brain. hBCATc in neurons sustains the neuronal glutamate pool using branched chain amino acids (BCAAs) and hBCATm in the brain endothelium works to recycle these BCAAs. Abbreviations: αKG — α-ketoglutarate; BCKA — branched chain keto-acids; EAAT—excitatory amino acid transporter; L1—large neutral amino acid transporter.

the vasculature of the human brain and would promote it as an energy source in these cells if glutamate is not used to restore BCAA in the model proposed earlier. In this model, transamination via hBCATm would produce both glutamate and a BCKA, leading to the oxidation of glutamate and BCKA as an energy source. However, these cell model studies were performed on cell culture models and are not necessarily comparable to *in vivo* transamination. In fact, BCAA utilisation as an energy source is a common occurrence in immortal or rapidly proliferating cells (Ichihara, 1975; Perez-Villasenor *et al.,* 2005).

In addition to the proposed role of hBCATm in endothelial cells, this isoform may operate in synergy with its cytosolic counterpart, hBCATc in neuronal cells, to maintain the glutamate pool. The role of BCAAs in glutamate production is based on studies that observed BCAAs labelling a significant proportion of the glutamate pool in neuronal cell cultures, which has also been demonstrated in *in vivo* rat retina models (Yudkoff *et al.,* 1983; Yudkoff *et al.,* 1994; Kanamori *et al.,* 1998; LaNoue *et al.,* 2001). However, this may not necessarily be the case and both isoforms may be operating independently of each other.

7.1.2 Localisation and proposed function of hBCATc in neurons

Similar to the rat studies by Garcia-Espinosa *et al.,* (2007) the majority of hBCATc-labelled neurons in the human brain were either GABAergic or glutamatergic, with GABAergic neurons observed to be more strongly immunopositive than glutamatergic neurons. However, many neurons that

were neither glutamatergic nor GABAergic also labelled for hBCATc. The distribution and the varied intensities of expression in the hippocampus, where labelling of the CA3 region was more intense than the CA1 region, mirrored the findings of Castellano *et al.,* (2007) who investigated mRNA expression of hBCATc in the postnatal and adult brains of mice.

The cell bodies of neuronal cells within the temporal and hippocampus showed intense labelling relative to the dendrite regions reflecting the possibility that the primary role of hBCATc is to contribute to the metabolic pool used to generate other neurotransmitters (i.e. GABA) rather than the neurotransmitter pool used during excitation (i.e. glutamate). Conversely, in the areas of the supraoptic tract, intense labelling along axons was noted, indicating an additional role of hBCATc transamination in glutamate production and release in this region. These findings are consistent with the rat metabolic studies, indicating that hBCATc transamination contributes to the pool of neurotransmitters and the glutamate required for release during excitation. However, contrary to the BCAA-BCKA shuttle it is suggested here that the direction of metabolism is in favour of glutamate generation rather than the generation of leucine. Metabolism of BCAAs in this model can generate all the major neurotransmitters, either glutamate, GABA or acetyl choline (Figure 7.2).

Magnocellular neuroendocrine cells were observed to express high levels of hBCATc, in the supraoptic nucleus and the paraventricular nucleus of the hypothalamus. In response to osmo-receptors in the magnocellular

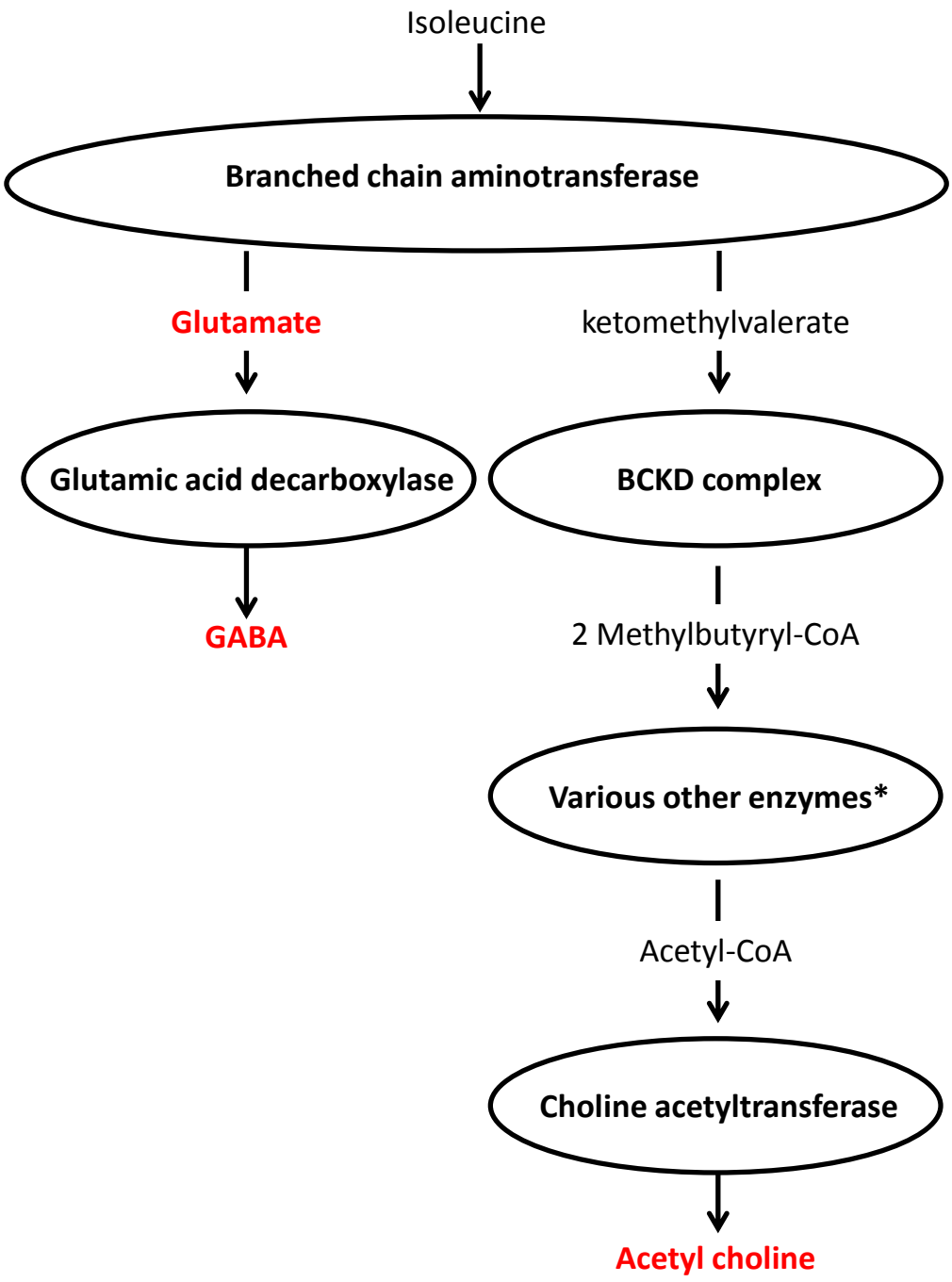

Figure 7.2 The metabolism of the branched chain amino acid isoleucine to produce neurotransmitters. The first step shows the transamination of isoleucine into its respective keto acids. This glutamate can be used as a neurotransmitter (glutamate is the main excitatory neurotransmitter in the human brain) or further metabolised to GABA (the main inhibitory neurotransmitter). The BCKA is metabolised further to the CoA product 2Methylbutyryl-CoA. This undergoes various other metabolic steps to produce Acetyl-CoA. Abbreviations: BCKD – branched chain keto acid dehydrogenase complex, GABA – γ-aminobutyric acid.
* These various enzymes are: 1) Acyl-CoA dehydrogenase, 2) Enoyl-CoA hydratase, 3) 3Hydroxy acyl-CoA dehydrogenase, and 4) Acetyl-CoA acyltransferase.

neuroendocrine cells, which are rich in AMPA and NMDA receptors, are stimulated by glutamate. Together with intrinsic membrane conductance, this controls the bursting pulsatile release of oxytocin and vasopressin (reviewed in Pak *et al.,* 2002). Precedence for BCAT metabolites in hormone secretion has been reported. Both KIC and leucine were demonstrated to be potent insulin secretagogues (Xu *et al.,* 2001; Zhou *et al.,* 2010). Although a number of mechanisms have been proposed recent data indicates that KIC induced secretion is dependent on the transamination of glutamate with KIC to leucine and αKG, which is further metabolized in the TCA cycle generating an increase in ATP, that stimulates insulin secretion (Zhou *et al.,* 2010).

This work proposes that similar mechanisms could operate in the magnocellular neurons and the substantia nigra that also show intense hBCATc expression in these dopaminergic neurons. Both dopamine and vasopressin release is inhibited through NO mediated interactions (Vacher *et al.,* 2003; Lu *et al.,* 2009). The hBCAT proteins have redox sensitive CXXC motifs, which are reversibly inhibited through S-nitrosylation (Conway, *et al.,* 2004; Conway, *et al.,* 2008; Coles *et al.,* 2009). Should transamination factor in vasopressin or dopamine release this work suggests that NO inhibition of hBCATc would reduce levels of αKG, leading to a drop in ATP and stopping the release of these hormones. These findings offer hints of intriguing new roles for hBCATc in hormone regulation that are intrinsically linked to the redox status of the cell.

Work by Sweatt *et al.,* (2003) observed that intense labelling for BCATm was in the serous-secretory epithelial cells throughout the digestive tracts, secretory cells of the exocrine pancreas, the theca interna secretory cells in the ovarian follicles and secretory epithelial cells of the endometrial glands located within the uterus (Sweatt *et al.,* 2003). It was concluded within this study that the role of hBCATm in the secretory context was to supply KIC or leucine to stimulate secretion. The presence of hBCATc in the neurons of the nucleus basalis of Meynert and the raphe nuclei raises a similar possibility of a role for hBCATc in acetylcholine and serotonin release or intermediate production (similar to that observed by Sweatt *et al.,* for BCATm).

Moderate labelling of hBCATc in pigmented cells, such as the neurons of the locus coeruleus, that strongly express melanin concentrating hormone (MCH), may be related to the role of BCAAs in melanogenesis: incubation of melanoma cells with BCAA inhibited the production of melanin (Cha *et al.,* 2011). The mechanism underpinning this inhibition was not determined. However, as MCH plays an important role in the regulation of energy homeostasis, the mechanism of action of the BCAA is likely to mimic that of leucine and its role as a nutrient signal (Li *et al.,* 2009). MCH deficient mice have reduced food intake and a lean phenotype (Rossi *et al.,* 1997; Shimada *et al.,* 1998). In support, increased levels of blood BCAA were reported to improve body weight control, increase glucose metabolism and adiposity (Doi *et al.,* 2007; Layman & Walker, 2006). Together these findings suggest that both MCH and the BCAA, particularly leucine, operate co-operatively and

behave as nutrient signals fine tuning energy homeostasis, having complementary but opposing effects. Therefore, in addition to the role of the hBCAT proteins in glutamate metabolism there is evidence of a role for hBCAT proteins in controlling hormone secretion in the brain.

A genome wide association study has identified a possible link between hBCATc and resting heart rate (Eijgelsheim *et al.,* 2010). This is explained by either hBCATc expression in cardiac tissue, hBCATc effect on hormone release or the control of heart rate by neurons containing hBCATc. It was observed in this work that neurons of the vagus nerve are positive for hBCATc and this relates to the findings of Eijgelsheim *et al.,*(2010). It could be suggested that genetically reduced expression of hBCATc within these vagus nerve cells would lead to a decrease glutamate and BCKA production resulting in decrease neurotransmitter and energy availability for the cell, and thus a decreased firing rate of the neuron. This opens up new areas of opportunities of regulating heart rate by hBCAT inhibitors (e.g. gabapentin) – it is also expected that individuals receiving gabapentin treatment should have an altered resting heart rate and would confirm this supposition.

7.1.3 Mapping of the redox proteins hPDI to the human brain

In addition to the role of hBCAT in the regulation of glutamate metabolism, recent studies by our group have established that these redox reactive proteins have thiol/ disulphide isomerase activity and can interact with hPDI *in vitro*. As hPDI has been implicated in protein misfolding in AD it was

important to investigate the brain distribution of hPDI to demonstrate a physiological relevance to *in vitro* interactions with hBCAT.

The hPDI protein was expressed to a larger degree than either hBCATc or hBCATm, with hPDI expression in almost all neurons, vasculature and some white matter oligodendrocytes. Previous work investigating the hPDI protein in the human brain has focused not on distribution but disease association. Tissue distribution was investigated in murine models but lacked in depth analysis within the brain (Marcus *et al.,* 1996). S-nitrosylated hPDI has been demonstrated in sporadic Parkinson's disease and AD. It is thought that this S-nitrosylation links oxidative stress with protein misfolding in neurodegeneration (Uehara *et al.,* 2006). Studies in other examples of neurodegeneration (Creutzfeldt-Jacob disease) have observed overexpression of the hPDI protein thought to be related to the cellular defence response against misfolded proteins (Yoo *et al.,* 2002).

Here, light microscopy of serial sections of the human brain demonstrates that hPDI and hBCATm are indeed present within the same cell. Electron microscopy work provides further evidence that not only is hBCATm and hPDI present within the same cell, but they can also co-localise within the same organelle (i.e. mitochondria). This is not the first work to demonstrate hPDI localisation to the mitochondria but it is the first to suggest a functional relevance over that of singular isomerase activity (Rigobello *et al.,* 2001). Colocalisation of the hBCATm protein with hPDI in neuronal cells was curious as hPDI is considered to be largely associated with the ER.

However, locating hPDI to mitochondria, and in close proximity to hBCATm, suggests that the *in vitro* dithiol-disulphide isomerase activity of hBCAT together with its differential interaction with hPDI in neuronal cells under stress may have physiological relevance. We propose that hBCATm and hPDI work in synergy through dithiol-disulphide exchange and that this colocalisation could bring about a more efficient isomerase response.

7.1.4 Localisation summary

In summary, this work provides the first direct evidence of transaminases in the endothelial layer that can potentially operate as a support network to astrocytes mediating the fine tuning of glutamate homeostasis. This work proposes that under normal physiological conditions the BCAAs are taken up by neuronal cells, where hBCATc controlled transamination acts as a key pathway to replenish the glutamate pool. This glutamate is cycled to the endothelial cell layer by either the ECF or astrocyte processes and subsequently transaminated to BCAAs that are returned to the neuron for *de novo* glutamate production. These findings may have implications for the understanding of the mechanisms of development and prevention of glutamate toxicity, an important contributor to a range of neurodegenerative and neurovascular diseases. The serum aminotransferase proteins, aspartate and alanine aminotransferase, have been suggested to play a therapeutic role in reducing brain glutamate. However, the possibility of transamination within endothelial cells was not considered and it may be transamination here rather than in the plasma that is buffering brain

glutamate (Boyko *et al.,* 2011). Further research is needed to decipher the role of BCAT in glutamate regulation and toxicity in the brain.

7.2 Overexpression and post-translational modification of hBCAT in AD

Given the role the hBCAT proteins play in brain glutamate regulation, it was proposed that these proteins could be altered in diseases where glutamate toxicity is a pathological mechanism. Consistent with this hypothesis it was observed that hBCATm (but not hBCATc) correlated with the Braak staging of AD to a higher degree than amyloid. Expression of hBCATc was increased in the hippocampus, frontal and temporal cortex of AD subjects, albeit non-significantly increased in the frontal and temporal cortex suggesting a more prominent role of the vasculature in AD pathology when related to BCAA metabolism. Distributional analysis did not observe a new cell type of hBCATc or hBCATm expression upon AD pathology (i.e. glial cells remained BCAT negative regardless of disease process).

Also, a multiple banding pattern was observed for hBCATm that was prominent in AD between 40 and 60 kDa. Banding patterns similar to those observed often reflect adduct formation (i.e. phosphorylation, oxidation, S-nitrosation, S-glutathionylation and glycosylation) and are commonly associated with AD pathology (O'Brien & Wong, 2011; Riederer *et al.,* 2011; Schedin-Weiss *et al.,* 2013; Sultana & Butterfield, 2010). Based on cell and animal models, it is proposed that overexpression of hBCAT has an initial neuroprotective role but as products of their metabolism are toxic, the role may turn from neuroprotective to neurotoxic, potentially contributing to neuronal cell death. These findings are discussed in further detail with respect to other protein alteration commonly observed in AD.

7.2.1 Factors significantly associated with expression of hBCAT and proposed functional role in AD

The progression of AD is assessed post-mortem using the Braak staging system. This method observes Tau pathology across the human brain. This work demonstrates that the Tau (%) observed in the temporal cortex correlates closely with Braak stage. Measurement of Tau (%) mirrors overall pathology better than Aβ average (%) due to the variability associated with Aβ pathology (Braak & Braak, 1991). In AD, there are many proteins with altered expression that predominantly have functions relating to neurotransmitter metabolism, cellular metabolism, antioxidants and apoptosis (Table 7.1) (Butterfield *et al.,* 2003; Papassotiropoulos *et al.,* 2006).

The most novel finding of this work was the increased expression of hBCATm in AD subjects and it was important to relate this increased expression with pathological features. It was further noted that hBCATc expression was increased within the hippocampus of AD subjects relative to controls, although this increase did not reach significance in the frontal and temporal cortex. Based on animal models, it is possible that this increase indicates a role for these proteins associated with cell survival in the early onset of disease. Kholodilov *et al.,* (2000) demonstrated that BCATc was upregulated in the substantia nigra of rats following developmental striatal target injury (Kholodilov *et al.,* 2000). Although this upregulation correlated with cell death induction, the neurons that labelled positive for BCATc showed normal morphology and rarely contained apoptotic chromatin. It was

Table 7.1 Overview of protein expressional changes in AD compared to controls. Papers were summarised that contained information about the expressional changes occurring in AD (Engidawork *et al*., 2001[a,b]; Frolich *et al*., 1998; Papassotiropoulos *et al*., 2006).

Protein	Alteration in AD
Neurotransmitter related proteins:	
Branched chain aminotransferase	Increased
Glutamate transporter EAAT2	Decreased (Butterfield, 2004)
Glutamine synthase	Decreased (Castegna *et al*., 2002)
α4 nAChR	Decreased (Martin-Ruiz *et al*., 1999)
Cellular metabolism related proteins:	
GLUT 1 and 3	Decreased (Liu *et al*., 2008)
Insulin receptors	Increased (Frolich *et al*., 1998)
ATP synthase α-chain	Decreased (Tsuji *et al*., 2002)
ATP synthase β-chain	Increased (Tsuji *et al*., 2002)
NADH-UO (24 kDa)	Decreased (Kim *et al*., 2001)
NADH-UO (75 kDa)	Decreased (Kim *et al*., 2001)
Antioxidant system related proteins:	
Antioxidant protein 2	Increased (Krapfenbauer *et al*., 2002)
Peroxiredoxin I, II and VI	Increased (Krapfenbauer *et al*., 2003)
Peroxiredoxin III	Decreased (Kim *et al*., 2000)
Superoxide dismutase	Decreased (Gulesserian *et al*., 2001)
Apoptosis process related proteins:	
ZIP kinase	Increased (Engidawork *et al*., 2001a)
Bim/BOD	Increased (Gulesserian *et al*., 2000)
p21	Increased (Gulesserian *et al*., 2000)
Procaspase 3	Decreased (Engidawork *et al*., 2001b)
Procaspase 8	Decreased (Engidawork *et al*., 2001b)
Procaspase 9	Decreased (Engidawork *et al*., 2001b)
Immune system related proteins:	
IL-6	Increased (Gonzalo-Calvo *et al*., 2010)
TNFα	Increased (Gonzalo-Calvo *et al*., 2010)

Abbreviations: ATP – adenosine triphosphate; Bim – Bcl-2 interacting mediator of cell death; BOD – Bcl-2 related ovarian death gene; EAAT – excitatory amino acid transporter; GLUT – glucose transporter; IL – interleukin; nAChR – nicotinic acetylcholine receptor; NADH – Nicotinamide adenine dinucleotide; TNF – tissue necrosis factor; UO – ubiquionone oxidoreductase; ZIP – zipper interacting protein.

proposed that BCATc upregulation might have a protective role in this form of neuronal damage or represent a return to normal function of the neuron.

Here, in human neuronal cells that label positive for hBCATc and vascular cells that label positive for hBCATm, cells were morphologically intact supporting this theory. And although brain glutamate is primarily controlled via the glutamate/glutamine cycle, recent studies supporting the importance of the endothelium in this regulation are gaining impetus (Helms *et al.,* 2012). Utilising a bovine endothelial/rat astrocyte co-culture model the K_m value of trans-endothelial glutamate efflux was estimated at about 138 µM (Helms *et al.,* 2012). Under pathophysiological conditions such as ischemia, traumatic head injury or neurodegenerative conditions (AD), where levels of glutamate are estimated to rise considerably in the interstitial fluid (up to 200 µM), the proposed BBB-efflux system could potentially assist in the control of glutamate ISF level. Therefore glutamate uptake and metabolism by hBCATm would be of benefit to glutamate homeostasis, when glutamate levels are toxic, indicating a possible role for this protein as an auxiliary neuroprotection mechanism.

Despite this, hBCATm shows greater expression with increasing Braak stages, increasing amyloid load (either soluble or insoluble), Tau load (%) and decreasing brain weight indicating that upregulation is persistent and correlates with many key aspects of the underlying pathology associated with AD. Perhaps over time, this hBCATm expression outlasts the neuroprotective role or becomes compromised by the accumulation of toxic

metabolites i.e. glutamate and the BCKAs. In support of this, increased number of fragmented vessels, increased capillary basement membrane thickening, increased collagen accumulation and reduced microvascular density are all observed in AD (Bailey *et al.,* 2004; Farkas & Luiten, 2001; Wu *et al.,* 2005).

Liu *et al*., (2008) demonstrated that a decrease in the glucose transporters GLUT1 and GLUT3 correlated with the hyperphosphorylation of tau and the density of neurofibrillary tangles in the human brain. It was proposed that this was causing a decreased glucose uptake in neurons and this in turn was leading to tau pathology. For hBCATm, the correlation with Braak stage may well relate to a similar energy requirement and demonstrates that hBCATm is correlating with disease pathology (i.e. Braak stage) rather than disease onset. Metabolism of the BCAAs produce BCKAs and glutamate – these BCKAs can then be transported to neurons to be further metabolised to produce Krebs cycle intermediates and provide energy for the cell. It is probable that this would be occurring alongside and working to counteract any decreased glucose utilisation within neurons. Similar to Braak pathology, the increased expression of hBCATm associated with decreasing brain weight may relate to the energy requirement of aging and AD pathology. As aging, and dementia progress there is a decrease in brain weight that is thought to reflect a functional loss of mental capacity (particularly in the case of dementia) (Double *et al*., 1996). This strongly implies that hBCATm is correlating with the underlying pathology of AD and serum hBCATm may

represent a novel marker of this providing there is release of hBCATm or there is turnover of these cells.

The BCKAs are unique products of BCAA metabolism and are not generated by other enzyme systems. The consequences of increased levels of BCKAs and BCAAs are manifested in the clinical condition MSUD (Silberman *et al.,* 1961). Mechanistically, high levels of these metabolites have been associated with an inhibition of cell proliferation and a prolonged increase in the G1 stage of the cell division cycle (Liao *et al.,* 1978). In fact in this work, increased exposure of neuronal cells to KIC resulted in a decrease in cell viability and a loss of cell morphology. Although dietary supplementation of BCAAs is widely used by professional sport people, thresholds for adverse effects in humans have yet to be determined (Fernstrom, 2005). A higher incidence of ALS (a chronic neurodegenerative condition, where glutamate toxicity features), has been reported in professional soccer players, where overuse of BCAAs, among other factors, has been implicated in the pathogenesis of this disease (Armon, 2007; Chio *et al.,* 2005; Piscopo *et al.,* 2011; Vanacore *et al.,* 2006).

The correlation of increased SVD score with increased hBCATm expression relates to the presence of hBCATm within the vasculature. It is possible that SVD and hBCATm are reflecting an overall decrease in vascular function. Greater than 30% of all AD cases exhibit significant vascular pathology and these are often accompanied by perivascular denervation – a causal factor in cognitive decline (Kalaria, 2002). In fact, klf-15 (the transcription factor

associated with hBCATm) is widely expressed in vascular cells and has been demonstrated to be an essential regulator of the vascular smooth muscle cells response to injury (Lu *et al*., 2010). In this model, after vascular injury klf-15 is down regulated and this acts as a negative feedback loop to inhibit proliferation of the vasculature. This is to prevent unrestrained smooth muscle cell activation which will lead to an increased proliferation of smooth muscle cells and a decreased vascular permeability (Lu *et al*., 2010). The fact that hBCATm expression is increased despite the vascular damage promotes the idea that there is a defect in this feedback loop. It is possible that the lack of klf-15 suppression leads to a decrease in vascular permeability due to the unrestrained smooth muscle cell activation. This decreased vascular permeability will lead to many pathological aspects associated with AD such as mild hypoxia, decreased BBB glucose uptake and glutamate toxicity.

Glutamate toxicity has been implicated in the pathogenesis of AD, here it is proposed that overexpression of hBCATc in neurons and hBCATm in the vasculature is initiated to promote cell survival in early AD but that this neuroprotective role becomes compromised with increased exposure to the metabolites, glutamate and BCKAs. The significant overexpression of hBCATm in the endothelial layer of the vasculature in the AD brain could also reflect the brains response to metabolise toxic levels of glutamate generated under pathogenic conditions.

7.2.2 Oxidation and S-nitrosylation in AD and the hBCAT protein

Oxidative stress is a key mediator of AD pathology and is associated with carbonyl, S-nitrosylation and S-glutathionylation adducts. Oxidative stress may cause reversible or irreversible modifications to sensitive proteins leading to alterations in structure, function or stability. In AD, the levels of antioxidants are decreased and therefore an increase in protein oxidation, lipid peroxidation, DNA oxidation and reactive oxygen species is observed (Pratico, 2008; Sultana *et al.,* 2010). Both senile plaques and NFTs are capable of generating oxidative stress through activation of glial cells (i.e. the immune response), protein binding (Aβ can bind to the NMDA receptor, increasing permeability) or channel formation (Aβ can produce calcium permeable channels in the cell membrane) all leading to an increasingly oxidative environment (Glabe, 2006; Gouras *et al.,* 2010; Jang *et al.,* 2009: Gotz *et al*., 2011). In addition to this, Aβ has a synergistic action with glutamate to induce neuronal damage through a nitric oxide (NO) dependent mechanism (Alberdi *et al.,* 2010; Li *et al.,* 2009).

One of the key mechanism by which oxidation regulates the hBCAT proteins is via their conserved CXXC motif. This CXXC motif is located 10 Å from the active site and under oxidizing conditions these cysteine residues can form a disulphide bond and thus act as a redox switch in the hBCAT protein. In facts, a loss of 40-45% hBCATc activity occurs upon air oxidation alone (Conway *et al*., 2008). In the study by Conway *et al*., (2002), H_2O_2 abolished hBCATm activity and resulted in the formation of an intra-subunit disulphide bond between Cys315 and Cys318. For hBCATm, the loss of a single thiol

group occurs rapidly and results in a 56% decrease in activity. The loss the second thiol occurs at a much slower rate and results in a further 35% decrease in activity. During non-oxidizing conditions the Cys315 residue plays a structural role by anchoring Tyr173 during catalysis and this is lost upon disulphide bond formation. The reducing agent DTT completely reversed this H_2O_2 induced disulphide bond and restored activity (Conway *et al*., 2002).

Further work demonstrated that mutation of the Cys315 residue resulted in a loss of sensitivity to H_2O_2, whereas mutation of the Cys318 residue maintained its sensitivity of H_2O_2 but loss of activity was irreversible despite addition of DTT. This was due to an over-oxidation of the Cys315 residue. It was proposed that Cys315 was the 'sensor' and Cys318 was the 'resolving cysteine' in the formation of the disulphide bond (Conway *et al*., 2004). If the higher bands observed in hBCATm Western blot are representations of various oxidation states then it stands to reason that these proteins have a decreased aminotransferase activity. In fact, work in rats observed a 41 and 43 kDa banding pattern for BCATm and it was only the 41 kDa BCATm that had activity associated with it, with the band at 43 kDa not associated with detectable BCAT activity (Perez-Villasenor *et al.,* 2005).

Other redox sensitive proteins similar to hBCAT, such as GAPDH (where protein expression is unaltered but the protein is oxidized), ATP synthase, triose phosphate isomerase, α-enolase and γ-SNAP are all heavily oxidised in post-mortem analysis of the AD hippocampus, whereas no difference

could be observed in the cerebellum. In the inferior parietal lobe studies also observed oxidation of excitatory amino acid transporter 2 (EAAT2), creatinine kinase (CK), glutamine synthase (GS), γ-enolase, neuropolypeptide h3 and lactate dehydrogenase (reviewed by Butterfield *et al.,* 2006; reviewed by Sultana *et al.,* 2006). This suggests that the post-translational modification of protein thiol oxidation is prevalent in the AD brain. Similar to the hBCAT proteins, many of these function in energy production pathways (GAPDH, ATP synthase, CK, enolase) or in neurotransmitter signalling (EAAT2, GS, neuropolypeptide h3).

Physiological NO is produced largely by arginine dependent NO synthetase (NOS), neuronal NOS, inducible NOS and endothelial NOS and like protein oxidation, protein S-nitrosylation has a fundamental physiological role exerted predominantly through functional regulation of proteins (Hess & Stamler, 2011). Reactive nitrogen species generated by calcium dependent NOS are not toxic (as such), but rather a signalling process. For example, lower concentrations of NO are considered anti-apoptotic, while higher concentrations are apoptotic (Pacher *et al.,* 2007).

Proteins with reactive cysteine residues are primary targets for NO modification. These effects are often transitory and a part of NO signalling. However, excessive NO or prolonged exposure can damage proteins and ultimately lead to cellular damage (Fang *et al*., 2007). Coles *et al*., (2009) demonstrated that GSNO (an NO donor) dose dependently inhibited hBCAT activity, with activity reduced by 50% and 77% for hBCATc and hBCATm,

respectively. It was further demonstrated that hBCATc is initially S-nitrosylated and that this leads to S-glutathionylation at increasing levels or duration of NO exposure. Although neither S-nitrosylation nor S-glutathionylation was observed for the hBCATm protein, full reactivation for both enzymes required a complete GSH/Grx reducing system implying both enzymes are sensitive to S-nitrosylation (Coles *et al*., 2009).

S-nitrosylation of tau occurs in AD, with S-nitrosylated tau present in pre-tangles, tangles and tau inclusions. As the nitration was prominent in pre-tangles it is assumed that tau nitration is an early event in AD pathology (Horiguchi *et al*., 2003). Specific nitration in the AD brain was also observed in α and γ-enolase, parkin, peroxiredoxin 2, protein disulphide isomerase, lactate dehydrogenase, triosephosphate isomerase, GAPDH, ATP synthase and voltage dependent anion channel protein 1 (Castegna *et al*., 2003; Sultana *et al*., 2006). Due to the fact that many of these are mitochondrial enzymes it was thought that the increased S-nitrosylation observed in AD is the result of mitochondrial leakage of electron equivalents and protein nitrating agents resulting in increased protein nitration (Castegna *et al*., 2003; Sultana *et al*., 2006). If the S-nitrosylation observed in AD is associated with the mitochondria it would explain why adducts are associated with hBCATm over hBCATc, due to the cellular distribution of the proteins.

Like the hBCAT protein, hPDI and other oxidoreductases also possess a reactive CXXC motif (Yennawar *et al*., 2006). The study by El Hindy *et al*., (2013) demonstrates that hBCAT proteins catalyse the insertion of disulphide

bonds into denatured RNase releasing active RNase, with the active-cysteines playing a central role in this mechanism. Other CXXC containing redox sensitive proteins similar to hBCAT, such as thioredoxin and platelet integrin alpha IIbbeta 3, have endogenous thiol isomerase activity (Langenbach *et al*., 1999; Lundstrom & Holmgren, 1990; O'Neill *et al*., 2000). It was further demonstrated by El Hindy *et al*., (2013) that the combination of hPDI and hBCAT demonstrated greater isomerase activity than that of the two proteins individually.

In relation to this work, the localisation of hPDI to mitochondria, as observed from electron microscopy, and in close proximity to hBCATm, demonstrates that they can colocalise within cells. This work further demonstrated *in vivo* localisation of hPDI and hBCAT to the same cell types within the human brain, with hPDI localised to the vasculature (in the same cells and hBCATm) and to neurons (in the same cells and hBCATc). This work suggests that the observed increase in *in vitro* dithiol-disulphide isomerase activity of hBCAT together with hPDI may have physiological relevance. It is therefore proposed that hBCATm and hPDI work in synergy *in vivo*, through thiol-disulphide exchange, and that hBCATm could potentially act as a chaperone to assist hPDI in facilitating protein folding.

The proposed interaction of hPDI and hBCAT will be governed by the redox state of the cell. Under cellular stress, oxidation of hBCATm by hydrogen peroxide will result in the formation of a disulphide bond between the reactive cysteine residues and not only render the protein catalytically inactive but

prevent further association with hPDI, as hPDI itself is redox sensitive. These are complex systems, and it remains to be determined whether hBCATm acts as a molecular chaperone similar to hPDI in its own right, to facilitate dithiol-disulphide exchange in misfolded proteins, or behaves like the Ero1 oxidase, providing a sink of exchangeable thiols for hPDI during catalysis. S-nitrosylation, like oxidation results in a decrease in hBCATm activity (Coles *et al*., 2009), but is also known to affect hPDI function. In hypoxia the accumulation of immature and denatured proteins results in ER dysfunction. There is an observed upregulation of hPDI associated with this and represents an adaptive response to hypoxia (Tanaka *et al*., 2000). In AD, it was observed that hPDI is S-nitrosylated and this will lead to a decreased activity of the protein. This in turn will lead to the accumulation of misfolded proteins and further activate the unfolded proteins response (Uehara *et al*., 2006).

In summary, both oxidation and nitrosation are key regulatory factors of the hBCAT and hPDI proteins. However, S-nitrosylation of hBCATm would not lead to a higher molecular weight band due to the reactive cysteines forming a disulphide bond rapidly under these conditions (Coles *et al*., 2009). However, it is possible that excessive S-nitrosylation may occur *in vivo*, particularly in pathology, despite what is observed *in vitro*. It is also possible that over oxidation of hBCATm would lead to an increased presence of irreversible sulphinic or sulphonic intermediates associated with the cysteine residues which would lead to an increased molecular weight. The oxidoreductase activity described for the hBCAT proteins would not operate

with over oxidation of the hBCAT protein. However, this associates hBCAT with the misfolded protein response and this raises the possibility that the upregulation of hBCAT observed in AD is not necessarily associated with the proteins aminotransferase activity.

7.2.3 S-glutathionylation in AD and the hBCAT protein

S-Glutathione (GSH) is the predominant antioxidant in the human brain reaching mM concentrations within cells. This GSH can react with free radicals to form oxidized GSH (GSSG) or react with reactive cysteine residues, like that observed in hBCAT, to produce S-glutathionylated proteins. This GSSG is subsequently recycled back to two GSH molecules by GSH reductase. In AD, peripheral lymphocyte GSH levels are decreased and GSSG levels are increased (Calabrese *et al.,* 2006). In fact, a direct correlation was demonstrated between increasing GSSG levels and decreased cognitive status as measured by the mini mental state examination (Lloret *et al.,* 2009).

It was previously discussed that S-nitrosylation of the hBCAT proteins lead to a reduction in activity of 50% and 77% for hBCATc and hBCATm respectively (Coles *et al*., 2009). It was demonstrated in this work that this S-nitrosylation leads to the S-glutathionylation of hBCATc in a mechanism similar to that described for the Trx and Grx proteins (Hashemy & Holmgrem, 2008; Hashemy *et al*., 2007). While hBCATm was inactivated by S-nitrosylation, neither S-nitrosylated nor S-glutathionylated products were detected (Coles *et al*., 2009). Similarly, GSNO mediated S-glutathionylation was not

observed for the mitochondrial Grx2 (Goto *et al*., 2005). It was proposed that this was due to an insensitive response to S-glutathionylation observed for hBCATm compared with hBCATc, with S-glutathionylation of hBCATm not observed until the redox environment was predominantly more oxidising (Conway *et al*., 2008). Both the hBCATc and hBCATm proteins show a reduced activity of 45% after S-glutathionylation, which is completely reversed upon reducing conditions (Coles *et al*., 2009; Conway *et al*., 2002; Conway *et al*., 2008).

Due to the chemically reactive nature of certain cysteine residues, there is the potential to generate other forms of modified cysteine groups that are either reversible (RSSR and Cys-SO) or irreversible (Cys-SO_2 and Cys-SO_3). This progression of oxidation of cysteine residues in redox sensitive proteins leads to the irreversible inactivation of the protein similar to that observed in the Cys318 mutant hBCATm protein discussed previously (Conway *et al*., 2004). Work by Islam *et al*., (2007) demonstrated that the reduced state of the CXXC motif was necessary for the binding of the hBCATm protein to the E1 subunit of BCKD. Thus, the CXXC motif of the hBCAT protein has a role not only in BCKA production but also the further metabolism of BCKAs (Islam *et al*., 2007). Similar occurrences were also observed for hBCATc in that the reduced state of the CXXC motif was necessary for the association of hBCATc with several neuronal proteins including tubulin β-chain, septin-4, sodium channel type 10 α-subunit and myosin-6 (Conway *et al*., 2008).

It was further observed in the work by Conway *et al*., (2008) that S-glutathionylation produced an altered banding pattern for hBCATc – with the appearance of two bands rather than one at a slightly decreased molecular weight (~43 kDa to ~40-43 kDa). This may relate to this work and explain some of the multiple bands associated with hBCATm. However, only two bands occurred with hBCATc S-glutathionylation even in the presence of physiologically excessive H_2O_2 concentrations (500 µM). And as hBCATc has four reactive thiols compared to the two of hBCATm, it can be assumed that S-glutathionylation would only account for two of the multiple bands observed for hBCATm.

It was demonstrated in this work that S-glutathionylated proteins were in fact decreased significantly by 35% in the frontal cortex, and non-significantly by 10% in the temporal cortex of AD subjects. In support of these findings another study demonstrated, utilising 3-(N-maleimido-propionyl) biotin binding, that although total SH groups were unaltered between AD and controls; there were decreased levels of protein bound SH groups (by 33%) in the AD hippocampus but not the cerebellum (Aksenov & Markesbery, 2001). This likely represents an increased consumption of free GSH in AD, with an inevitable decline in overall levels – similar to that demonstrated by Calabrese *et al.,* (2006). This study further demonstrated an increased level of glutathionylated protein with increasing age in the frontal and temporal cortex and decreasing Braak stage in the frontal cortex. It is appreciated that levels of GSH are decreased in diseases where oxidative stress is a pathological mechanism (such as AD) in addition to an overall decrease

occurring with age (Bermejo *et al.,* 2008). Proteins sensitive to oxidation (such as hBCAT) are therefore at increased risk of S-glutathionylation and that is observed in this work. Although it is not clear whether increased S-glutathionylation of proteins represents (i) a regulatory mechanism to inactivate proteins, (ii) a protective mechanism to prevent irreversible oxidation of residues critical to protein function, or (iii) a 'redox sink' mechanism to conserves cellular GSH under conditions of increasing oxidative stress.

It is further proposed in this work that the decreased association of S-glutathionylated protein observed with increasing Braak stage relates to an increased consumption of GSH, similar to that proposed in AD. S-glutathionylation of tubulin has been associated with the repair of damaged cytoskeletal proteins, of which tau is a member (Landino *et al*., 2007). It is thus suggested that proteins like tau and hBCAT that require S-glutathionylation are at increased risk of irreversible oxidative damage in AD due to the decreased presence of free GSH. It is likely that free GSH decreases with age due to an increasingly oxidative environment associated with the aging process which is demonstrated in this work (Bermejo *et al.,* 2008).

In summary, previous work demonstrates the importance of the CXXC motif in hBCAT function. In addition to demonstrating a decreased activity upon S-glutathionylation of 45% and an irreversible loss of activity upon complete oxidation of the cysteine residues (Coles *et al*., 2009; Conway *et al*., 2002;

Conway *et al*., 2008). Increased S-glutathionylation of the hBCAT protein is likely to occur with increasing brain age, however it is not clear whether this is to protect protein function or a 'redox sink' for GSH. It is proposed in this work that the decreased S-glutathionylated protein observed with increasing Braak stage and AD represents a consumption of total GSH within the cell due to excessive oxidative processes – such as that generated by senile plaques and NFTs (Glabe, 2006; Gouras *et al.,* 2010; Jang *et al.,* 2009: Ittner & Gotz, 2011).

7.2.4 Phosphorylation in AD and relation to BCAT

Phosphorylation is the process of adding a phosphate group to a protein and regulates protein function. This regulation can either be inhibition, activation, improved protein-protein interaction or increased protein degradation and is the best characterised adduct associated with AD (Hanger *et al.,* 2009). For example; phosphorylation of src tyrosine kinase induces a conformational change that results in the kinase domain becoming inaccessible and therefore inhibits kinase activity, whereas phosphorylation of NADPH oxidase subunits allows these proteins to interact with each other to generate superoxide, and phosphorylation of inhibitory protein of the κB family (α) promotes degradation by the ubiquitin proteasome pathway (Cole *et al.,* 2003; Liu & Chen, 2010). Both hBCAT isoforms have phosphorylation sites, with a possible 8 phosphorylation sites for hBCATc (protein kinase C (PKC), tyrosine kinase, cAMP and cGMP dependent protein kinase and casein kinase II sites) and a possible 8 phosphorylation sites for hBCATm (protein kinase C, tyrosine kinase and casein kinase II sites) (Figure 7.3).

hBCATc:
MKDCSNGCSAECTGEGGSKEVVGTFKADLIVTPAIVTPATILKEKPDPNNL
VFGTVFTDHMLTVEWSSEFGWEKPHIKPLQNLSLHPGSSALHYAVELFE
GLKAFRGVDNKIRLFQPNLNNMDRMYRSSAVRATLPVFDKEELLECIQQL
VKLDQEWVPYSTSASLYIRPTFIGTEPSLGVKKPTKALLFVLLSPVGPYFS
SGTFNPVSLWANPKYVRAWKGGTGDCKMGGNYGSSLFAQCEAVDNGC
QQVLWLYGEDHQIT**EVGTMNLFLYWINEDGEEELATPPLDGIILPGVTRR**
CILDLAHQWGEFKVSERYLTMDDLTTALEGNRVREMFGSGTACVVCPVS
DILYKGETIHIPTMENGPKLASRILSKLTDIQYGREERDWTIVLS

hBCATm:
MAAAALGQIWARKLLSVPWLLCGPRRYASSSFKAADLQLEMTQKPHKKP
GPEPLVFGKTHTDHMLMVEWNDKGWGQPRIQPFQNLTLHPASSSLHYS
LQLFEGMKAFKGKDQQVRLFRPWLNMDRMLRESAMRLXLPSFDKLELL
ECIRRLIEVDKDWVPDAAGTSLXVRPVLIGNEPSLGVSQPRRALLFVILCP
VGAYFPGGSVTPVSLLADPAFIRAWVGGVGNYKLGGNYGPTVLVQQEAL
KRGCEQVLWLYGPDHQLT**EVGTMNIFVYWTHEDGVLELVTPPLNGVILP**
GVVRQSLLDMAQTWGEFRVVERTITMKQLLRALEEGRVREVFGSGTAC
QVCPVHRILYKDRNLHIPTMENGPELILRFQKELKEIQYGIRAHEWMFPV

Red = CXXC
Green = Phosphorylation sites
Underlined = N-Myrisoylation sites
Purple = Glycosylation sites
Bold = Aminotransferase site

Figure 7.3 Protein sequence of the hBCAT proteins and known motifs. Protein sequences were entered into ProSite and relevant motifs were noted. Both BCAT proteins have equal phosphorylation sites, CXXC motifs and glycosylation sites. It is interesting to note that hBCATc has more numerous N-myristoylation sites with one of these sites localised to a phosphorylation site, suggesting that phosphorylation may regulate the availability of this N-myristoylation site.

Metabolism of the BCAAs has already been demonstrated to be affected by phosphorylation as phosphorylation regulates the BCKD complex. Low levels of KIC mediate the phosphorylation of the BCKD complex and this inhibits further metabolism of BCKAs (Paxton *et al*., 1984). Unpublished work by El Hindy *et al*., (2013) has demonstrated that hBCAT is phosphorylated by several PKC isoforms (particularly PKCα). Phosphorylation by PKC correlated with a loss of hBCAT transaminase activity *in vitro*. Reduced conditions were optimal for hBCAT phosphorylation and this related to the CXXC motif associated with the hBCAT proteins. Lee *et al*., (2004) demonstrated that either blocking with the thiol-specific reagent, N-ethylmaleimide (NEM) or mutating active cysteines was sufficient to block phosphorylation of platelet derived growth factor β (Lee *et al*., 2004).

Similar to this work, NEM labelling and site-directed mutagenesis studies, demonstrated that the N-terminal cysteine of hBCAT was required for optimal phosphorylation (El Hindy *et al*., 2013). Furthermore, hBCATm associates with the E1 subunit of BCKD and this association is prevented upon mutation of either thiols of the CXXC motif or phosphorylation of the E1 subunit of BCKD (Islam *et al.,* 2007). Without the shuttling of BCKAs to BCKD this may result in BCKA accumulation. Permanent phosphorylation of the E1 subunit of BCKD would allow BCKAs to accumulate without further metabolism. The detrimental effect of accumulated BCKAs is apparent from diseases such as MSUD and would lead to severe cognitive dysfunction (Muelly *et al.,* 2013). Therefore, with the association of phosphorylation with the disease

processes of AD, phosphorylated hBCATm could feasibly contribute to this pathology.

It is not yet apparent whether the hyper-phosphorylation observed in AD is the pathological event as hyper-phosphorylation of the tau protein, at similar sites to that observed in AD, also occurs in foetal neurons. Also, neurons positive for NFTs can survive for up to 20 years (Hanger *et al.,* 2009; Lee *et al.,* 2005). Due to the correlation between hyper-phosphorylated tau, NFTs and neuronal death a causative relationship is an attractive hypothesis. Additionally, mutations in the tau genes themselves are enough to facilitate hyper-phosphorylation, aggregation and the onset of disease. These neurodegenerative diseases occur in the absence of amyloid pathology (Iqbal *et al.,* 2010; Johnson & Bailey, 2002). There is also a combination of both views where it is considered that the NFTs are not toxic but could protect against degeneration in a similar way to aggregates observed in HD and it is the intermediates (i.e. partially phosphorylated tau) that are toxic (Arrasate *et al.,* 2004). Tau is increased in the CSF in AD subjects both in the phosphorylated and un-phosphorylated form, and is currently the only successful diagnostic marker for AD so far (Buerger *et al.,* 2006; Montine *et al.,* 2010). Phosphorylated hBCATm would decrease BCAA or glutamate transamination within the mitochondria of vascular cells. This would lead to a decreased energy production within these cells or a decreased glutamate removal.

Mitochondria have an altered population of phosphatases and kinases when compared to the cytosol, with the BCKD kinase entirely mitochondrial specific (Pagliarini *et al.,* 2005; Tso *et al.,* 2013). This is important as it explains the differential regulation of the hBCAT proteins by phosphorylation and may explain why hBCATm was observed to have modifications (as observed by Western blot), relative to hBCATc. For mitochondrial specific phosphorylation there would need to be a mitochondrial specific isoform of either protein kinase C, tyrosine kinase or casein kinase II or an as of yet unknown kinase that specifically targets hBCATm. It has already been noted, however that PKC levels in the post-mortem AD brain are decreased which would imply a decreased capacity to phosphorylate hBCATm (Alkon *et al*., 2007). It has further been demonstrated that increased intracellular calcium (such as that observed in AD) activates kinases rather than phosphatases (Foster *et al*., 1997). Therefore, although hBCAT does have potential for hyperphosphorylation it is not apparent how hBCATm would be phosphorylated over hBCATc and why the mitochondria would favour phosphorylation over the cytosol. These questions are important to answer and will form the basis of further studies.

7.2.5 Glycosylation in AD and possible relation to BCAT

Glycosylation is where the amino group of a protein, particularly N-terminal amino groups and side chains of lysine and arginine, reacts with a monosaccharide. This process does not necessarily require enzymes and is predominantly with glucose, fructose, hexose-phosphates and trioses (reviewed by Moremen *et al.,* 2012). Some proteins only fold correctly once

they have been glycosylated and the majority of proteins within the rough ER are glycosylated in addition to 30% of all cytosolic protein in the human brain (Kanninen *et al.,* 2004). Other proteins are glycosylated to add stability, for example when a protein is secreted (Varki *et al.,* 2009). These forms of glycosylation are enzyme dependent in nature. Glycosylation and glycation tend to be used interchangeably; however glycosylation refers to an enzymatic process whereas glycation does not. However, the end result is the same, although glycation is considered to be an uncontrolled process.

Proteins that are subject to enzymatic glycosylation have a motif consisting of asparginine, followed by anything but proline, followed by serine or threonine, followed by anything but proline. However, for glycation hydrophobic or uncharged side chain amino acids such as alanine, valine, leucine and serine were identified as occurring most frequently in peptides from proteins glycated *in vivo* (Zhang *et al*., 2011). Both hBCATc and hBCATm have a single glycosylation site, with hBCATm containing a large alanine rich tail that is a possible target of glycation (Figure 7.3). However, it is important to note that no research has been performed on the hBCAT proteins to demonstrate whether these glycosylation sites are functional and what effect this may have on activity.

In diseases such as AD, glycosylation adducts can undergo further oxidations and dehydrations to produce advanced glycation end-products (AGEs). Pathological effects of AGEs are increased oxidative damage, inhibition of neuronal signalling and inhibition of protein turnover (Srikanth *et*

al., 2011). Formation of AGEs is largely considered to be irreversible and causes protease resistant cross linking of proteins. These proteins become largely inactive but have a propensity to aggregate. For example, increased extracellular AGE formation has been demonstrated in amyloid plaques in AD. Further studies observed that primitive plaques, coronas of old plaques, NFTs and some glial cells were positive for AGEs (Sirkanth *et al.,* 2011). With AGEs playing a significant role in vascular complications it is entirely possible that the multiple hBCATm bands observed in this work are the result of advanced glycation (Basta, 2008; Yamagishi, 2011). These glycations will likely result in hBCATm becoming inactive and prone to phosphorylation and aggregation.

In summary, glycation is a prominent pathological mechanism in AD and it is possible that the hBCATm protein is glycated. It is noted that mitochondrial proteins may be specific targets of glycosylation in addition to having a higher risk of glycation due to the highly oxidizing environment of the mitochondria (Pun & Murphy, 2012). This may give rise to the additional bands observed for hBCATm and not hBCATc. However, AGE labelling of the vasculature was not a consistent occurrence in AD, with some authors citing labelling and others an absence (reviewed by Srikanth *et al.,* 2009). Therefore the effect of glycosylation and glycation needs to be demonstrated for the hBCAT proteins *in vitro* and *in vivo* in order to provide evidence for this occurrence in AD.

7.2.6 Protein expression and modification summary

It is unlikely that the increased expression in hBCATc and hBCATm described here is unrelated to AD pathology – particularly with increasing hBCATm expression correlating with increasing Braak staging, decreasing brain weight, increasing tau load, soluble Aβ, insoluble Aβ, APOE genotype and SVD score. However, a correlative effect does not lead to conclude causation and it is likely that AD pathology is occurring along with hBCAT associated changes. This work, for the first time, implicates altered BCAA metabolism as a pathological feature of AD and it is likely that this is also occurring in a number of dementias (such as MND). Metabolism of the BCAAs is an (as of yet) untargeted area of AD intervention so this work provides a whole system of novel targets for the treatment of AD, not just the hBCAT proteins themselves.

This alteration in hBCAT expression gives a key insight into the pathological basis for AD; however, it is not clear how this increased expression relates to other alterations within the disease. The decreased expression of GS along with increased oxidation of the protein will result in an accumulation of glutamate in astrocytes, leading to a decreased astrocytic uptake and accumulation of glutamate within the synaptic cleft. The reduced expression of the glutamate transporter EAAT2 also decreases glutamate removal from the synaptic cleft. The pro-excitotoxic conditions are exacerbated by decreased functional nicotinic acetylcholine receptors and decreased GABA (inhibitory) neurons present leading to an overall excitotoxic environment. Increased hBCATm could be a response to this environment and operate to

remove excess glutamate. However, the reverse may also occur and operate to increase BCKAs and glutamate levels for energy production within the brain, working counterproductively to disease pathology.

Decreased functional ATP synthase and NADH-ubiquinone oxidoreductase leads to a decreased energy production within the cell (Lau & Tymianski, 2010). With the synaptic cleft in continuity with the extracellular space within the brain, if neurons lose the ability to produce energy, glutamate will leak out of the cell (Szydlowska & Tymianski, 2010). This will exacerbate excitotoxic glutamate signalling. Antioxidant proteins are predominantly increased along with immune factors such as IL-1 and TNFα implying an oxidative environment possibly brought on by activation of the immune system. Finally, the alterations of apoptotic proteins imply cell death is the result of the above changes to cellular protein expression. The increased expression of hBCATc and hBCATm described in this work has implications for both energy and neurotransmitter production, with metabolism of BCAAs providing Krebs cycle intermediates in addition to glutamate and the metabolism of glutamate providing BCAAs for signalling and αKG.

The altered banding pattern for hBCATm observed in AD is undoubtedly the result of post-translational modifications to the hBCATm proteins. It has been observed in other work that S-glutathionylation of the hBCAT protein results in a dual banding pattern and may explain some of the banding observed in this work (Conway *et al*., 2008). It has been further discussed here that phosphorylation and glycosylation may also play a role in the

modifications of hBCATm observed in AD. Further work is necessary to elucidate what post-translational modifications of hBCATm are occurring in AD and to demonstrate *in vitro* and *in vivo* what the result of these modifications will be on both the aminotransferase and isomerase function of the hBCATm protein.

7.3 Insights into hBCAT function and relation to AD utilising the neuroblastoma cell line IMR32

This work has demonstrated that hBCATc and hBCATm are ubiquitously expressed throughout the human brain and that both hBCATc and hBCATm levels are increased in AD brain. Based on these findings it was proposed that altered expression of hBCAT observed in this work is likely the result of pathological features associated with AD, such as hormones and immune components of the inflammatory response. Cell imaging revealed a time and dose dependant negative effect of KIC and glutamate on neuronal morphology and viability. The effect of hormones (insulin and oestrogen), inflammatory markers (IL1 and TNFα) and hBCAT substrates (leucine and glutamate) demonstrated a sensitivity of hBCAT expression to inflammatory markers, but a comparative difference was noted between hBCAT expression and aminotransferase activity in the IMR32 cells.

For the first time, a receptor function of the hBCATc proteins is described. This receptor function was demonstrated to be sensitive to key hBCAT metabolites and this is discussed in relation to cell signalling (i.e. mTOR). This also suggests a physiological relevance of previous work that demonstrated an hBCATc redox-sensitive association with sodium channel type 10 α-subunit, as part of a receptor unit (Conway *et al*., 2008). A further novel finding of this work was the colocalisation of hBCATm and hPDI to the same organelle in addition to the same cell type. These findings are discussed in relation to the proposed hBCAT shuttle in the human brain or the association of the hBCAT proteins with AD pathology.

7.3.1 The effect of hBCAT metabolites on cell morphology and viability

The BCKAs are unique products of BCAA metabolism and are not generated by other enzyme systems. The true consequences of increased levels of BCKAs and BCAAs are manifested in the clinical condition MSUD (Silberman *et al.,* 1961). Mechanistically, high levels of these metabolites have been associated with an inhibition of cell proliferation and a prolonged increase in the G1 stage of the cell division cycle (Liao *et al.,* 1978). In addition, KIC inhibits pyruvate and hydroxybutyrate transport to the mitochondria resulting in a decreased utilisation of these metabolites for glutamate and energy production (Halestrap *et al.,* 1974; Land *et al.,* 1974). Further to this, KIC stimulates whole brain glutamate oxidation and depletion within astrocytes by up to 50% (Yudkoff *et al.,* 1994[a,b]; Zielke *et al.,* 1997). Finally, KIC is capable of inhibiting αKG dehydrogenase selectively, with no alteration to other citric acid cycle enzymes where KIC acts as an uncoupler of oxidative phosphorylation (Amaral *et al.,* 2010).

It is possible that hBCATc is also metabolising neuron glutamate in the instance of increased BCKAs due to a shift in enzyme direction to convert the increased KIC to the relatively physiologically less toxic leucine. In this study there was a clear time and dose dependant detrimental effect of KIC on cell morphology and viability. These studies, along with the work described here, suggest that KIC inhibition of neuronal growth is through a decreased energy production within the cell – with KIC reducing intracellular glutamate levels in addition to inhibiting Kreb cycle turnover.

Glutamate toxicity is a well described mechanism where the excessive stimulation of excitatory glutamate receptors (predominantly NMDA receptors) causes a prolonged post-synaptic influx of sodium and calcium ions (Dong *et al.,* 2009; Hynd *et al.,* 2004; Arundine & Tymianski, 2003). The calcium ions eventually exceed the capacity of the regulatory mechanisms such as calcium binding proteins and the storage of calcium in the ER. This produces an inappropriate activation of calcium dependent enzymes such as calpain or nitric oxide synthase. These bring about processes that directly damage neurons by cytoskeletal breakdown or toxic reaction product formation, like peroxynitrite ($ONOO^-$), that ultimately lead to cell death (Olney, 1978[a,b,c]; Rothstein, 1996; reviewed by Sattler & Tymianski, 2000). The sodium ions entering the neuron also result in osmotic damage due to the influx of chloride ions and water that swell the cell and interfere with signalling (Olney, 1978[a,b,c]; Olney, 1994; Lau & Tymianski, 2010).

Glutamate treatment of neuronal cells resulted in a decrease in morphology and viability that varied depending on the metabolic environment. Decreasing serum from 20% or 10% to 0% in the media had a detrimental effect on IMR32 cell number and morphology, as did the addition of 2 mM glutamine. However, the use of EMEM over RPMI demonstrated little effect on overall cell morphology. It is likely that factors present in the serum were able to either sequester glutamate or prevent apoptosis. It is further proposed here that the addition of glutamine reduced the conversion of glutamate to the relative physiologically inert glutamine (via glutaminase).

This would result in a decrease in the cells ability to remove excess glutamate (le Chatelier's principle).

If BCKAs or glutamate are increased in AD – the detrimental effect of neuron survival is evidenced not only by this work, but by the clinical manifestation of increased BCKA levels observed in MSUD and the clinical manifestation of increased glutamate levels observed in epilepsy. Future work will investigate whether these metabolites are altered in the serum, CSF and brain tissue of individuals affected by AD.

7.3.2 Hormone and hBCAT metabolites significantly associated with the expression and activity of the hBCATc protein.

Recent work from Lei *et al.,* (2012) demonstrated that BCAA transamination was altered by hormonal influences in bovine mammary epithelial cells. Elevated levels of growth hormone dose-dependently inhibited leucine transamination, whereas cortisol and glucagon increased transamination with prolactin and insulin showing no change (Lei *et al.,* 2012). In cows, lactation is associated with an increase in plasma concentrations of cortisol, prolactin and glucagon, but a reduction in insulin, 17β oestradiol and growth hormone (Chew *et al.,* 1984a; de Boer *et al.,* 1985; Marinelli *et al.,* 2007; Sartin *et al.,* 1985; Whitely *et al.,* 1984). Insulin levels within the human brain have been observed to decrease with age. In AD, these insulin levels are further decreased and it is likely that this is associated with a decreased neuronal glucose utilisation. This decrease in insulin occurs alongside a decrease in

insulin-like growth factors, therefore it was proposed that AD represents a neuroendocrine disorder (Steen *et al*., 2005).

Here, using a neuronal cell model an increase in transamination in response to insulin was observed, together with a marked increase in expression (and transamination) when treated in combination with leucine. This implies that the decreased levels of insulin observed in AD may lead to a decrease in hBCATc expression and transamination – further exacerbating the increase in hBCATm expression occurring within the vasculature. However, 17β oestradiol showed no effect on hBCAT expression. This could be due to many factors such as cell type, absent hormone interactions, species differences or the protein expression profile of immortal IMR32 cells.

Other results from this work observed that hBCATm expression was higher in females to males. It is still unclear whether this will predispose females to AD pathology or whether the females in this cohort had a higher level of overall pathology. The role of sex in AD has been debated for some time and it is still not clear whether there is any effect. More women suffer from AD when compared to males but some research suggests that this is down to the increased longevity associated with females, and not due to underlying processes (Bachman *et al.,* 1993; Plassman *et al.,* 2007; Rocca *et al.,* 1998). However, larger studies dispute these findings and demonstrate that the increase of AD in females is still apparent when longevity is taken into account (Launer *et al.,* 2007).

Glutamate levels have been observed to be higher in the hippocampus of females relative to males. In fact, glutamate levels decline with normal ageing in both the hippocampus and the anterior cingulate cortex, and this is more prominent in women than men (Hadel *et al.,* 2013). In our cell model, glutamate caused a minor decrease in aminotransferase activity but a large increase in hBCATc expression; this may be the mechanism by which the hBCAT protein is involved in a toxic gain in function. Initially, high glutamate concentrations raise hBCAT expression to compensate and remove excess glutamate, however there remains an overall decrease in transamination due to the oxidative stress associated with increased glutamate levels and the sensitivity of the hBCAT protein to oxidation. When this glutamate is removed, abnormally high levels of the BCAT protein possibly remain. This remaining hBCAT would leave the brain vulnerable to alterations of glutamate as a result of fluctuations in serum BCAAs due to dietary intake.

7.3.3 Immune factors significantly associated with the expression and activity of the hBCAT protein

The involvement of the immune system in AD is well described (Lambert *et al*., 2010), therefore this work aimed to investigate if common pro-inflammatory components of the immune system (TNFα and IL1α) affect the expression and activity of hBCAT. It was demonstrated that both TNFα and IL1α dose-dependently decreased the expression of hBCATc. The activity was assessed with TNFα, where a mild decrease in activity was observed. This effect may create a feedback loop capable of causing signal cessation of TNFα. In this model, immune system activation leads to an increased

TNFα concentration within the human brain, this TNFα subsequently leads to a decrease in hBCATc expression and a decrease in BCAA conversion to glutamate. Finally, increased BCAA concentration has been proposed to decrease the production of TNFα (Bassit *et al.,* 2002). This will complete a negative feedback loop and allow individual neurons to effect the local immune system (Figure 7.4).

The concentrations of TNFα and IL1α used in this experiment were high compared to elderly control serum levels (2.49 ± 1.27 pg/mL), although possible in septic shock and still effective within the physiological range (Alvarez *et al.,* 2007). It is also important that immune synapses between neurons and glial cells may well allow microenvironments of effective concentrations. This work proposes that the effect of TNFα on hBCATc expression relates immune system function with cognition. In animal models, hBCATc is responsible for 30% de novo glutamate synthesis (LaNoue *et al*., 2001). With TNFα decreasing hBCATc expression in neurons this may (in theory at least) lead to a decreased glutamate production and therefore a decrease in the signalling of glutamatergic (or even GABAergic) neurons. This decreased signalling will affect the function of the local neurons and thus the cognition of the individual.

The presence of hBCATc on the surface of dead immune cells raises the question of function in this event. It is noteworthy that the cells with cell death expressions of hBCATc were all myeloma cell lines (RPMI, Jim3 and U261). With proposed models in the mammalian brain promoting hBCATc as

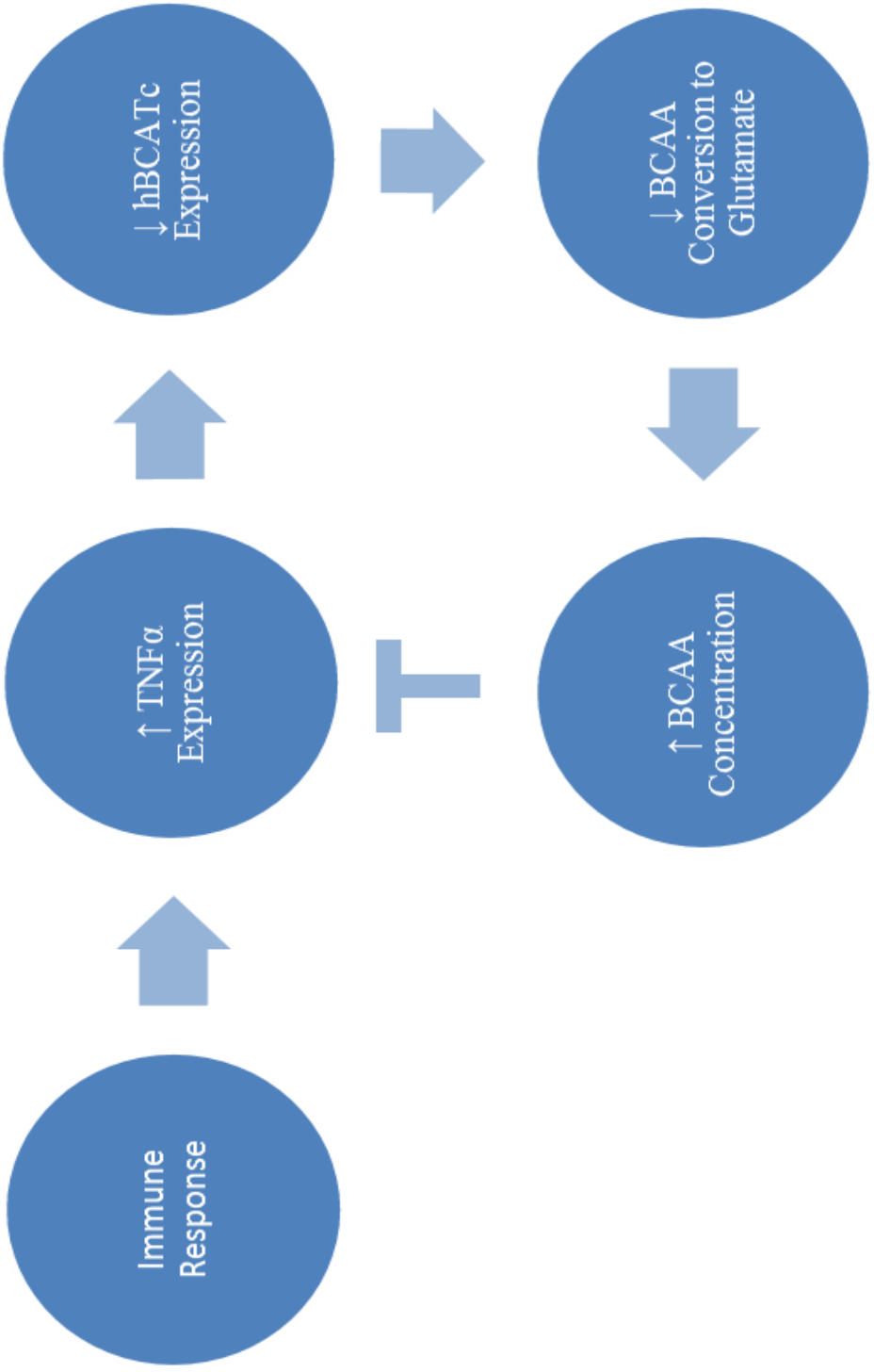

Figure 7.4 Proposed relationship between TNFα and hBCATc. Immune activation leading to an increase in TNFα expression will, in turn, decrease hBCATc expression. This will lead to a decrease in BCAA related glutamate production and an overall increase in BCAA concentration. Increases in BCAA concentration will then negatively feedback causing a decrease in TNFα expression. It can also be speculated that a similar mechanism may exist for IL1α (Bassit *et al.*, 2002).

anti-apoptotic this cell surface death induced expression of hBCATc is contrary to this. It has been proposed that hBCATc is a novel target for chemotherapy as hBCATc gene expression is associated with a higher rate of metastasis and hBCATc inhibitors have been patented in the treatment of such conditions (Radlwimmer *et al.,* 2012; Zhou *et al.,* 2013). Annexin V is another protein that is externalised upon apoptosis, this relates to hBCATc in that this protein is kept on the intracellular side of the membrane to be externalised upon cell death. This would imply that the hBCATc is not pro or anti-apoptotic as such but is the result of apoptosis (or cell death). On the intracellular cell surface hBCATc would be capable of maintaining hBCAT metabolite concentrations in order to regulate mTOR activity – a vital part in immune function.

7.3.4 Novel functions of the hBCAT proteins in cell signalling

Conway *et al.,* (2009) demonstrated that hBCATc associates with myosin 6 and sodium channel type 10α subunit under reducing conditions and could not bind under oxidized conditions. It was proposed that this may play a role as a protective mechanism for these proteins but work here demonstrates that hBCATc may actually be part of a functioning receptor. This work demonstrates that treatment with glutamate caused an initial (30 minutes treatment) decrease in cell surface hBCATc expression but an increase after 24 hours. Leucine treatment (24 hours) also caused a long term increase in hBCATc cell surface expression. It is proposed here that hBCATc is operating as a metabolic sensor for its metabolites (particularly glutamate),

the immediate effect causes an internalisation and probable G-protein signalling (similar to other receptors) (Figure 7.5).

The hBCATc protein was demonstrated to associate with both myosin 6 and sodium channel type 10α subunit (Conway *et al*., 2009). Myosin 6 is proposed to transport endocytic vesicles into the cell but also functions in cell migration and mitosis (Buss *et al*., 2004). In addition, the sodium channel type 10α subunit mediates voltage dependant sodium ion permeability on excitable membranes. The protein forms a sodium-selective channel through which sodium ions can pass in accordance with the gradient (Rabert *et al*., 1998). As both of these proteins are responsible for key cell surface processes this work suggests that the association of these proteins with hBCATc is part of a functional receptor.

This cell surface expression (however) may be a reserve function for toxic glutamate insult (e.g. in stroke), as concentrations required to produce a response were physiologically high. Under toxic insult hBCATc can operate to remove excess glutamate and work with mTOR to promote cell survival (Figure 7.5). The isomerase and reductase hPDI also has cell surface expression and it was proposed that this is to maintain cell surface thiols in the reduced state but may also assist in clot formation within the vasculature (Essex, 2009; Jiang *et al.,* 1999; Popescu *et al.,* 2010; Willems *et al.,* 2010). It is entirely possible that both isomerase and aminotransferase functions of hBCATc are occurring but this work provides additional evidence for the metabolic receptor role rather than the redox role as it is both

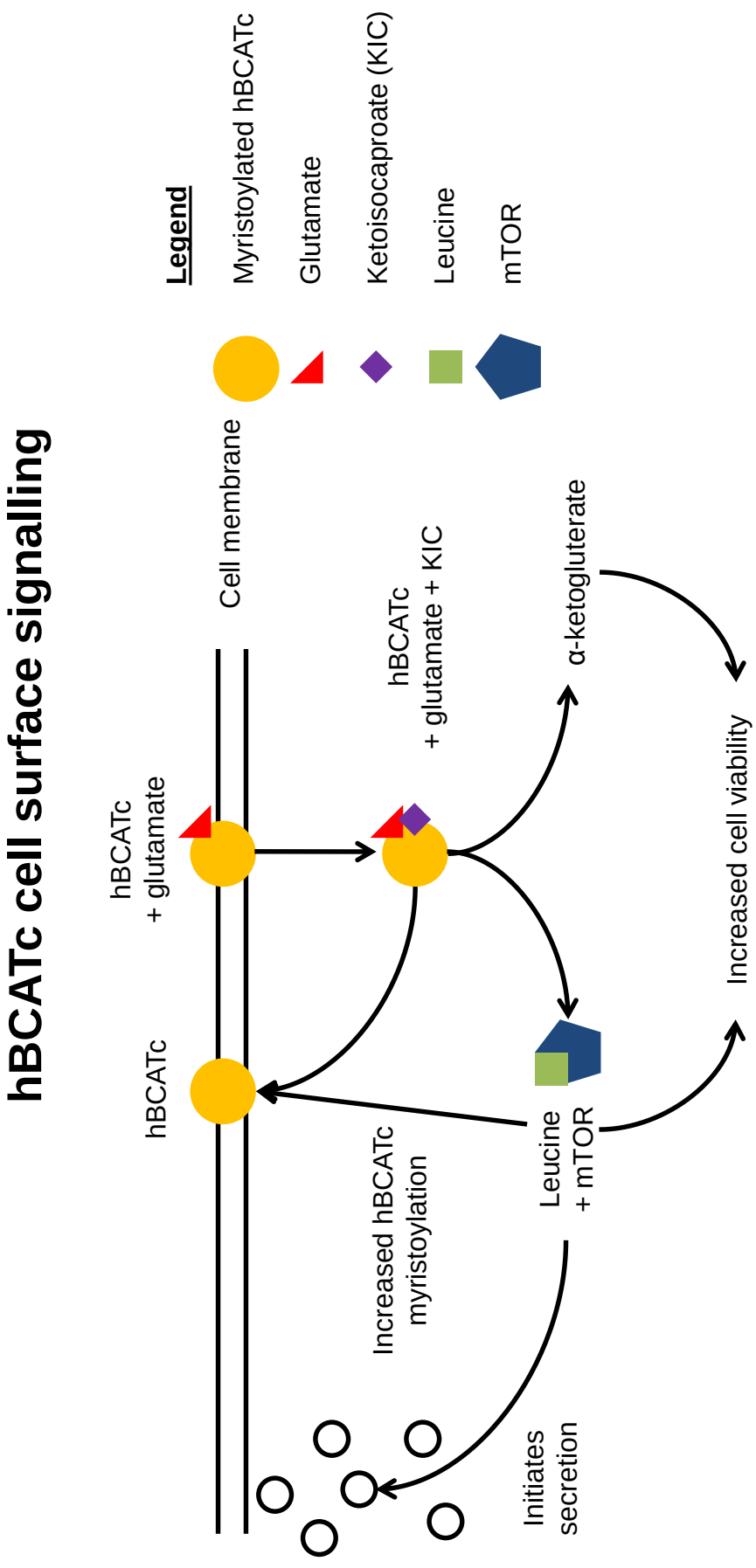

Figure 7.5 Proposed mechanism of hBCATc cell surface signalling. Myristoylated hBCATc rests at the cell surface acting as a receptor for glutamate (and perhaps the BCKAs). Upon binding the hBCATc protein is internalised (or associates with mTOR close to the cell surface) and transaminates glutamate and KIC (found either externally or internally) to leucine and α-ketoglutarate. This leucine then stimulates mTOR to improve protein production and increase cell survival. mTOR activation may also increase the myristoylation of hBCATc making cell surface expression increase over time. The α-ketoglutarate can also be used in mitochondria energy production, further increasing cell viability.

responsive to leucine and glutamate. However, the 24 hour toxic glutamate levels used here would create an oxidative environment so the increase in cell surface expression may well be a response to this but would not explain the immediate decrease in cell surface expression after a 30 minutes treatment.

7.3.5 Novel colocalisation of the hPDI and hBCATm proteins, chaperone mediated functions

Electron microscopy work presented here demonstrates a co-localisation of the hBCATm and hPDI protein. In addition to this, immunohistochemistry demonstrated a colocalisation of these proteins to the same cell type (i.e. the vasculature) of the human brain. Further electron microscopy work has localised hBCATm with the autophagic proteins LC3 and hPDI, particularly under conditions of oxidative stress ($+H_2O_2$) and creates a role for hBCATm in the autophagic process. Multiple functions of hBCAT have already been described by Cooper *et al.,* (2003) who demonstrated that hBCAT are cysteine S-conjugate β-lyases with turnover leading to inactivation. In higher plants (*Vigna unguiculata*), BCAT2 is upregulated in response to sucrose starvation related to the stimulation of autophagy and amino acid catabolism. It is thought that this BCAT upregulation in plants enhances autophagy by regulating the BCAA levels (Kaneko *et al.,* 2013) and this is conceivably also occurring in humans.

Additional functions have been further investigated by El Hindy *et al.,* (2013) where it was observed that the hBCAT proteins have thiol disulphide

isomerase activity that is enhanced by the presence of hPDI. The proposed interaction of hPDI and hBCAT will be governed by the redox state of the cell so that cellular stress will not only render hBCATm catalytically inactive but will also prevent further association with hPDI (Conway *et al*., 2002). S-nitrosation, like oxidation results in a decrease in hBCATm activity (Coles *et al*., 2009), but is also known to affect hPDI function, as S-nitrosation of hPDI has been reported to be physiologically relevant in AD pathology and links oxidative stress to misfolded proteins (Uehara *et al*., 2006). However, it remains to be determined whether hBCATm acts as a molecular chaperone similar to hPDI in its own right, to facilitate dithiol-disulphide exchange in misfolded proteins, or behaves like the Ero1 oxidase, providing a sink of exchangeable thiols for hPDI during catalysis, assisting in its neuroprotective role.

7.3.6 Summary of cell work and implications for AD treatment and pathology

Given the proposed role hBCAT enzymes play in the pathogenesis of AD it is important to relate these findings to potential treatment applications and pathological mechanisms. Currently, there are two successful targets for treatment in AD, the cholinesterase inhibitors (that make up the bulk of approved drugs) and NMDA receptor antagonist (a relatively new single approved drug called Memantine). These drugs are notoriously ineffective, are not efficacious in all instances, (may even exacerbate deterioration,) and do not reverse the course of the disease, merely slow progression. Altered

BCAA metabolism and glutamate production represents a novel area of drug research for dementia not just targeting the hBCAT proteins.

This work proposes, for the first time, novel functions of the hBCAT proteins. The relationship between immune factors (TNFα and IL1α) and BCAA metabolism will undoubtedly be complex; however, the work here proposes that the two systems are interrelated and work as part of a feedback loop – allowing the neuron to control local immune cells through metabolite production. Cell surface expression of hBCAT, controlled by BCAT metabolites and insulin, offers a role for these protein in cell signalling, which will contribute to the role of leucine as a nutrient signal. Finally, the co-localisation of hBCATm and hPDI suggests a role in chaperone mediated thiol disulphide exchange for the hBCATm protein. All these factors are likely related to the increased expression observed in the AD brain.

Here, we propose that the upregulation of hBCATm observed in the brain of patients with AD relative to controls may initially be a protective mechanism. Either due to the hBCAT proteins isomerase activity, the receptor function of the hBCATc protein or the hBCATm proteins proposed role in autophagy. This increased expression may outlive the protective function and become pathogenic (i.e. a toxic gain of function similar to that of tau) or the aminotransferase activity of the hBCAT proteins may be inseparable from their protective roles. This work demonstrates toxicity of the metabolites of hBCAT (glutamate and KIC) and it is likely that this is mediating the toxicity of the proteins. However, the toxicity of glutamate seems to be attenuated with

the addition of serum and exacerbated by the presence of glutamine. This is likely due to the presence of protective factors within serum and the presence of glutamine likely makes it difficult to remove additional glutamate by conversion to the relatively physiologically inert glutamine. This suggests that attenuating glutamate toxicity is a viable possibility for treatment of AD as factors in serum improve cell survival.

In conclusion, work on cell models provides numerous possible causes of altered hBCAT expression in addition to providing new mechanistic insights into the proteins regulation and function. Although it is unlikely that a single cause will ultimately be demonstrated as the initiating factor in AD pathology or hBCAT expression, this work provides initial insight on the early stages of AD pathology.

8 Conclusion

The distributional analysis of the hBCAT protein in the human brain observed a unique difference of hBCATm localisation in the human brain to that of animal and cell models. For the first time, hBCATm has been observed in the vasculature of the human brain. A new model for hBCAT metabolism in the human brain is therefore proposed in this work based on that described by Hutson *et al*., (1998). The presence of hBCATc in many secretory cells of the human brain implies another role for hBCATc and its metabolites in this context. This will be through either intermediates for neurotransmitter production or mTOR activation for neurotransmission and secretion (Figure 8.1).

For the first time, Western blot analysis of the hBCAT proteins in the frontal and temporal cortex of AD subjects demonstrated an increase of 117% and 143% respectively for the hBCATm protein. It is therefore probable that the hBCATm protein is playing a significant role in the glutamate pathology of AD. Immunohistochemistry also observed an increase in hBCATc expression occurring within the hippocampus. It was further demonstrated in this work that hBCATm expression correlated with key pathological features associated with AD such as Braak stage, Tau (%) and soluble and insoluble Aβ. This work proposes a novel role of BCAA metabolism in AD pathology and reveals new drug targets for the possible treatment of AD.

Work using the IMR32 cell line lead to the proposal of several novel functions of the hBCAT proteins. The relationship between immune factors (TNFα and

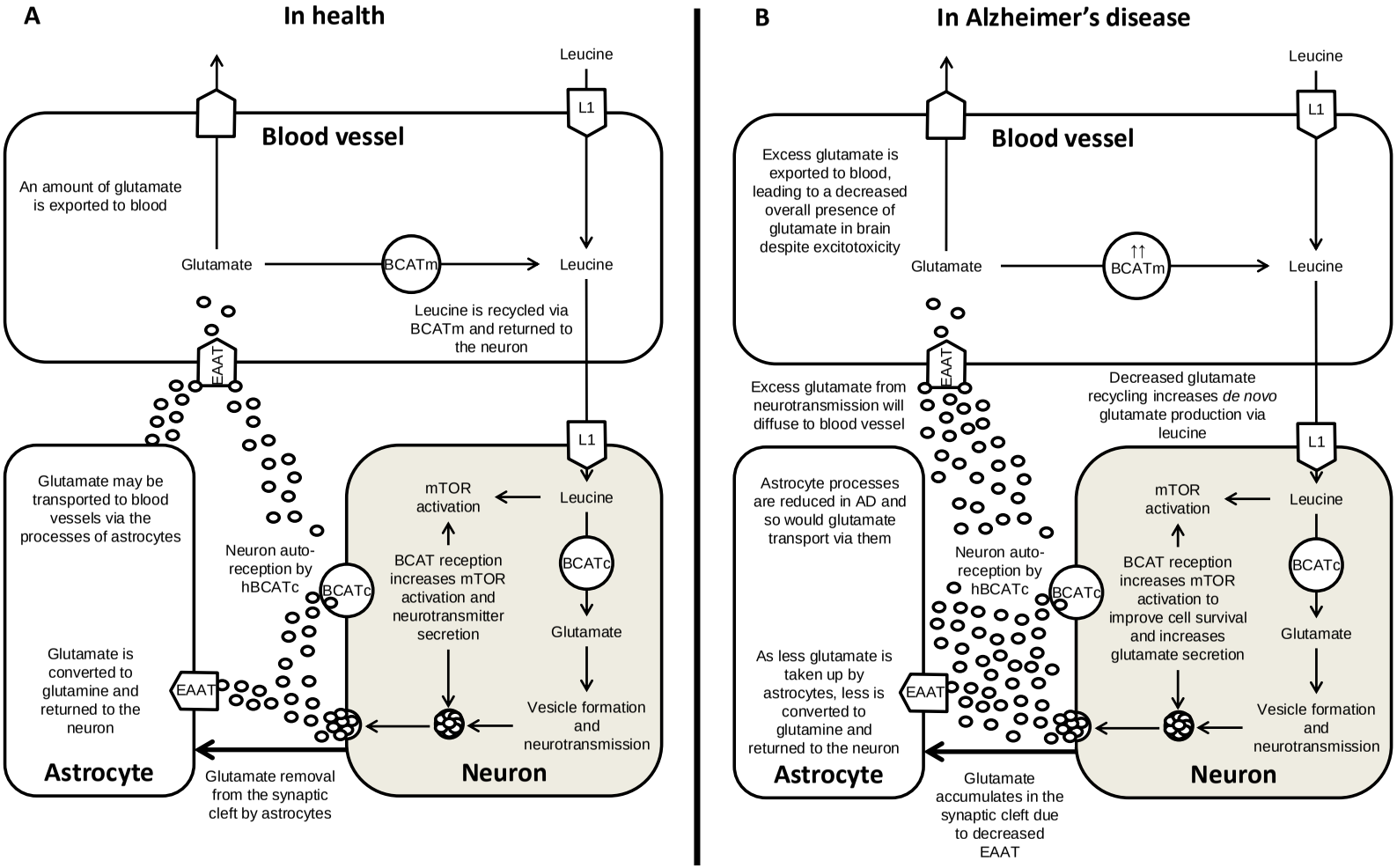

Figure 8. 1 Model proposed for hBCAT signalling in the human brain in health (A) and AD (B). A: hBCATc in neurons sustains the neuronal glutamate pool using branched chain amino acids such as leucine and hBCATm in the brain endothelium works to recycle these BCAAs. B: In AD, the excess glutamate signalling and the decreased glutamate recycling require additional de novo glutamate production. The increased expression of hBCATm observed in this work likely relates to the increased requirement of leucine. Abbreviations: BCAT – Branched chain aminotransferase; EAAT – excitatory amino acid transporter; L1 – large neutral amino acid transporter; mTOR – mammalian target of rapamycin.

IL1α) and BCAA metabolism (along with other published work) suggests that the two systems are interrelated and work as part of a feedback loop. Flow cytometry work demonstrated cell surface expression of the hBCATc protein that was sensitive to hBCAT metabolites. This work posits that this is related to mTOR signalling and metabolite sensing on the cell surface of neuronal cells (Figure 8.1). Finally, the colocalisation of hBCATm and hPDI in this work (along with other work) suggests a role for hBCATm in chaperone mediated thiol disulphide exchange. Here, we propose that the upregulation of hBCAT observed in the Western blot or immunohistochemistry analysis of AD subjects relative to controls may initially be a protective mechanism. This increased expression may outlive the protective function and become pathogenic (i.e. a toxic gain of function similar to that of tau) or the aminotransferase activity of the hBCAT proteins may be inseparable from their protective roles.

This work proposes two new treatment options for use in AD including BCAA supplementation or hBCAT inhibition (either hBCATc, hBCATm or both). Current patents have already produced several hBCATc specific inhibitors and have even patented their use in dementia, but none so far have produced any specific inhibitors for hBCATm and it is unclear whether modern hBCATc specific inhibitors will outperform gabapentin. This work suggests that an hBCATm inhibitor may well be a disease specific modifier in the AD brain however it is worth noting that hBCATm is by far the dominant isoform in the body, so side effects for any hBCATm inhibitor may make it an

unreasonable drug target. Despite this, hBCATm knock-out mice are reported to be physiologically healthy.

9 Future work

To further characterise the role of hBCATc and hBCATm in AD in addition to any alterations of these proteins to physiological differences, the following investigations should be considered:

1. To evaluate the role of hBCATm in the vasculature and in the blood-brain barrier a new vascular cell line to study protein expression of hBCATm in response to various stimuli will be assessed.

2. Using this vascular cell line expressing hBCATm, both metabolites and immune components will be studied for their effect on expression and activity. Also, other immune factors and hormones will be considered for both hBCATc and hBCATm. Metabolism of BCAAs is different in males compared to females and that is caused by hormonal differences. Future work should assess the effect of testosterone, growth hormone, other oestrogens, progesterone and cortisol in addition to disease specific factors such as hyper phosphorylated tau, soluble amyloid and insoluble amyloid.

3. The relationship between hBCATc and mTOR could be investigated with knock-down models of hBCATc. In theory, the knock-down of hBCATc will decrease mTOR activity in response to glutamate or leucine. Over expression would also be predicted to increase the cellular response to BCAA metabolites on the activation of mTOR.

4. Further characterisation of other aminotransferase proteins in the human brain will also be a promising area or research. Both alanine aminotransferase and aspartate aminotransferase have not been adequately investigated in the human brain. Furthermore, other proteins such as the BCKD complex also need to be mapped to the human brain and require investigation to complete the investigation of BCAA metabolism within the human brain.

5. Assessment of BCAA concentrations in the CSF will be important to understand metabolite uptake under pathogenic conditions. This investigation will also include measurement of the BCKAs. These metabolites are toxic and may reveal an MSUD like syndrome occurring in the AD brain. Other common metabolites will also be investigated in the post-mortem tissue to try and define the metabolic profile of AD.

6. The activity of the hBCAT proteins in post-mortem tissue, CSF and serum are of high importance due to the possible use as a biomarker for AD. This will also demonstrate the activity of hBCAT so will confirm whether hBCATm increased expression is a functional one.

7. The work described here in the MND motor cortex is promising and should be extended to include a larger cohort similar to that done with AD.

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11 Appendix

11.1 Figures from results section 5.2 not included in main text

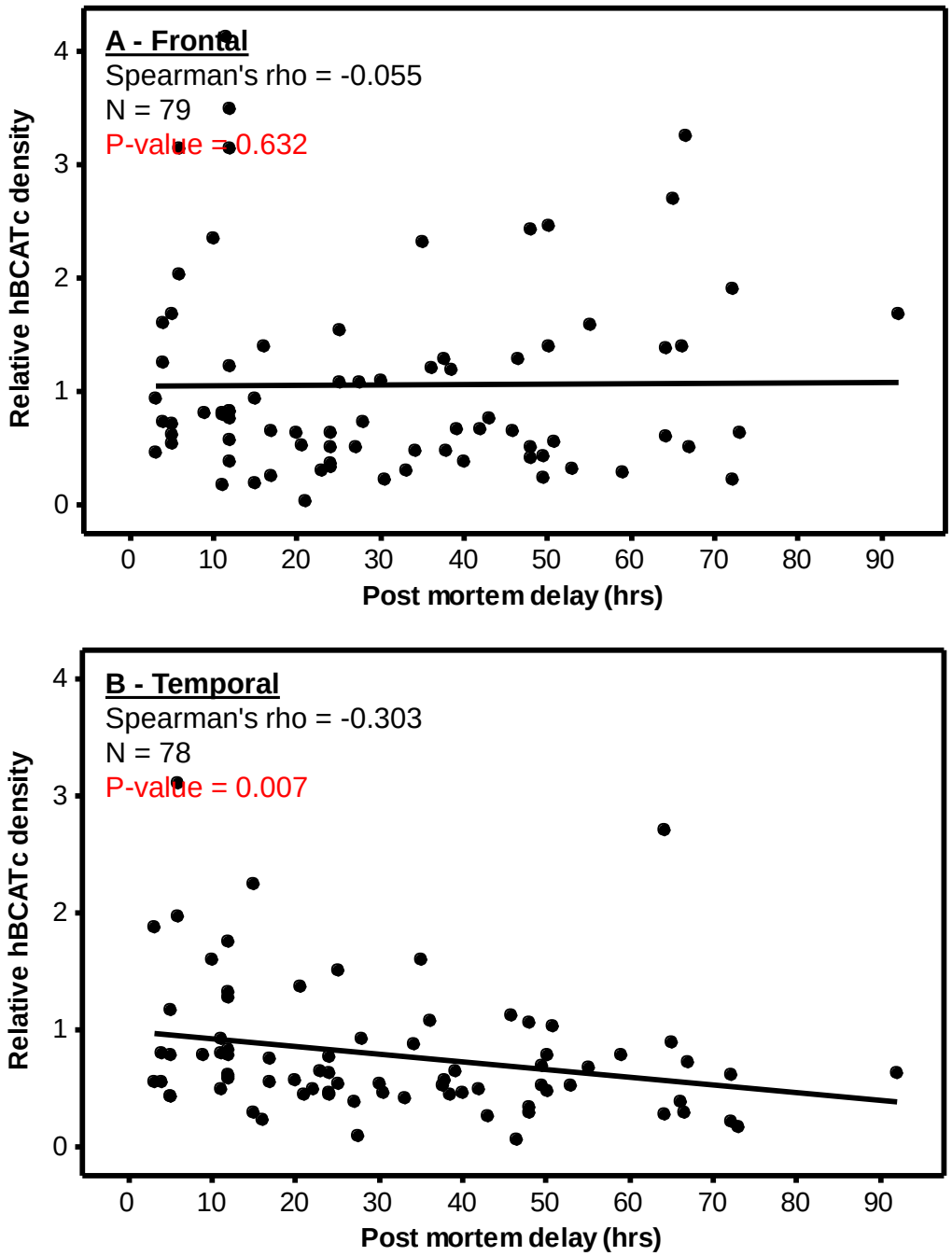


Figure 11.1. Scatterplots of frontal and temporal hBCATc protein levels correlated with post-mortem (PM) delay. The density of bands were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

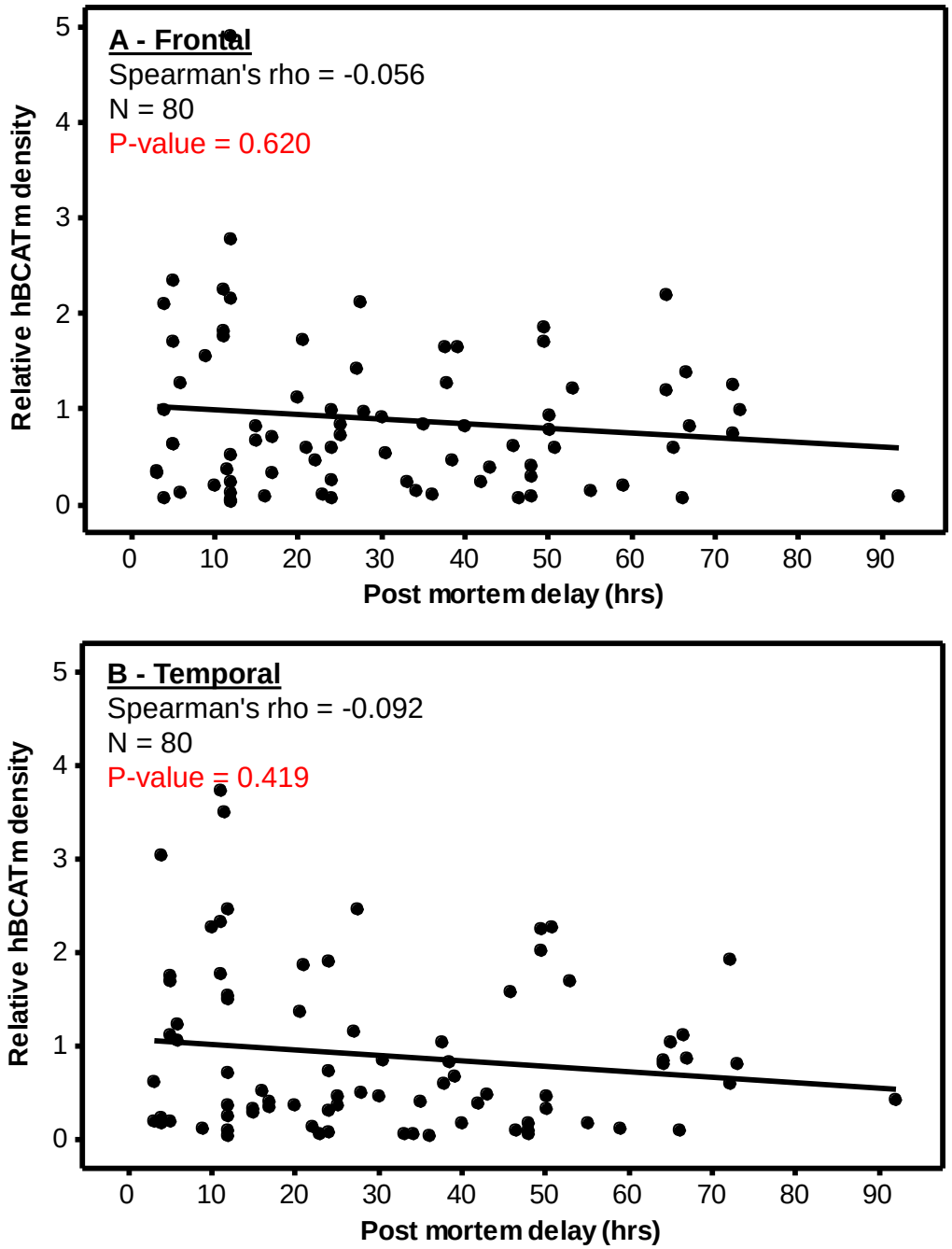


Figure 11.2. Scatterplots of frontal and temporal hBCATm protein levels correlated with PM delay. The density of bands were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

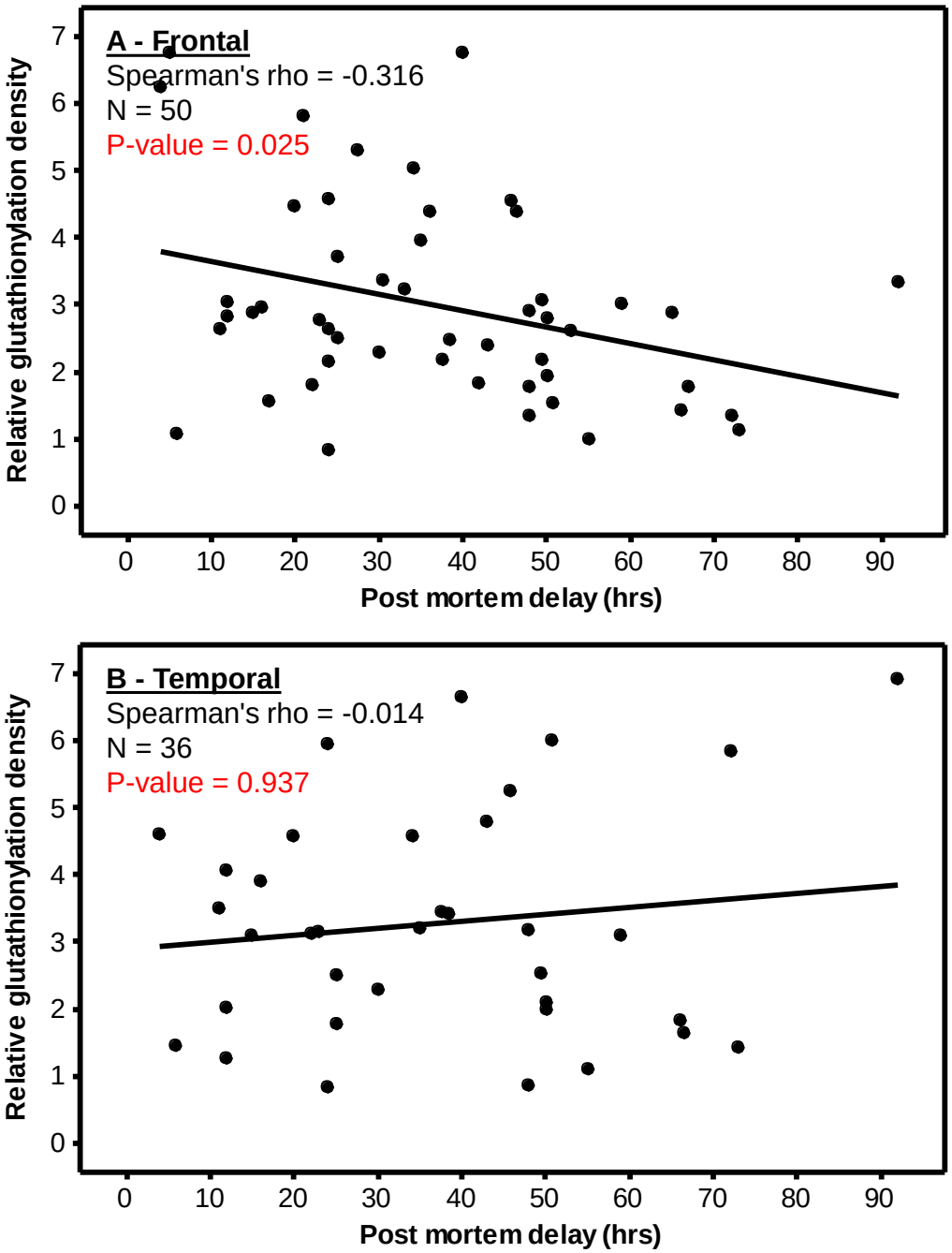


Figure 11.3. Scatterplots of frontal and temporal glutathionylated protein levels correlated with PM delay. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

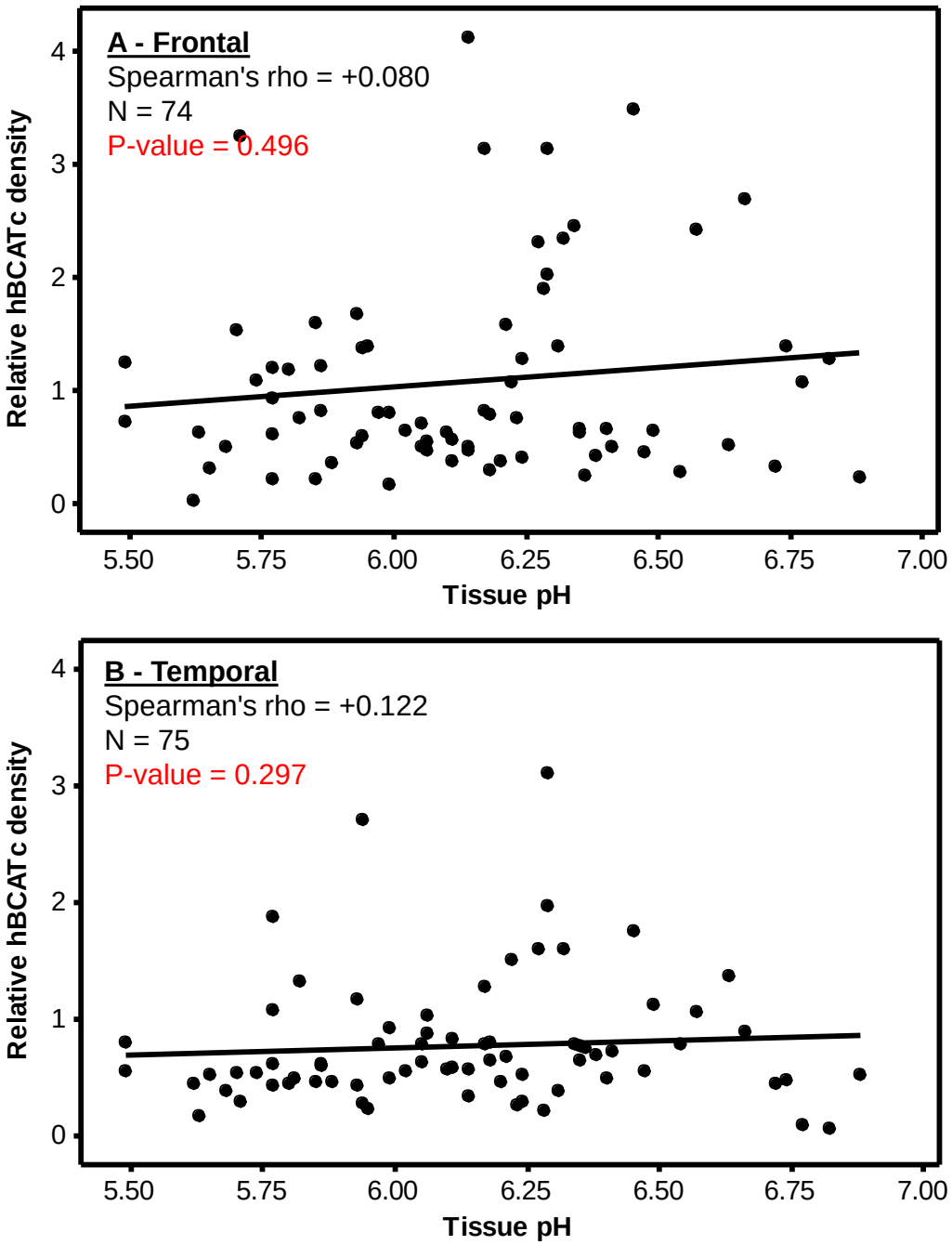


Figure 11.4. Scatterplots of frontal and temporal hBCATc protein levels correlated with frontal tissue pH. The density of bands were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

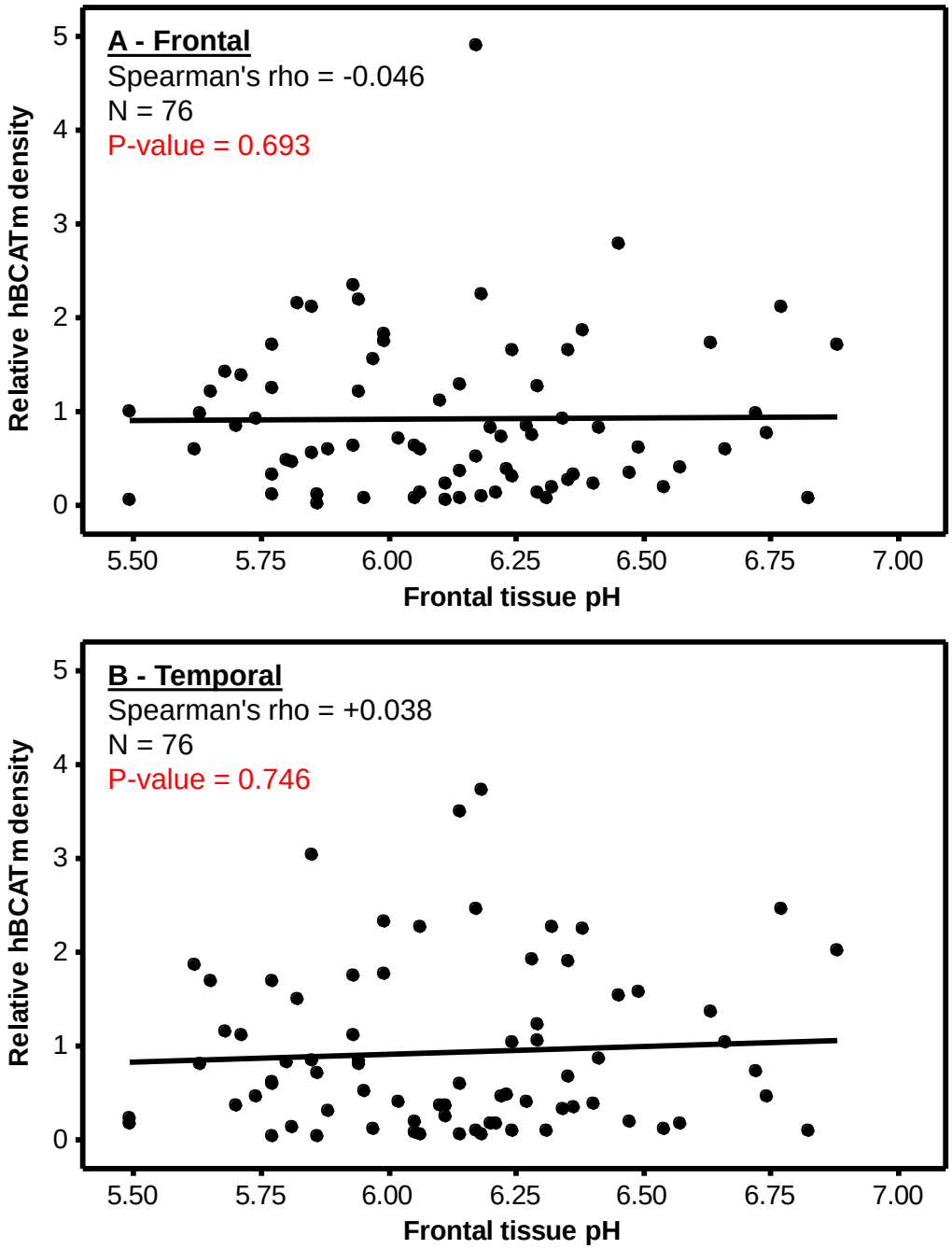


Figure 11.5. Scatterplots of frontal and temporal hBCATm protein levels correlated with frontal tissue pH. The density of bands were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

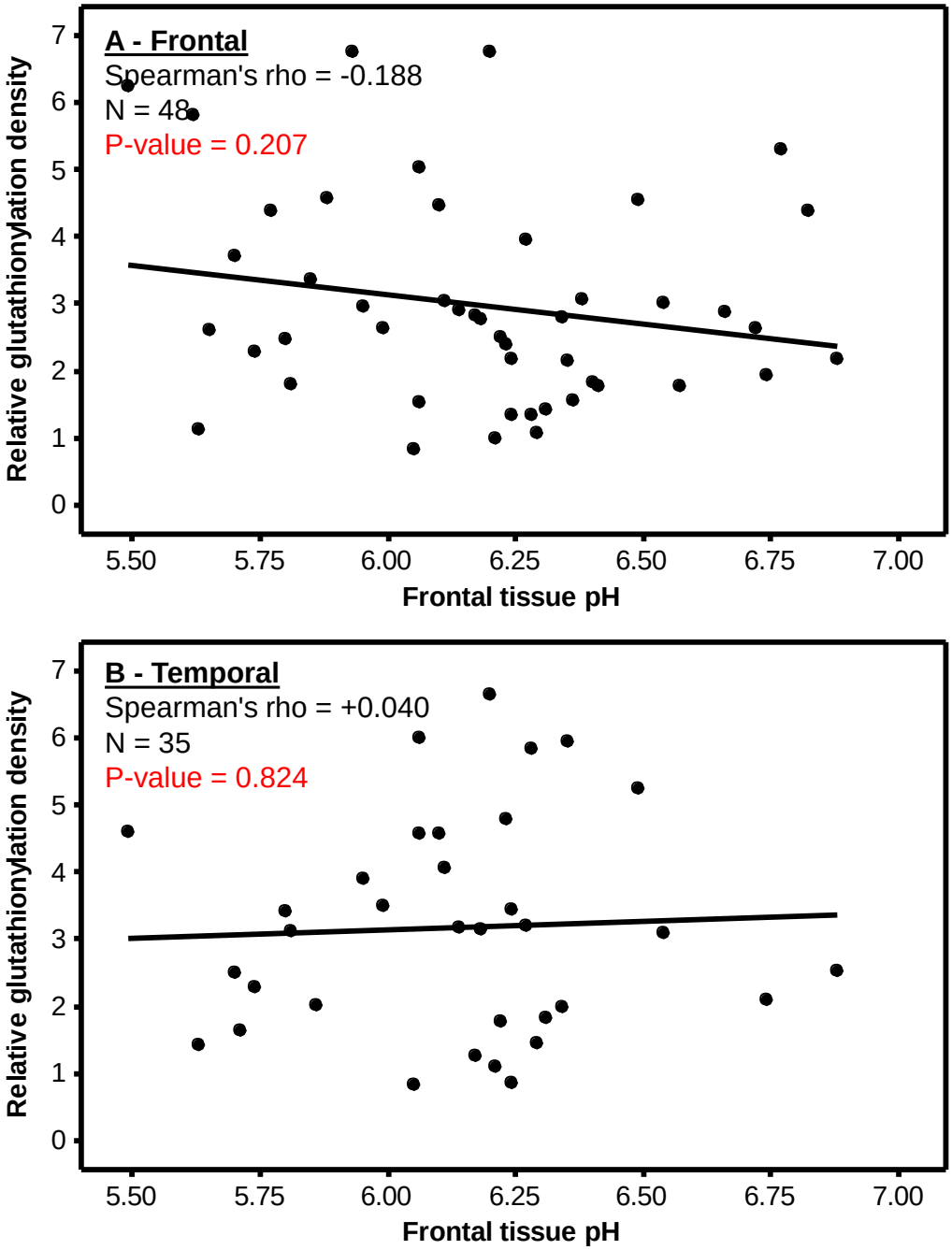


Figure 11.6. Scatterplots of frontal and temporal glutathionylated protein levels correlated with frontal tissue pH. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

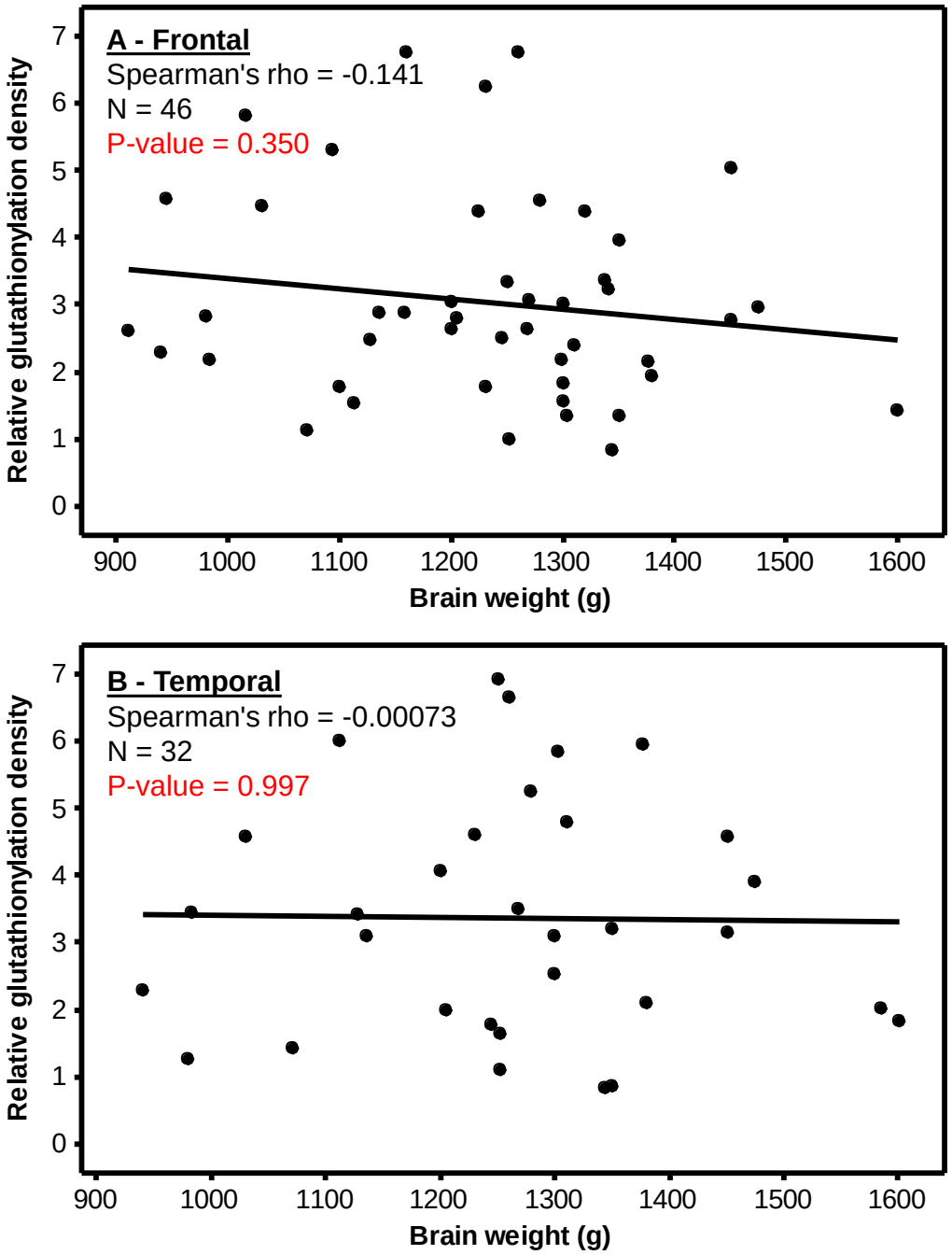


Figure 11.7. Scatterplots of frontal and temporal glutathionylated protein levels correlated with brain weight. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

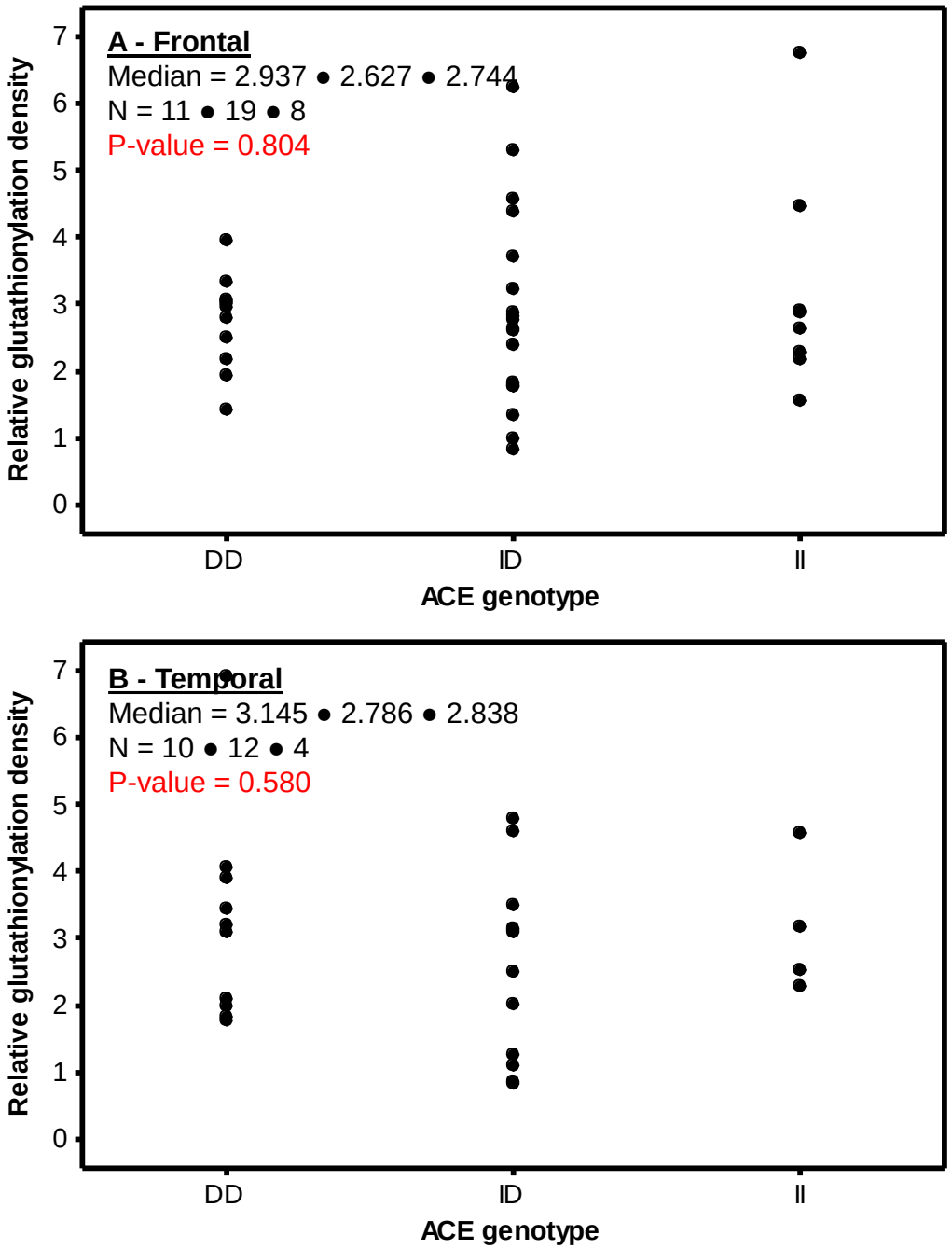


Figure 11.8. Individual value plots of frontal and temporal glutathionylated protein levels with ACE genotype. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Kruskal-wallis test using Minitab™.

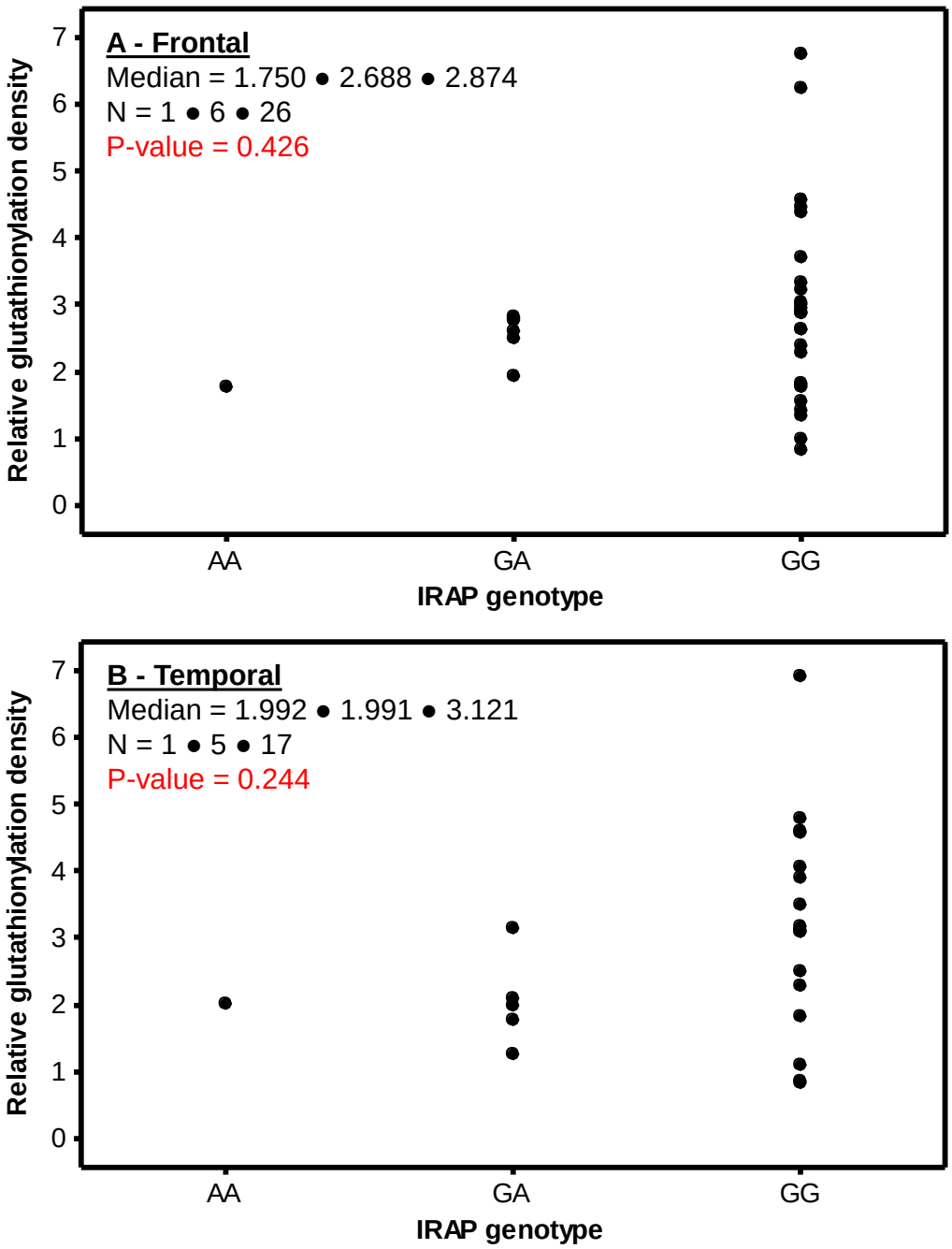


Figure 11.9. Individual value plots of frontal and temporal glutathionylated protein levels with IRAP genotype. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Kruskal-wallis test using Minitab™.

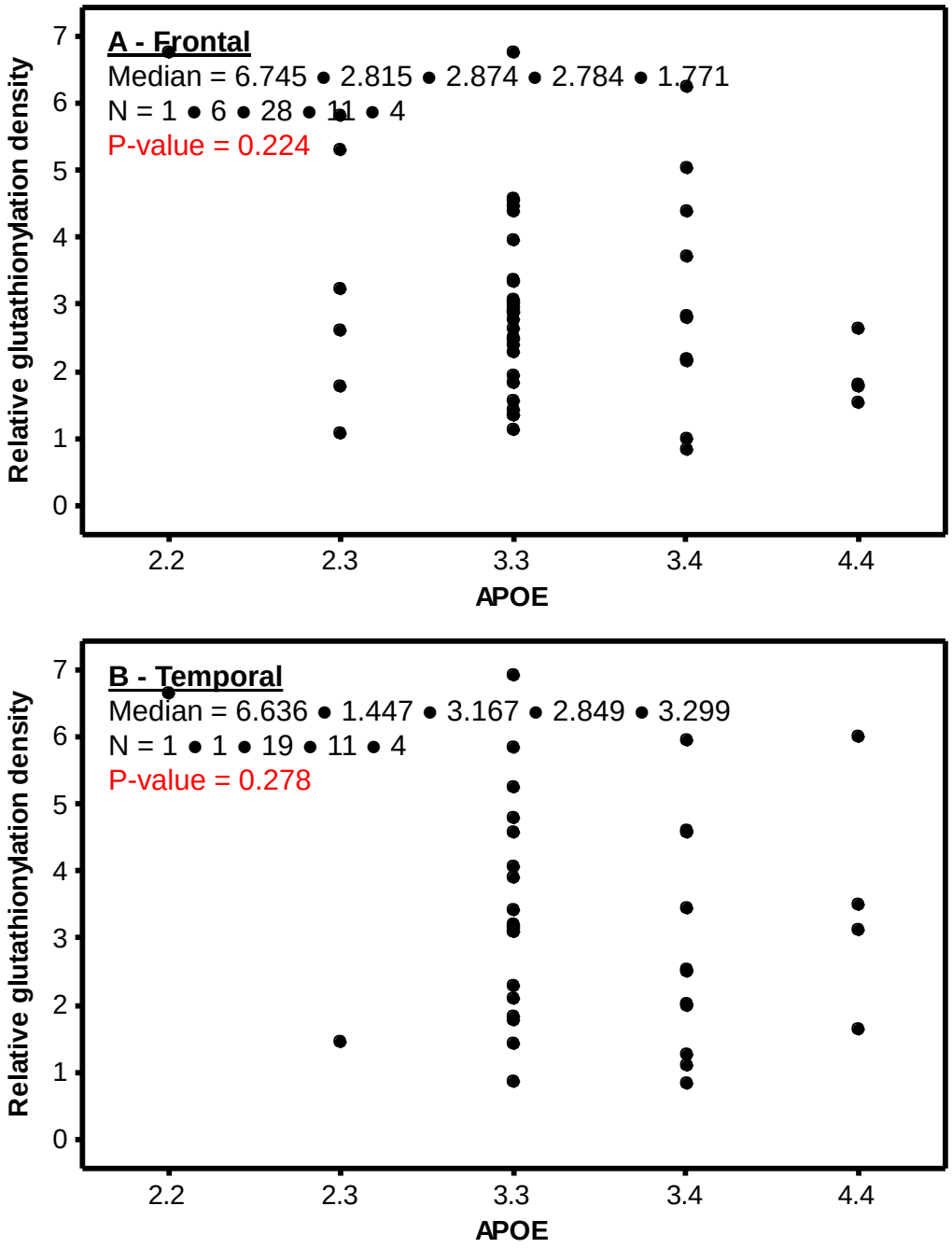
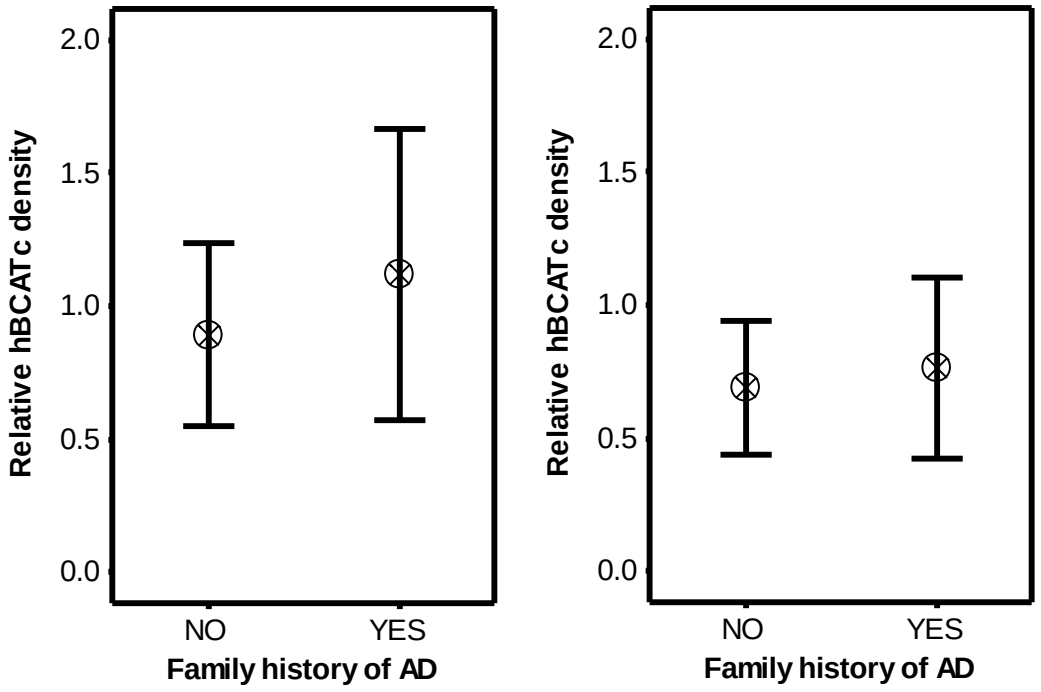


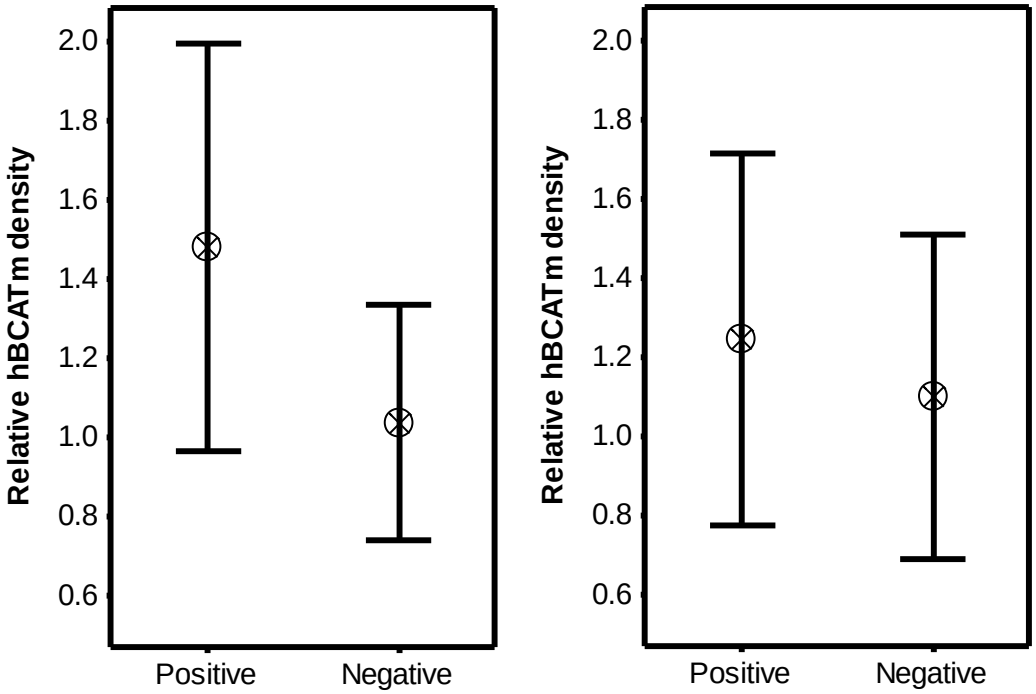
Figure 11.10. Individual value plots of frontal and temporal glutathionylated protein levels with APOE genotype. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Kruskal-wallis test using Minitab™.



A - Frontal
Mean = 0.892 ● 1.118
N = 22●9
P-value = 0.448

B - Temporal
Mean = 0.687 ● 0.760
N = 22●9
P-value = 0.705

Figure 11.11. Interval plots of frontal and temporal hBCATc protein levels in individuals with a positive family history of AD and individuals with a negative family history of AD. The density of bands was measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Mann-Whitney U test using Minitab™. Panels show 95% confidence interval and the mean.



A - Frontal
Mean = 1.482●1.037
N = 9●22
P-value = 0.133

B - Temporal
Mean = 1.246●1.099
N = 9●22
P-value = 0.372

Figure 11.12. Interval plots of frontal and temporal hBCATm protein levels in individuals with a positive family history of AD and individuals with a negative family history of AD. The density of bands was measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Mann-Whitney U test using Minitab™. Panels show 95% confidence interval and the mean.

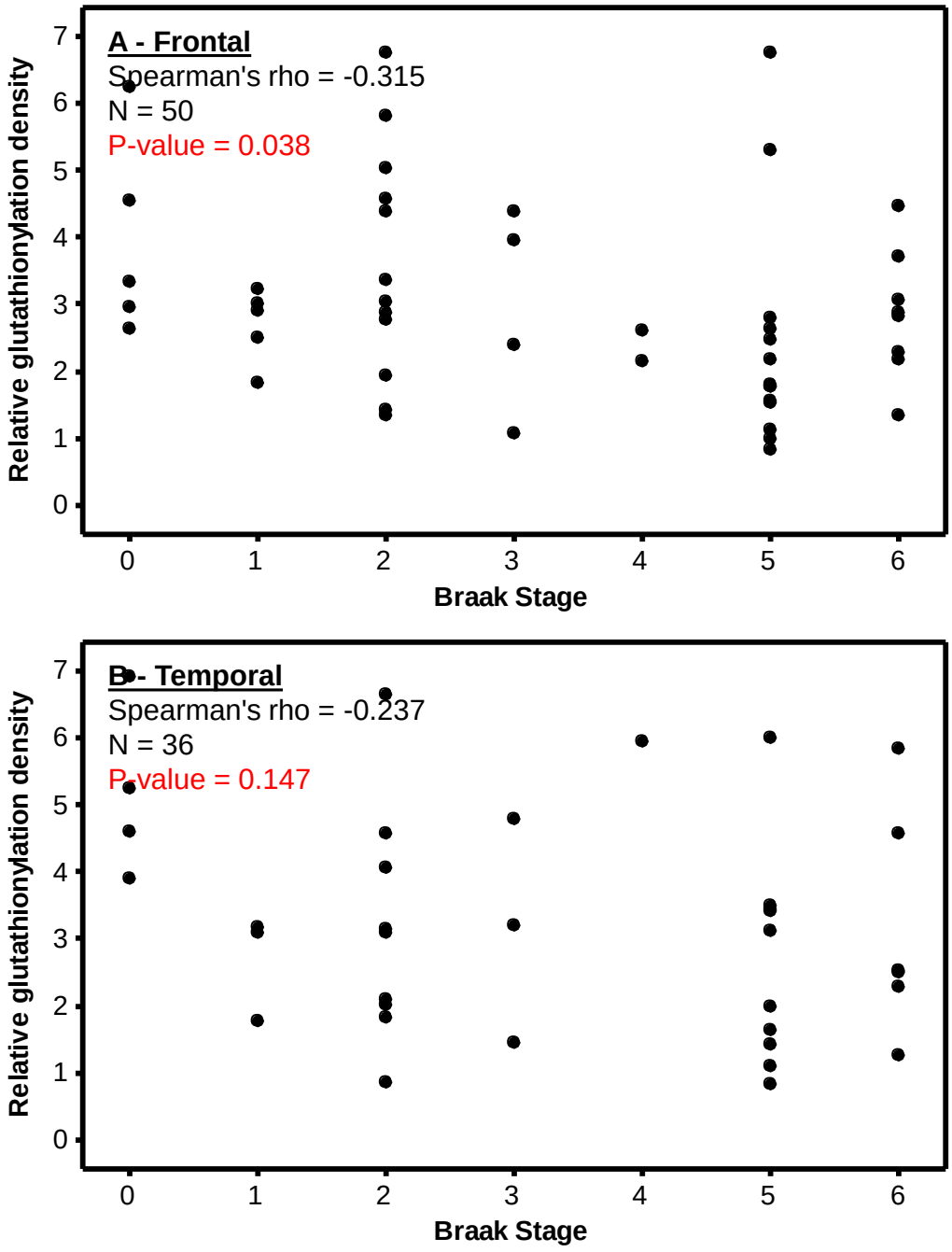


Figure 11.13. Scatterplots of frontal and temporal glutathionylated protein levels correlated with Braak stage. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

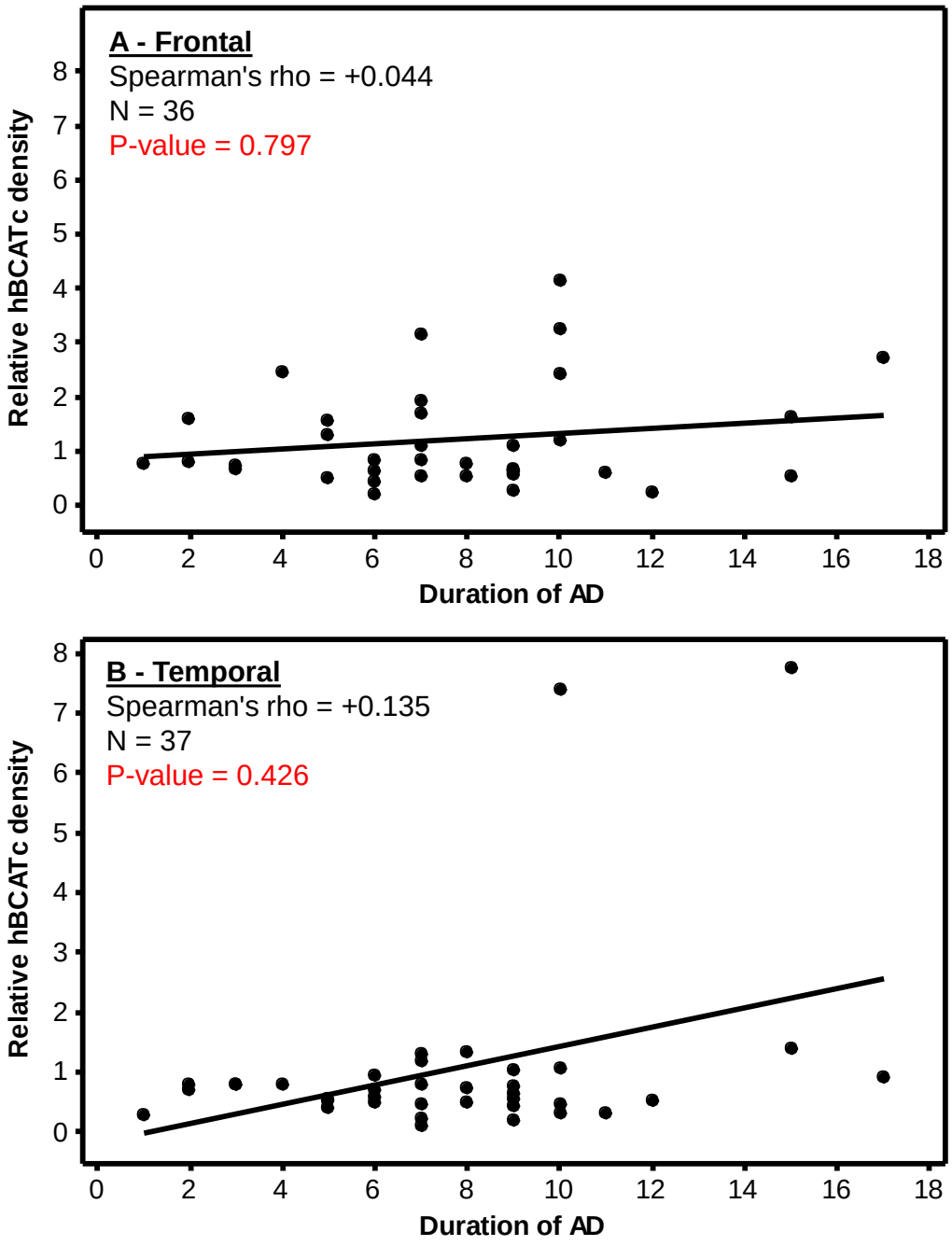


Figure 11.14. Scatterplots of frontal and temporal hBCATc protein levels correlated with duration of AD. The density of bands were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

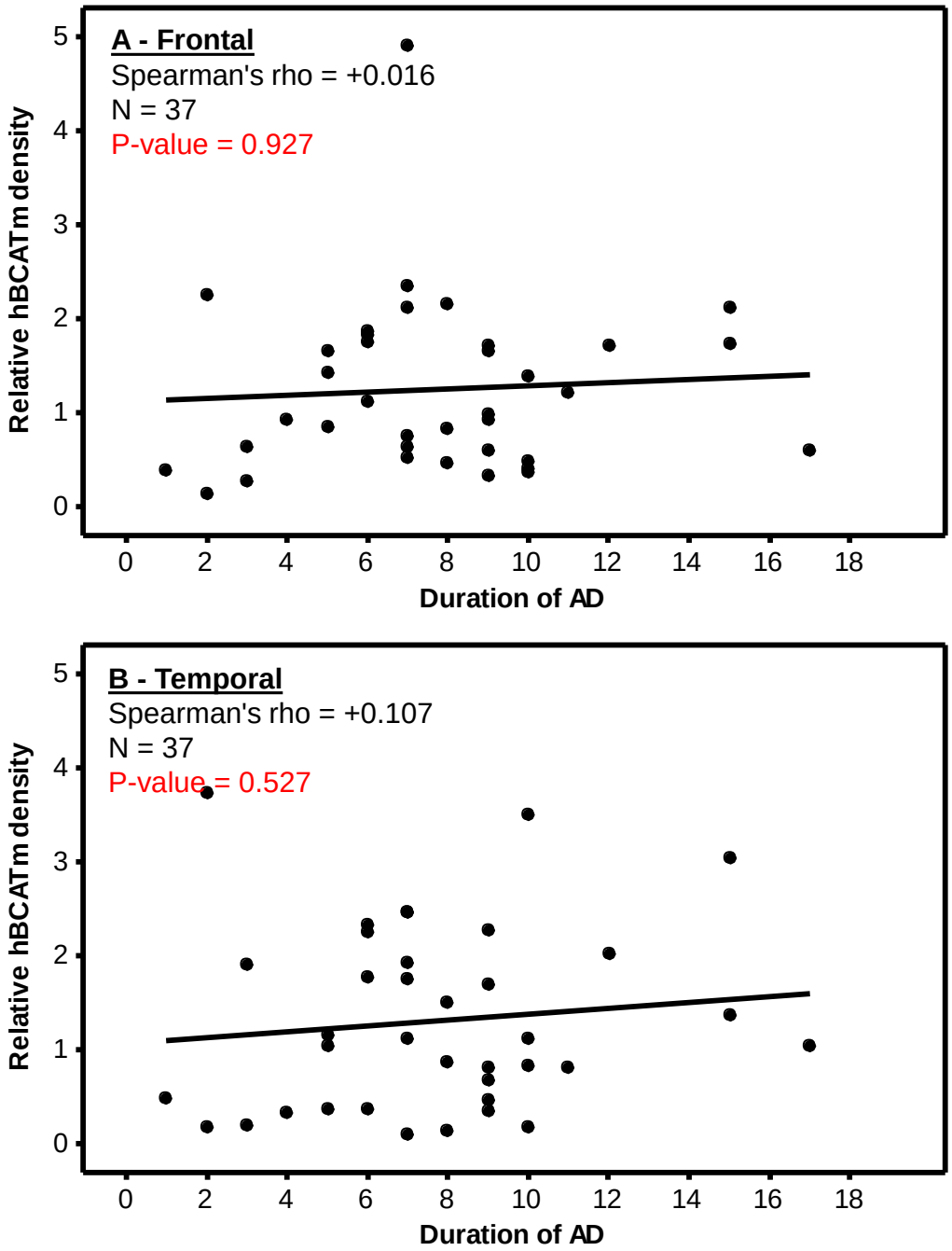


Figure 11.15. Scatterplots of frontal and temporal hBCATm protein levels correlated with duration of AD. The density of bands were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

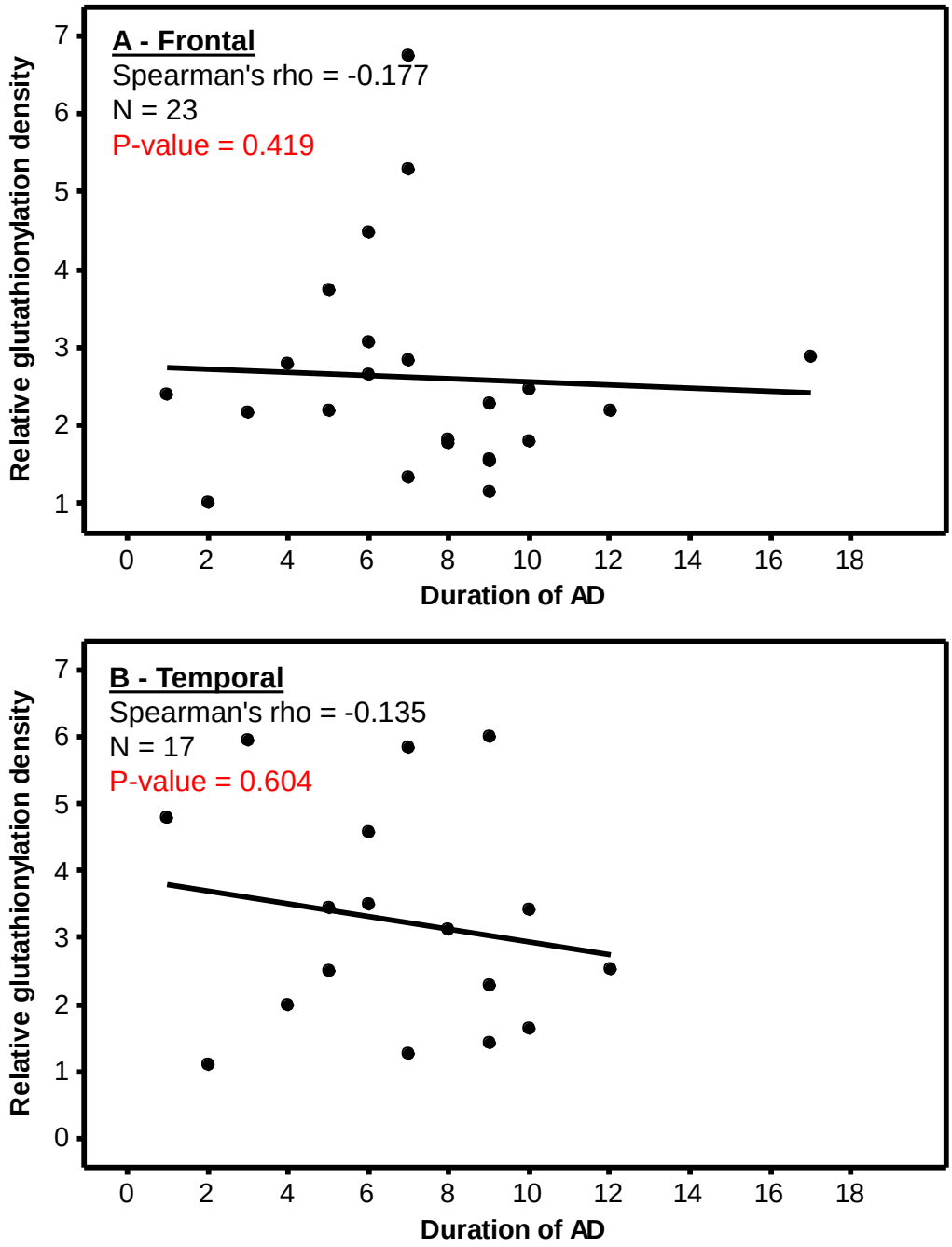


Figure 11.16. Scatterplots of frontal and temporal glutathionylated protein levels correlated with duration of AD. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

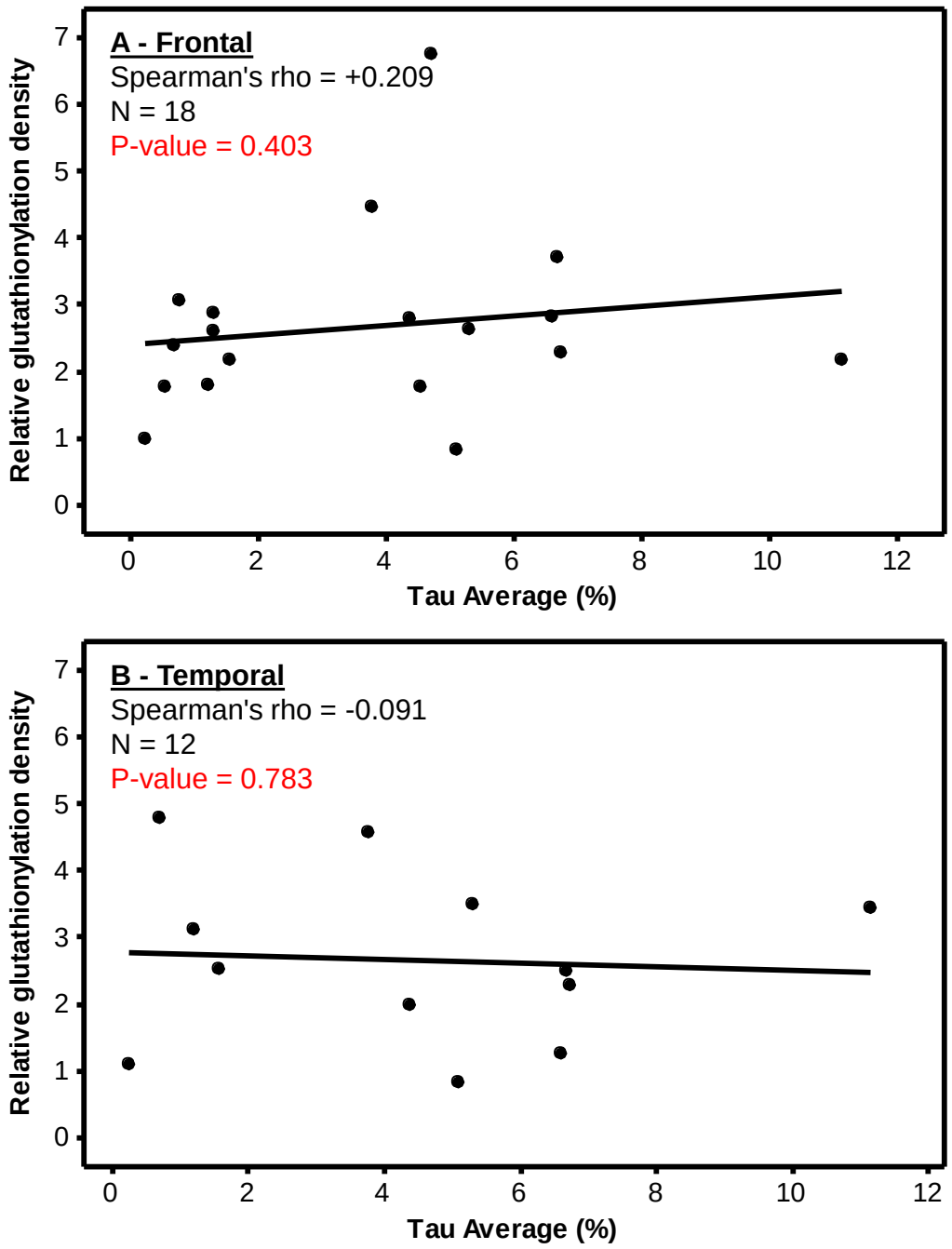


Figure 11.17. Scatterplots of frontal and temporal glutathionylated protein levels correlated with Tau average (%). The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

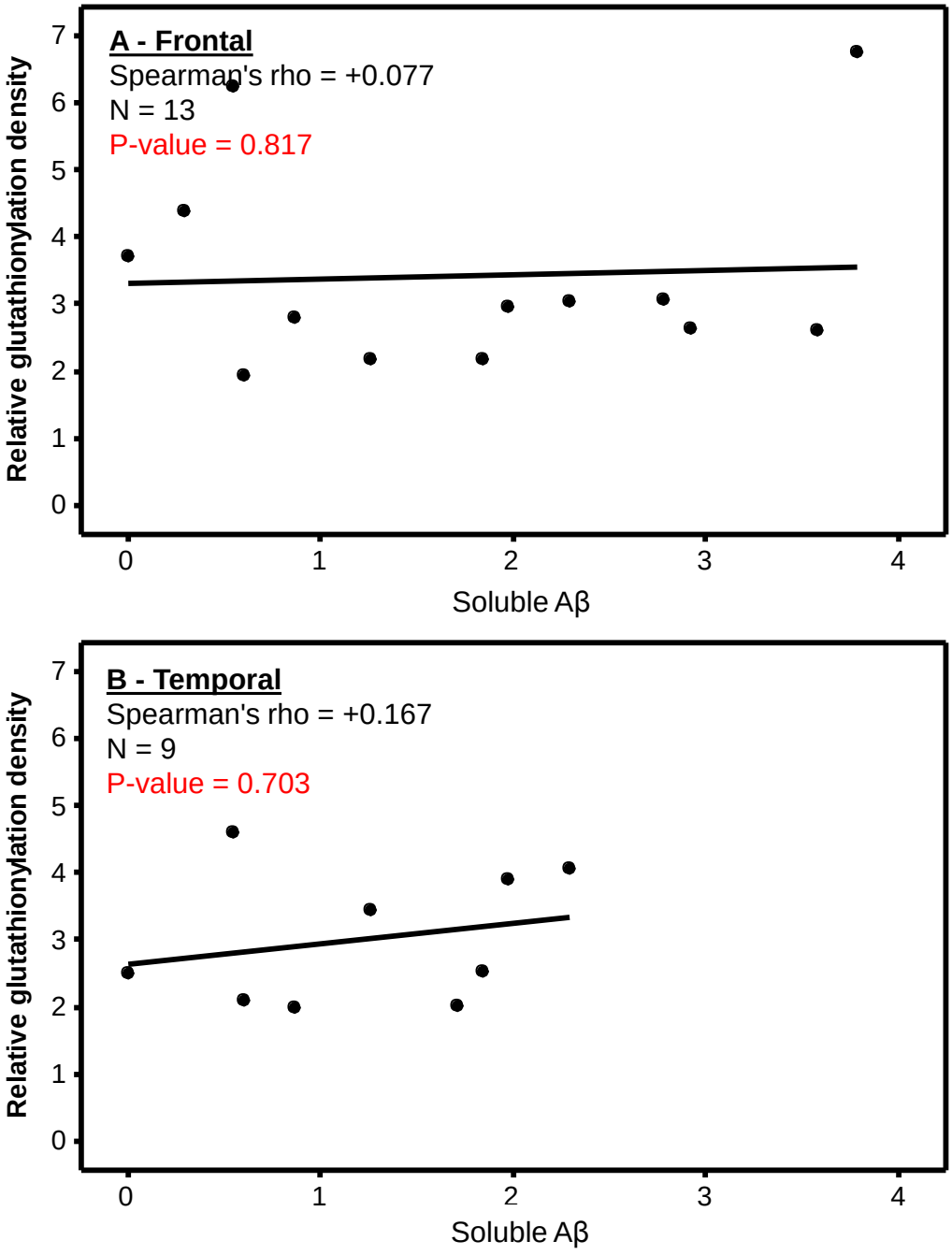


Figure 11.18. Scatterplots of frontal and temporal glutathionylated protein levels correlated with soluble Aβ. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

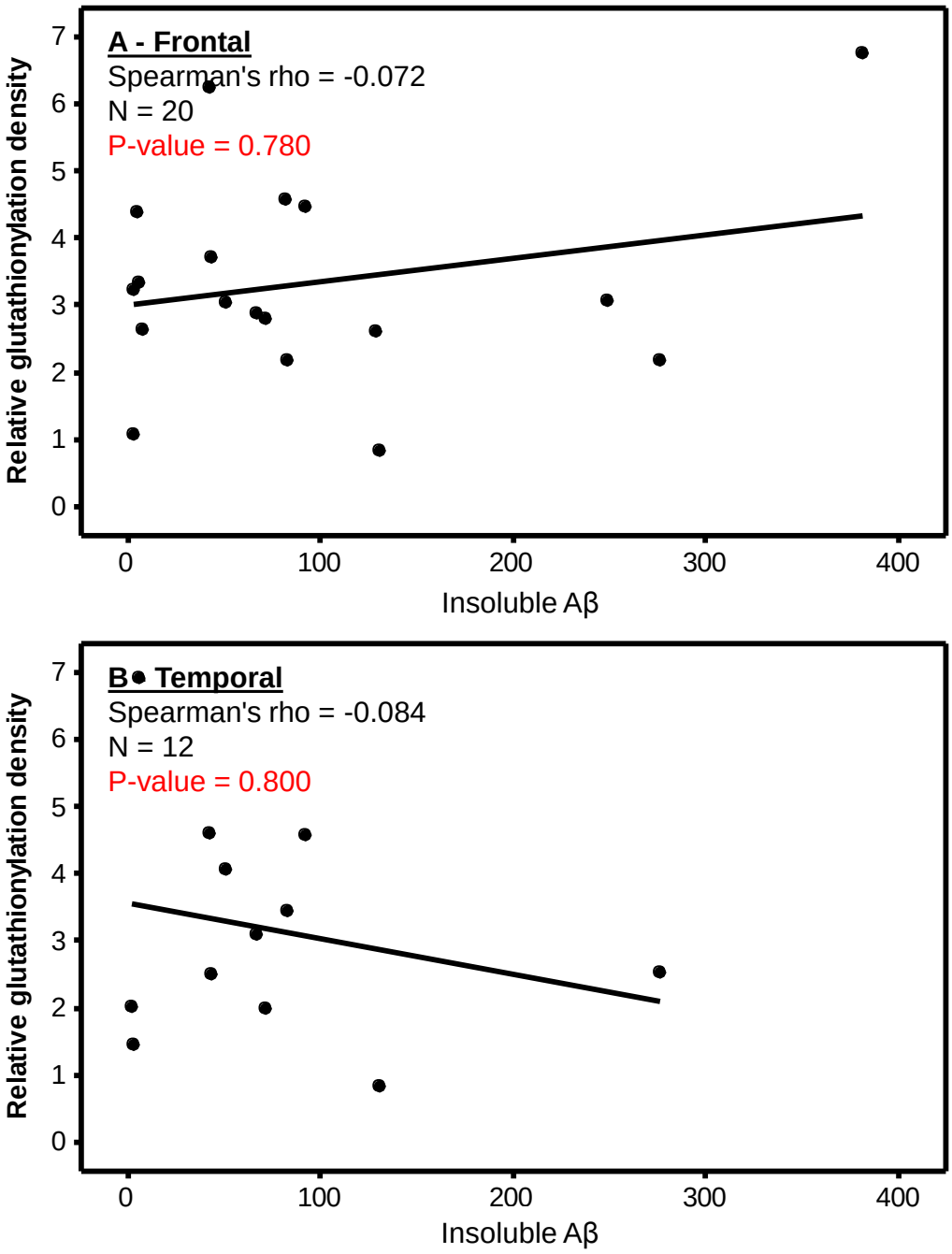


Figure 11.19. Scatterplots of frontal and temporal glutathionylated protein levels correlated with insoluble Aβ. The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

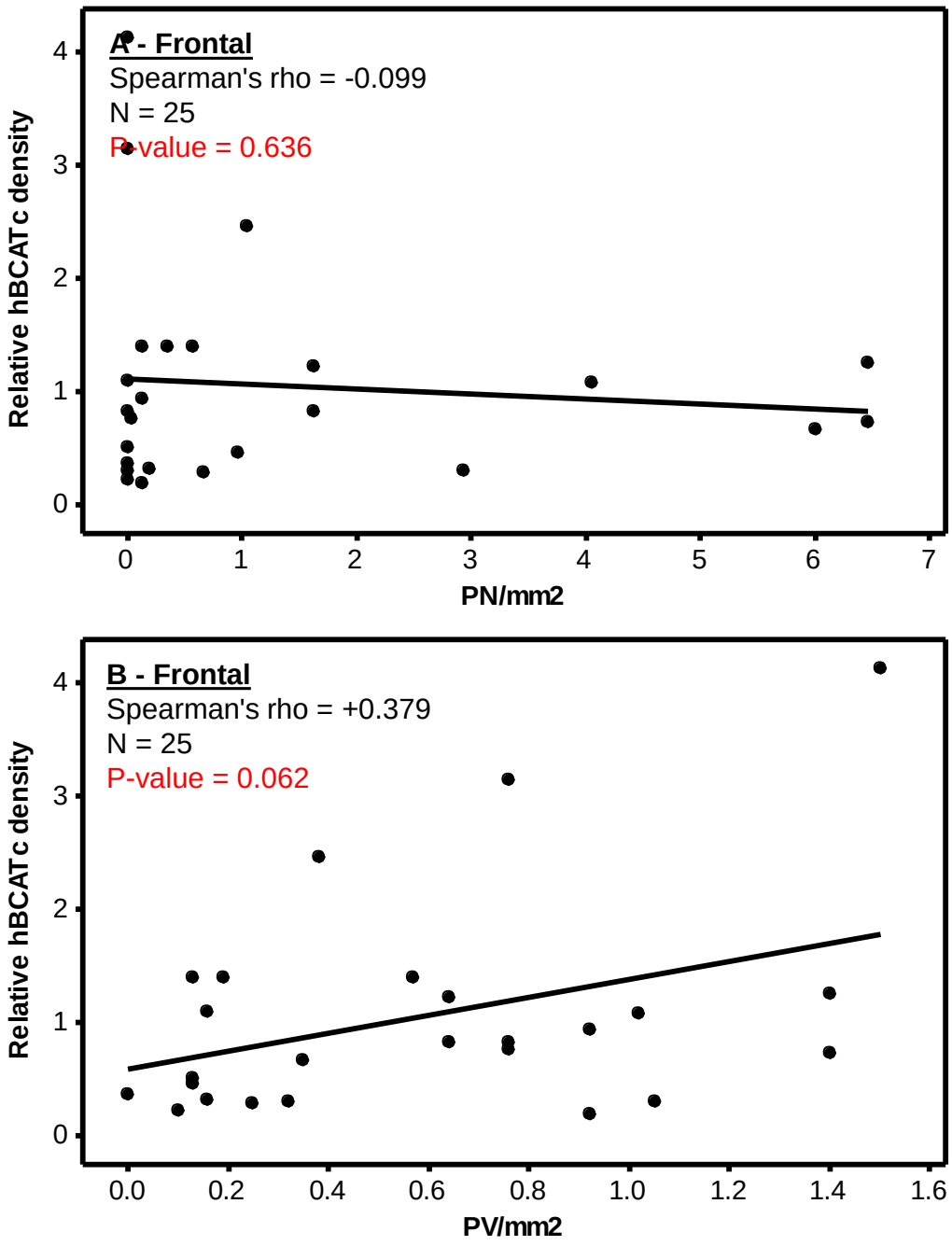


Figure 11.20. Scatterplots of frontal and temporal hBCATc protein levels correlated with perineuronal net (PN) and parvalbumin positive neurons (PV). The density of bands were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

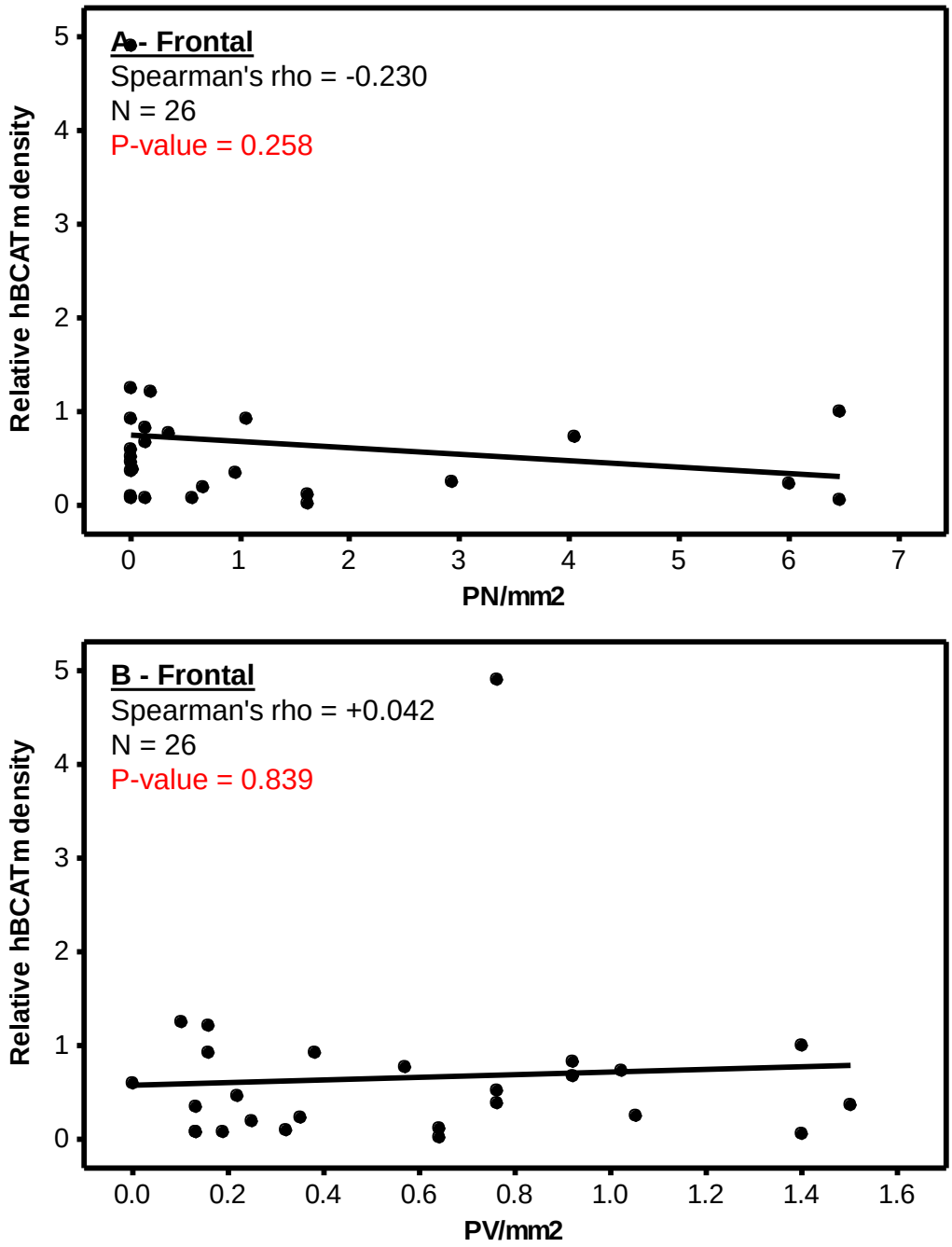


Figure 11.21. Scatterplots of frontal and temporal hBCATm protein levels correlated with perineuronal net (PN) and parvalbumin positive neurons (PV). The density of bands were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

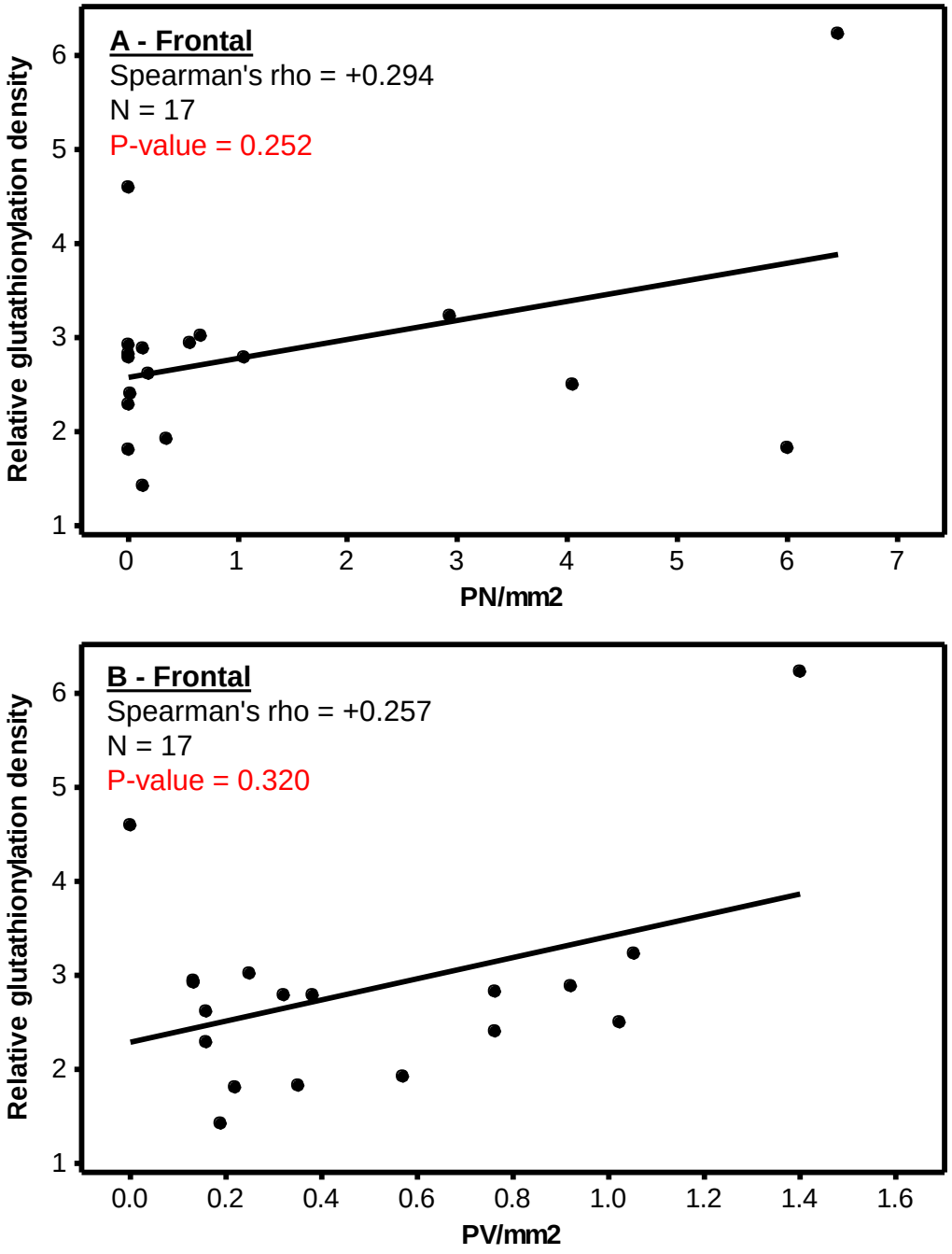


Figure 11.22. Scatterplots of frontal and temporal glutathionylated protein levels correlated with perineuronal net (PN) and parvalbumin positive neurons (PV). The density of lanes were measured using ImageJ™ software (Wayne Rasband, National Institute of Health, USA) and expressed relative to the density for GAPDH. Data was then analysed for significance using Spearman's rho test using Minitab™.

11.2 Expressional alteration of the BCAT enzymes in the AD brain, poster presentation, UWE (2012.01.13)

Expressional alteration of the branched chain aminotransferase enzymes in the AD brain

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†This study was supported by bursary from BRACE (Bristol Research into Alzheimer's and Care of the Elderly) awarded to M.E.C. at the University of the West of England.

Introduction

The chemical neurotransmitter, glutamate is essential for memory and learning. However, as levels of glutamate rise it becomes toxic to the cell ultimately leading to neuronal degeneration as described for patients with Alzheimer's disease (AD)[1,2]. The human cytosolic branched chain aminotransferase enzyme (hBCATc) is a brain specific enzyme involved in the control of glutamate levels in the brain. The cytosolic isoform is neuronal specific and considered to play a prominent role in replenishing glutamate levels in neuronal cells[3,4], whereas the mitochondrial isoform, hBCATm, identified in the endothelial cells of the vasculature, are proposed to support astrocytes in the fine tuning of glutamate levels in the brain (Figure 1). As glutamate toxicity contributes to neuronal destruction, the strategic location of these enzymes and their functional role in contributing to glutamate brain metabolism led to the hypothesis that expression of these enzymes may be altered and influence the pathogenesis of AD.

Aims: To investigate alterations in the expression of the BCAT enzymes in AD brain homogenates compared to age and gender matched controls. Immunohistochemistry (IHC) was used to detect if the cell type responsible for the up-regulation could be described.

Results

Here, hBCATc expression was significantly upregulated by 28% in the frontal and temporal cortex in AD individuals compared to controls (Figure 2A & 3A and B). This upregulation was neuronal in nature but other cells also contributed (Figure 4A-F). Significant upregulation of hBCATm by 160% and 170% in the frontal and temporal cortex in AD individuals compared to controls, respectively (Figure 2B & 3C and D). And that this increased expression seemed predominantly endothelial (**P-value of 0.025**) in nature (Figure 5). This study also correlated the Braak pathological scoring system with hBCATm but not hBCATc levels in the frontal (**P-values of 0.034 and 0.605, respectively**) and temporal cortex (**P-values of 0.016 and 0.482, respectively**) found by western blot (Figure 6).

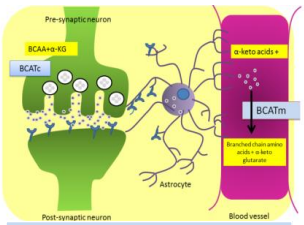

Figure 1. Proposed hypothesis of the hBCAT cycle. The hBCATc enzyme produces glutamate that is functionally used either as a neurotransmitter or as a precursor in neurons. The hBCATm replenishes brain branched chain amino acids for the further production of glutamate.

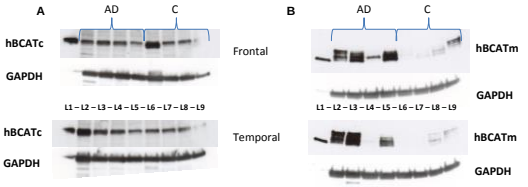

Figure 2. Western blot analysis of hBCATc and hBCATm in patients with AD relative to controls. Lane 1 (L1) on each gel contained a constant 30 ng of purified hBCATc or hBCATm and densitometry was carried out relative to this. Lanes 2-5 contained AD patients and lanes 6-9 contained age and sex matched controls. GAPDH is also shown to display equal loading.

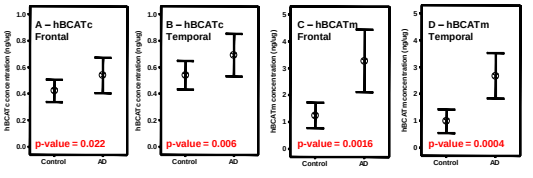

Figure 3. Statistical evaluation of hBCAT expression in AD compared to control subjects. Densitometry of Western blot analysis was used to determine hBCAT expression. Panel A/B, statistical analysis of 30 AD and control patients in the frontal and temporal cortex, respectively. Panel C/D, statistical analysis of 30 AD and control patients in the frontal and temporal cortex.

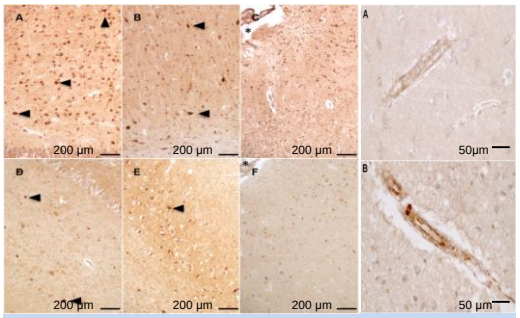

Figure 4. Staining of hBCATc in the hippocampus and temporal of AD and control subject. Panels A-C: CA4, CA1 and temporal cortex in an AD patient. Panes D-F: CA4, CA1 and temporal cortex in control patient. Large arrows show neuronal staining. * marks the collateral sulci of the temporal lobe.

Figure 5. Vessel staining of hBCATm in AD and controls. Panel A and B: hBCATm staining in control compared with AD

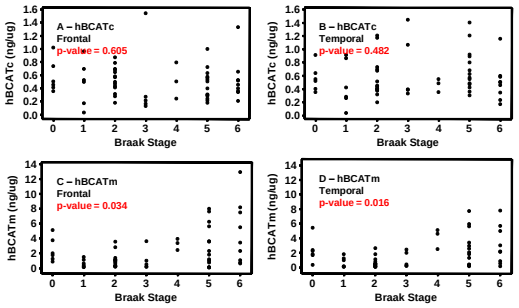

Figure 6. Scatter plot of hBCAT concentration against Braak stage. It can be seen that there is no relationship or correlation between hBCATc concentration and Braak stage in either the frontal or temporal cortices. It can also be seen that there is a consistent increase of hBCATm concentration with increasing Braak stage in both the frontal and temporal cortices.

Discussion

Increased expression of the hBCAT proteins seen here in AD patients would metabolically cause an imbalance in glutamate metabolism. This will either cause an over production of glutamate (as hBCATc is proposed to be responsible for glutamate production in neuronal cells) or it may be an adaptive response by the mitochondrial isozyme, which is hypothesized to work in synergy with astrocytes to remove excess glutamate from synapses. However, increased oxidative stress that is reported during the pathogenesis of AD can also modify the function of receptive enzymes[5]. The mitochondrial hBCATm protein is redox regulated and should hBCAT become oxidatively modified and 'turned off' glutamate and the keto acids (toxic metabolites) would increase.

Altered levels of hBCAT intermediates are known to cause neurological problems e.g. maple syrup urine disease (MSUD). Recent work by Mochel *et al.*, reported that BCAA levels are decreased in Huntingons disease. Interestingly, the decrease of the BCAA leucine precedes clinical features of the disease promoting them as novel biomarkers[6]. Leucine is a known activator of mTor (mammalian target of rapamycin) whose inhibition causes autophagic proteolysis, therefore decreased levels of leucine may promote autophagic proteolysis in diseases where altered BCAA metabolism is a pathological feature[7,8]. This also suggests that dietary alteration of BCAA (either supplementation or restriction) may be a novel therapeutic avenue for neurodegenerative disease reported in MSUD and Phenylketonuria, hepatic cirrhosis and neurological disorders such a bipolar disorder[9,10]. Understanding these pathways and mechanisms will open new avenues for targeted treatment for AD potentially slowing down or halting the progression of AD.

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11.3 Pilot study: Expressional changes of hBCATc in the IMR-32 cell line, implications for neurological disease, poster presentation, UWE (2012.12.19)

Pilot study: Expressional changes of hBCATc in the IMR-32 cell line, implications for neurological diseases

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Introduction: Glutamate is the major neurotransmitter of the human brain, with neurons utilising glutamate either as a neurotransmitter or a metabolite for other neurotransmitter production [1]. The human branched chain aminotransferase (hBCAT) proteins are key contributors to the brain glutamate pool with the cytosolic hBCAT (hBCATc) showing neuronal specificity and the mitochondrial hBCAT (hBCATm) form restricted to the vascular cells of the brain (Fig 1) [2, 3]. Alterations in glutamate signalling are seen in many neurological disorders (e.g. Alzheimer's disease and motor neuron disease) and occur via a pathological mechanism referred to as glutamate toxicity [4]. This occurs via the over activation of post-synaptic calcium channels (i.e. NMDA receptors) leading to an excess of intra-cellular calcium and inappropriate activation of calcium dependent enzymes (e.g. calpain or calcium dependent phosphatases) [4]. There is mounting evidence that pro-inflammatory cytokines such as TNFα and IL1α can exacerbate this glutamate toxicity [5, 6, 7].

Aim: The aim of this study was to determine what factors commonly found in disease, as well as key metabolites for the hBCATc enzyme, would effect hBCATc protein expression in the human neuroblastoma cell line IMR-32.

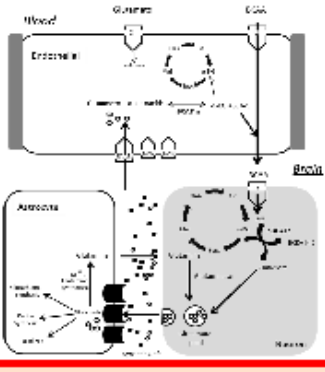


Figure 1: Model proposed for hBCAT signalling in the human brain. hBCATc in neurons sustains the neuronal glutamate pool using branched chain amino acids (BCAAs) and hBCATm in the brain endothelium works to recycle these BCAAs. Abbreviations: αKG — α-ketoglutarate; BCKA — branched chain keto-acids; EAAT—excitatory amino acid transporter; L1—large neutral amino acid transporter.

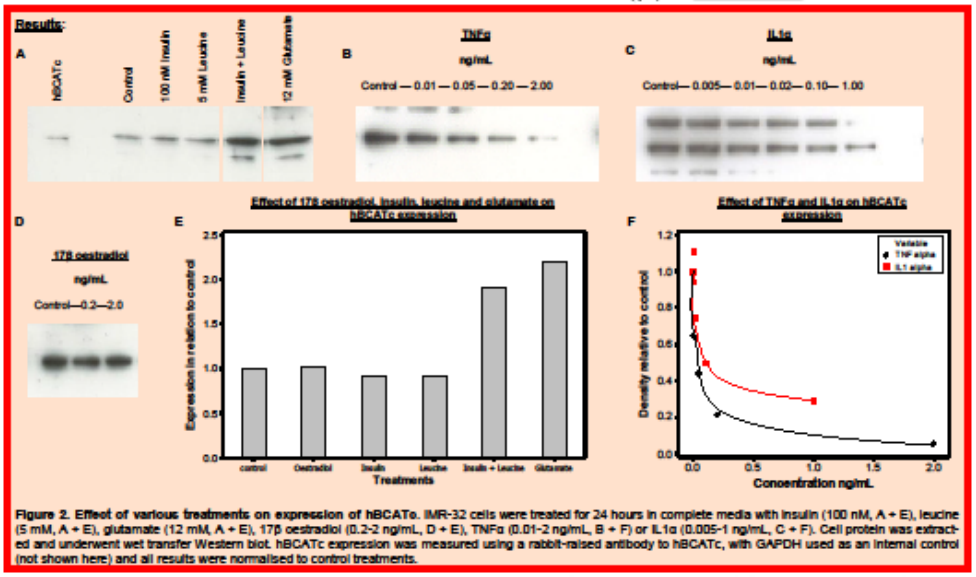


Figure 2. Effect of various treatments on expression of hBCATc. IMR-32 cells were treated for 24 hours in complete media with insulin (100 nM, A + E), leucine (5 mM, A + E), glutamate (12 mM, A + E), 17β oestradiol (0.2-2 ng/mL, D + E), TNFα (0.01-2 ng/mL, B + F) or IL1α (0.005-1 ng/mL, C + F). Cell protein was extracted and underwent wet transfer Western blot. hBCATc expression was measured using a rabbit-raised antibody to hBCATc, with GAPDH used as an internal control (not shown here) and all results were normalised to control treatments.

Conclusion: The effectiveness of IL1α (figure 2C + F) and TNFα (Figure 2B + F) in altering hBCATc expression raises an interesting question about the relationship they share in the brain immune response. With the branched chain amino acids decreasing TNFα expression in certain models [8] a possible negative feedback mechanism seems feasible (Fig 3). This will allow neurons to control the level of immune involvement in the brain, however this may also lead to an increased vulnerability of these neurons to glutamate insult via decreased removal capacity [7, 9]. The effect of leucine and insulin (Figure 2A + E) implies that a synergistic effect is used to alter hBCATc expression, most likely through synergistic activation of the mTOR pathway [10]. Further studies will be carried out in the presence of Rapamycin, an inhibitor of mTOR, to confirm this. Increased expression of hBCATc with 12 mM glutamate (Figure 2A + E) is most likely a response to pathological levels of glutamate, with the hBCATc working to remove excess glutamate. Future work will investigate these treatments and other treatment combinations associated with disease. hBCATc activity will be investigated to see if it correlates with observed expression.

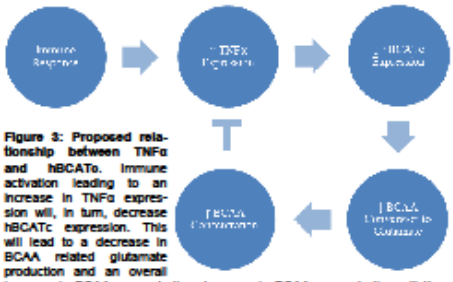


Figure 3: Proposed relationship between TNFα and hBCATc. Immune activation leading to an increase in TNFα expression will, in turn, decrease hBCATc expression. This will lead to a decrease in BCAA related glutamate production and an overall increase in BCAA concentration. Increase in BCAA concentration will then negatively feedback causing a decrease in TNFα expression. It can also be speculated that a similar mechanism may exist for IL1α.

References: [1] Meldrum, (2000). *J. Nutr.*; 130:1007S-1015S. [2] LaNoue, *et al.* (2001). *Journal of neuroscience research*; 66:914-922. [3] Hull, *et al.* (2012). *J. Neurochem*; [e-pub ahead of print]. [4] Seltzer & Tymianski, (2000). *Journal of molecular mechanisms*; 78:3-13. [5] Clark, *et al.* (2010). *Pharmacology and therapeutics*; [e-pub ahead of print]. [6] Griffin, *et al.* (1995). *Journal of neuropathology and experimental neurology*; 54(2):276-281. [7] zao & Crews, (2004). *Brain research*; 11-24. [8] Terakura, *et al.* (2012). *Carcinogenesis*; 33(12):2499-2506. [9] Kholodilov, *et al.* (2001). *Molecular Brain Research*; 75:281-286. [10] Gran & Cameron-Smith, (2011). *BMC physiology*; 11:10.

11.4 Co-localisation of hBCATm protein with LC3-II using confocal and electron microscopy: relation to AD pathology, poster presentation, ARUK conference (2013.02.26)

Co-localisation of hBCATm protein with LC3-II using confocal and electron microscopy: relation to AD pathology

Jonathon Hull, Farah El Amraoui, Andrew Wilson, Mohammed Hezwani, Maya El Hindy, David Corry, David Patton, Martin Hassler, Abbe Mansbridge, Pat Kehoe, Seth Love and Myra Conway

Introduction

The human branched chain aminotransferase (hBCAT) proteins are key metabolic enzymes that catalyse the transamination of the branched chain amino acids (BCAAs) leucine, isoleucine and valine to their respective α-ketoacids and glutamate.[1,2] Furthermore, the hBCAT proteins are unique among the aminotransferases in that they have a redox active CXXC motif that regulates protein function through oxidation and nitrosation.[3] Understanding the role of hBCAT in cellular repair or protein folding is essential as oxidative modification and accumulation of mis-folded proteins are hallmarks of Alzheimer's disease (AD), as well as other neurodegenerative diseases (e.g. Parkinson's disease).[4] Autophagy is one of the key processes by which irreversibly modified and aggregated mis-folded proteins are removed from within the cell, and involves the breakdown of these cellular components through lysosome activity. In health, autophagy ensures the degradation and recycling of cellular components and is inhibited by the hBCAT metabolite leucine through mTOR activation.[5] It is thought that some aspects of this process are dysfunctional in neurodegenerative diseases.[6] Taken together, as brain-localised hBCAT proteins are significantly increased (>100%) in the brains of patients with AD[7] our hypothesis is that this up-regulation may relate to a) hBCATs role as a transaminase (i.e. regulation of excess BCAAs or glutamate) or b) hBCATs redox role within the cell.

Aims: This study aimed to answer these following questions: (i) Can hBCAT refold proteins? (ii) Is there a link between hBCAT and autophagy?

Results and Discussion

Frontal and temporal post-mortem tissue of 30 AD and 30 control subjects showed a clear up-regulation of the hBCATm protein (by >100%), that was seen using both immunohistochemistry (Figure 1) and Western blot analysis. To investigate the cause of this increased expression the isomerase ability of hBCAT as well as its association with the key autophagy marker LC3-II was investigated. It was found that the hBCAT proteins have dithiol-disulphide isomerase activity (Figure 2A) that is mediated through an S-glutathiolylated mechanism (Figure 2B & C). Mutation of a single cysteine of the CXXC motif ablates isomerase activity (Figure 2D), with activity returning upon addition of GSH/GSSG or GSNO (Figure 2E). Electron and confocal microscopy showed that hBCATm co-localises with LC3-II (Figure 3, Figure 4). Electron microscopy showed hBCATm localising with LC3 positive phagosomes under control, 100 nM Rapamycin and 100 μM H_2O_2 conditions, with this association being less common in 5 mM leucine treatment (Figure 3A, B, C & D). This work also raises the possibility of the phagosome membrane being derived from the mitochondria as well as the endoplasmic reticulum which has been previously described.[8] Confocal microscopy showed that under hydrogen peroxide induced cell death, co-localisation between LC3-II and hBCATm appeared to increase (Figure 4).

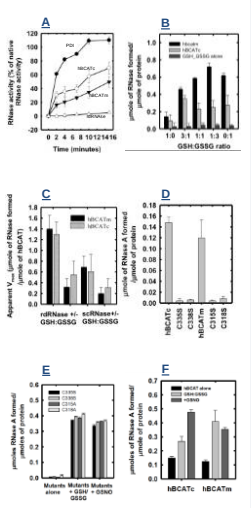


Figure 2. Isomerase activity of the hBCAT proteins and the influence of the redox environment. Reduced and denatured rdRNase (30 μM) was incubated with PDI, hBCATm or hBCATc (5 μM, unless otherwise stated) in 0.1 M Tris-HCl, pH 7.4 and 1 mM EDTA and then monitored for the effect of either time, concentration, GSSG and/or GSH. Refolding was determined using the RNase activity assay monitoring the increase in absorbance at 284 nm. Data are the mean ± SEM, n=3. **Panel A**: Dithiol-disulphide exchange activity for PDI (●), hBCATc (○), hBCATm (▼), and rdRNase alone (Δ), showing that the hBCAT proteins have isomerase function but with a lower activity of PDI. **Panel B**: RNase was incubated with hBCATm or hBCATc at 10 μM +/- varying GSH/GSSG ratios (1:0, 3:1, 1:1, 1:3 and 0:1) over 24 hours. Controls included incubating the unfolded rdRNase with the same ratios of GSH/GSSG with exception to the hBCAT proteins and rdRNase with hBCAT alone. **Panel C**: Varying concentrations of rdRNase or scRNase (5-60 μM) were incubated with hBCATc and hBCATm at 10 μM, respectively +/- a glutathione buffer (3:1 GSH:GSSG) over 24 hours. Lineweaver-Burk plots were used to determine the apparent Vmax in μmoles min-1 which was converted to μmoles of active RNase formed/μmole of each respective hBCAT protein. **Panel D**: Active RNase formed by native hBCAT relative to their mutant proteins, showing a complete removal of isomerase activity upon CXXC mutation. **Panel E**: Active RNase formed by mutant proteins +/- a glutathione redox buffer or +/- 1 mM GSNO, respectively. **Panel F**: Active RNase formed of native hBCAT +/- a glutathione redox buffer or +/- 1 mM GSNO.

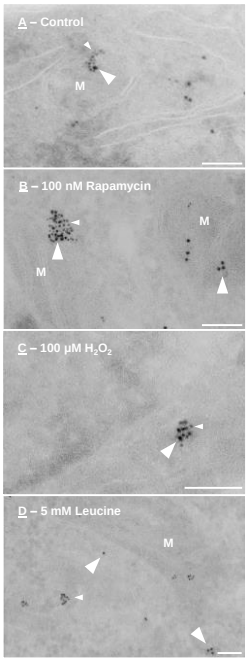


Figure 3. Electron Microscope Images of IMR-32 cells. IMR-32 cells were treated for 30 minutes (control, Rapamycin and H_2O_2) or 24 hours (leucine). Cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde and processed according to the Tokuyasa method for cryosectioning.[9] Gold labeled secondary antibodies were used to visualize the primary antibodies, hBCATm (10nm, large arrow) and LC3-II (6nm, small arrow) were present in all treatment conditions. Uranylacetate was used as a counterstain. Sectioning and imaging was carried out at Bristol University (Wolfson Bioimaging Facility). **M** denotes the mitochondria when present on the images. Scale bars: 100 nm.

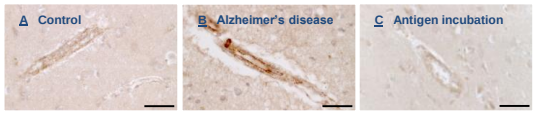


Figure 1. Vessel staining of hBCATm. **Panel A**: hBCATm staining in a control subject. **Panel B**: hBCATm staining in an AD subject. **Panel C**: Antigen incubation at 200X molar excess. Representative images are shown of 30 control and 30 AD subjects. Western blot analysis was also carried out and showed the same pattern. Slides were incubated overnight at 60°C before being de-waxed in clearene and ethanol. Endogenous peroxidase was quenched (using H_2O_2 and methanol) before citrate pre-treatment and serum block. Primary antibodies were incubated overnight, secondary antibodies were peroxidase linked and DAB was used to visualise the secondary antibody. Haematoxylin and eosin were used as a counter stain and the slides were dehydrated in ethanol and clearene, then mounted in clearium. Images were acquired on a Nikon eclipse 80i. Scale bar: A, B and C, 50 μM.

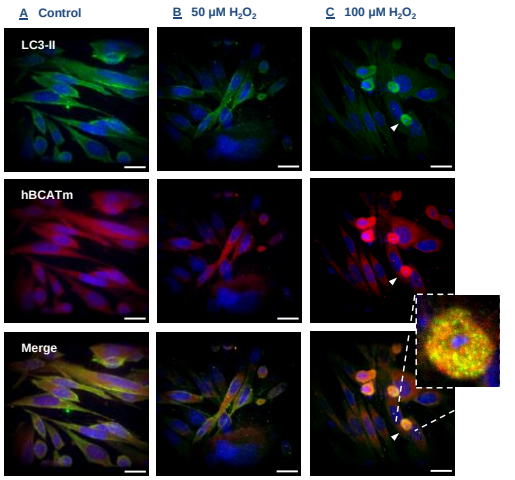
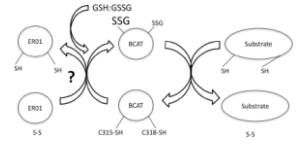


Figure 4. Confocal Images of IMR-32 cells. IMR-32 cells were grown on glass coverslips (treated with poly-L-lysine) and then treated with either control media (**Panel A**) or media containing H_2O_2 (50 μM **Panel B**, 100 μM **Panel C**) for 30 minutes (37°C humid incubator). Cells were fixed with 0.25% glutaraldehyde and permeabilised with Triton X. Alexafluor 568 (red) was used to label hBCATm antibodies (Insight biotechnology) and alexafluor 488 (green) was used to label LC3-II antibodies (Santa-Cruz). Cells were mounted using a DAPI containing hard-set medium (Vector labs). Mander's correlation coefficient (Mx values) was used to measure correlation statistically. Slides were imaged using a Perkin Elmer Ultraview FRET H spinning disk confocal microscope.Scale bars: 20 μm.



Scheme 1. Isomerase activity of hBCAT. Under nucleophilic attack the reactive thiolate anion of RNAse forms an inter-disulphide bond with S-glutathionylated hBCAT. Together with the C terminal cysteine the second thiol of RNAse forms a disulphide bond, releasing RNAse. Without the thiol at the C terminal position the mixed disulphide intermediate cannot be released and remains fixed the N-terminal residue (Figure 2E).

Conclusion

- hBCAT has been identified as a dithiol-disulphide isomerase that can refold proteins (Scheme 1).
- hBCATm co-localises with LC3-II, a key protein of autophagy. This is similar to work done with PDI[10], that localised PDI to the early phagosome – possibly associated with chaperone mediated autophagy.
- Expression of hBCATm in AD is increased by over 100%.
- This increased expression is likely related to the isomerase function or role in autophagy associated with hBCATm, however what effect this has on hBCAT aminotransferase activity is under investigation.

References [1] Conway *et al.* (2003) *Biochemica Et Biophysica Acta* **1647**: 61-65; [2] Hull *et al.* (2012) *Journal of Neurochemistry* **123**: 997-1009; [3] Conway *et al.* (2004) *Biochemistry* **42**: 7356-7364; [4] Dobson (2003) *Nature* **426**: 884-890; [5] Larsen & Sulzer (2002) *Histology and Histopathology* **17**: 897-908; [6] Levine & Kroemer (2008) *Cell* **132**: 27-42; [7] Hull *et al.* (2013) *In preparation*; [8] Dunn (1990) *Journal of Cell Biology* **110**: 1923-1933; [9] Tokuyasu (1973) *Journal of Cell Biology* **57**: 551-565; [10] Rich *et al.*, (2003) *Cellular Microbiology* **5**: 455-468.

11.5 Distribution of the branched chain aminotransferase proteins in the human brain and their role in glutamate regulation, published paper (2012)

11.6 The branched chain aminotransferase proteins: novel redox chaperones for protein disulphide isomerase, published paper (2013)

11.7 Upregulation of the BCAT protein in the brains of patients with Alzheimer's disease: implications in glutamate toxicity (2014)