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1 **Distribution of the branched-chain α-ketoacid dehydrogenase complex E1α**
2 **subunit and glutamate dehydrogenase in the human brain and their role in**
3 **neuro-metabolism**[†]

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18

Abstract

Glutamate is the major excitatory neurotransmitter of the central nervous system, with the branched-chain amino acids (BCAAs) acting as key nitrogen donors for *de novo* glutamate synthesis. Despite the importance of these major metabolites, their metabolic pathway in the human brain is still not well characterised. The metabolic pathways that influence the metabolism of BCAAs have been well characterised in rat models. However, the expression of key proteins such as the branched-chain α-ketoacid dehydrogenase (BCKD) complex and glutamate dehydrogenase isozymes (GDH) in the human brain is still not well characterised. We have used specific antibodies to these proteins to analyse their distribution within the human brain and report, for the first time, that the E1α subunit of the BCKD is located in both neurons and vascular endothelial cells. We also demonstrate that GDH is localised to astrocytes, although vascular immunolabelling does occur. The labelling of GDH was most intense in astrocytes adjacent to the hippocampus, in keeping with glutamatergic neurotransmission in this region. GDH was also present in astrocyte processes abutting vascular endothelial cells. Previously, we demonstrated that the branched-chain aminotransferase (hBCAT) proteins were most abundant in vascular cells (hBCATm) and neurons (hBCATc). Present findings are further evidence that BCAAs are metabolised within both the vasculature and neurons in the human brain. We suggest that GDH, hBCAT and the BCKD proteins operate in conjunction with astrocytic glutamate transporters and glutamine synthetase to regulate the availability of glutamate. This has important implications given that the dysregulation of glutamate metabolism, leading to glutamate excitotoxicity, is an important contributor to the pathogenesis of several neurodegenerative conditions such as Alzheimer's disease.

Keywords: BCKD, GDH, BCAA, glutamate.

Running title: BCKD and GDH in the human brain.

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1 **Abbreviations**

2 AD, Alzheimer's disease; BCAA, branched-chain amino acids; hBCAT, human
3 branched-chain aminotransferase; BCKA, branched-chain α-keto acid; BCKD,
4 branched-chain α-keto acid dehydrogenase complex; CA, cornu ammonis area; DAB,
5 3.3'-diaminobenzidine; DAPI, 4',6-Diamidino-2-Phenylindole; DPX, distyrene
6 plasticiser xylene; EDTA, ethylenediaminetetraacetic acid; GABA, gamma-amino
7 butyric acid; GDH, glutamate dehydrogenase; GFAP, glial fibrillary acidic protein;
8 hBCATc, human cytosolic branched-chain aminotransferase; hBCATm, human
9 mitochondrial branched-chain aminotransferase; PBS, phosphate buffered saline; RT,
10 room temperature; VWF, Von Willebrand factor.

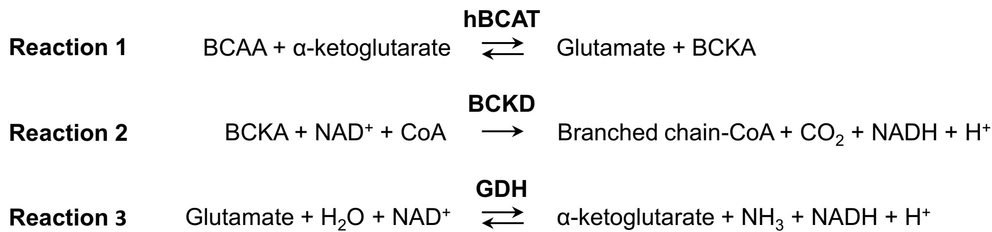
11

Introduction

The three-essential branched chain amino acids (leucine, isoleucine and valine) are key nitrogen contributors for the *de novo* synthesis of glutamate. Studies on the rat retina demonstrated that inhibition of the cytosolic branched-chain aminotransferase (BCATc) decreased *de novo* glutamate synthesis by 30% (LaNoue *et al*., 2001; Lieth *et al*., 2001). In humans, the first step in BCAA metabolism is catalysed by the branched-chain aminotransferases (hBCAT, EC 2.6.1.42), mitochondrial (hBCATm) and cytosolic (hBCATc). These proteins mediate the transfer of the α-amino group from the BCAA to α-ketoglutarate to produce glutamate and the respective branched-chain α-keto acid (BCKA – ketoisocaproate, ketomethylvalerate and ketoisovalerate; Scheme 1, reaction 1). Studies in human tissue have demonstrated hBCATm expression within the vasculature, with hBCATc predominantly expressed in neurons (Hull *et al*., 2012). The second step in BCAA metabolism is considered the rate-limiting step, as the BCKAs are irreversibly oxidised by the branched-chain α-keto acid dehydrogenase (BCKD, EC 1.2.4.4) complex. This produces branched-chain acyl Co-A derivatives (Scheme 1, reaction 2), generating the Kreb's cycle substrate acetyl-CoA, and the Kreb's cycle intermediate succinyl-CoA (Harris *et al*., 1986; Hutson *et al*., 2005).

The BCKD complex is primarily regulated by phosphorylation and dephosphorylation of the E1 subunit by the BCKD kinase and BCKD phosphatase proteins, with phosphorylation inactivating the complex (Damuni *et al*., 1984; Damuni *et al*., 1987; Popov *et al*., 1991; Popov *et al*., 1992; Shimomura *et al*., 1990; Shimomura *et al*., 2001; Wynn *et al*., 2000). The distribution of the BCKD complex has yet to be determined in the human brain. However, work in rat has demonstrated BCKD-E1α within neurons, where it is thought to contribute to the BCAA-BCKA shuttle and energy production (Cole *et al*., 2012). The activity of the BCKD complex is influenced by the activity of the two glutamate dehydrogenase isoforms (GDH1 and GDH2; EC 1.4.1.3), which catalyse the conversion of glutamate into α-ketoglutarate (Scheme 1, reaction 3) (Islam *et al*., 2010).

Scheme 1



GDH1 is expressed in the liver and was previously detected in astrocytes within the human brain. Conversely, the GDH2 protein is absent from liver and expressed in the human testes, kidney and brain, where it was also observed in astrocytes in the human cortex (Nissen *et al*., 2016; Spanaki *et al*., 2010; Spanaki *et al*., 2014; Spanaki & Plaitakis, 2012). Both GDH isoforms are considered mitochondrial, however GDH activity has also been associated with the nucleus (di Prisco *et al*., 1968; Lai *et al*., 1986) and the endoplasmic reticulum (Colon *et al*., 1986; Lee *et al*., 1999) of rat tissue. The GDH proteins link glutamate metabolism with the Kreb's cycle, and whilst the reaction is potentially reversible, it is accepted that in the brain this reaction is predominantly directed to metabolise glutamate (Lorin *et al*., 2013; Yudkoff *et al*., 1994; Li *et al*., 2012). GDH is regulated through many factors including the availability of purine nucleotides (ADP, ATP, GTP and NADH), steroid hormones and L-leucine. GTP is the predominant negative regulator of GDH1, activating the protein when cell energy production is reduced. GDH2 lacks such a regulator and is constitutively active in a metabolically active cell (Spanaki *et al*., 2010). The predominant positive regulators of both enzymes are ADP and L-leucine (increasing activity by up to 14-fold and 9.7-fold, respectively), resulting in acute sensitivity of these proteins to the activity of hBCAT and the BCKD complex (Erecinska & Nelson, 1990; Mastorodemos *et al*., 2005; Plaitakis *et al*., 2013; Smith & Stanley, 2008; Tomita *et al*., 2011).

Previous work by Islam *et al.* (2007, 2010) using pure protein and rat models, demonstrated that hBCATm is able to form a protein complex with the BCKD subunit E1α and GDH1. This complex allows channelling of the BCKAs to the E1α subunit, increasing BCKA decarboxylation 12-fold. The GDH1 protein then binds to the pyridoxamine-5'-phosphate form of BCATm and increases the recycling of the BCAT cofactor pyridoxal-5'-phosphate, also replenishing α-ketoglutarate (Islam *et al*., 2007;

Islam *et al*., 2010). Despite the importance of BCAA and glutamate metabolism in the human brain, it has not been established whether a similar process occurs within this tissue. Establishing a complete understanding of BCAA and glutamate metabolism is of particular relevance to diseases where these metabolites contribute to the pathological process, such as maple syrup urine disease and Alzheimer's disease, respectively.

Our aim in this study was to examine the distribution of the GDH protein and the BCKD complex in the human brain, to ascertain whether the previously described BCAA shuttle (Hull *et al*., 2012) could be extended to include these key metabolic proteins. This previous work provided the first evidence of transaminases (hBCATm) in the endothelial layer operating as a support network to astrocytes mediating the fine-tuning of glutamate homeostasis. We further proposed that under normal physiological conditions, the BCAAs are taken up by neuronal cells, where hBCATc controlled metabolism acts as a pathway to replenish the glutamate pool.

For the first time, the BCKD complex E1α subunit and the GDH proteins were mapped to the human brain. In agreement with findings of Cole *et al*., (2012) in the rat, we report that BCKD complex E1α is in neurons, widely distributed throughout the brain in a variety of neuronal types. However, in addition to observations in the rat, our findings highlight the expression of the BCKD complex E1α in endothelial cells throughout the brain vasculature. In line with previous work (Spanaki *et al*., 2014), immunolabelling for GDH was predominantly expressed in astrocytes; however, labelling was also evident within the vasculature with weak labelling of cerebellar Purkinje cells. We discuss the impact of these findings with respect to the human BCAA-BCKA shuttle and the regulation of glutamate production in the human brain.

Materials and Methods

Materials

Antibodies. Rabbit polyclonal antibodies to BCKD-E1α (ab126173) and GDH1/GDH2 (ab154027), goat polyclonal secondary antibody to rabbit Alexafluor® 488 (ab150077) and mouse Alexafluor® 568 (ab175473), mouse monoclonal antibody to glial fibrillary acidic protein (GFAP), a marker of astrocytes (ab10062) and β3-tubulin, a neuronal marker (ab78078) were purchased from Abcam (Cambridge, UK). Mouse monoclonal antibody to von Willebrand factor (vWF), a marker of vascular endothelial cells (M0616), was purchased from Dako (Cambridge, UK).

Immunohistochemistry. Haematoxylin was purchased from SurgiPath (Peterborough, UK). 4',6-Diamidino-2-Phenylindole (DAPI)-containing hard-set mounting medium, peroxidase substrate 3,3'-diaminobenzidine (DAB), goat serum (S-1000), biotin-labelled secondary antibody raised to IgG, and avidin (Vectastain ABC kit) were purchased from Vector labs (Peterborough, UK). All other materials were purchased from Fisher Scientific (Loughborough, UK).

Tissue preparation

The study was approved by North Somerset and South Bristol Research Ethics Committee. All brain tissue used in this study was from brains donated to the South West Dementia Brain Bank, University of Bristol. The right hemi-brain had been fixed in 10% buffered formalin for 3 weeks before tissue blocks were cut and embedded in paraffin wax for detailed neuropathological assessment. For this study, we examined 18 brains, from people who were older than 60 years of age, had no history of dementia, and minimal or no neuropathological abnormalities as determined by Braak stage (Table 1) and small vessel disease score (Supplementary Table 1).

Serial sections 7 µm in thickness were taken from multiple regions of the brain, including the frontal, temporal, parietal and occipital lobes, basal ganglia, midbrain, cerebellum and pons, and collected on APES coated glass slides. Sections were

1 incubated at 60°C overnight to aid adhesion prior to immunolabelling as described
2 below.

3

4 **Immunohistochemistry**

5 The sections were dewaxed in histoclear (2 x 5 min) and dehydrated in 100% ethanol
6 (2 x 3 min). Endogenous peroxidase was quenched in 0.09% hydrogen
7 peroxide/methanol solution for 30 min at room temperature (RT). The slides were pre-
8 treated with citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). The slides
9 were then washed (2 x 3 min) in phosphate buffered saline (PBS – 0.154 M NaCl, 1.86
10 mM $NaH_2PO_4.2H_2O$, 7.48 mM $Na_2HPO_4.12H_2O$, pH 7.1) and non-specific binding sites
11 were blocked with 10% horse serum in PBS (20 min, RT). Sections were incubated
12 overnight (20 h, 4°C) with primary antibody (1:3000 for GDH1/GDH2 and BCKD-E1α)
13 in PBS containing 3% Marvel (GDH) or 3% BSA (BCKD-E1α). The sections were
14 washed in PBS (2 x 3 min) then incubated with biotinylated antibody to IgG for 20 min
15 (Vectastain ABC kit). The slides were washed again in PBS (2 x 3 min) and incubated
16 with the avidin-biotin complex in PBS for 20 min (Vectastain ABC kit). Slides were
17 developed with DAB/H_2O_2 in distilled water (DAB substrate kit) for 10 min prior to
18 immersion in copper sulphate solution (16 mM $CuSO_4.5H_2O$, 0.123 M NaCl) for 4 min,
19 and subsequently counterstained with Harris's haematoxylin (25% Gill haematoxylin).
20 The slides were dehydrated in 100% ethanol (2 x 5 min), cleared in 100% clearene (2
21 x 3 min) and mounted in Clearium. Sections were viewed and imaged on a Nikon
22 Eclipse 80i.

23

24 **Immunofluorescence**

25 The sections were dewaxed in histoclear (2 x 5 min) and dehydrated in 100% ethanol
26 (2 x 5 min), followed by deionised water (2 x 5 min). Sections were pre-treated with
27 EDTA (1 mM, pH 8.0) for 30 min at 95°C, and washed (2 x 5 min) in deionised water
28 followed by PBS (2 x 5 min). Non-specific binding sites were blocked with 10% goat
29 blocking serum in PBS for 20 min at RT. Slides were incubated overnight (20 h, 4°C)
30 with primary antibodies (1:500 for β3-tubulin; 1:100 for BCKD-E1α; 1:500 for

1 GDH1/GDH2; 1:500 for GFAP; 1:25 for VWF) in PBS containing 1% goat serum. The
2 slides were subsequently washed in PBS (2 x 5 min) and incubated with secondary
3 antibody (1:100 for Alexafluor® 488 and Alexafluor® 568) for 1 h, washed in deionised
4 water (2 x 5 min) and further blocked with Sudan black (0.3% Sudan black in 70%
5 ethanol) for 2 min. Slides were rinsed 8 times with PBS and washed in PBS (2 x 5
6 min), mounted with DAPI and allowed to set for 30 min. Images were acquired using
7 Andor IQ software and a Nikon Eclipse TE300.

8

Results

Antibody specificity for BCKD-E1α and GDH was tested by western blot analysis on pure protein and human brain homogenates (Figure 1A-B). No cross reactivity with unrelated proteins was detected at the antibody concentrations used in these experiments. For commercial antibodies raised to BCKD-E1α and GDH, a negative antibody control was included with each experiment. For further confirmation, antigen absorption was also performed (at 200X molar excess) during IHC (Figure 1C-F). Labelling was completely prevented by prior incubation of the antibody with antigen. The figures are representative of the labelling observed in all 18 brains (Table 1).

Distribution of BCKD-E1α in the human brain

Labelling of BCKD-E1α was predominantly in neuropil and neurons throughout the brain (Figure 2A). However, endothelial cells were also labelled (Figure 2B). Certain astrocytic populations (notably Bergmann astrocytes) showed labelling but this was not a consistent feature (4/8 brains examined). BCKD-E1α was detected in all regions of the brain examined, in both neurons and endothelial cells. Labelling of neurons was stronger than that of endothelial cells.

Within the hippocampus, neuronal labelling was strongest within the cornu ammonis area 4 (CA4), and weaker in successive CA regions through to CA1 (Figure 3A-C). In the cerebral cortex, larger pyramidal cells were strongly immunopositive (Figure 3D, large arrows). Vasculature labelling was observed throughout the cerebral cortex (Figure 3E) with only weak astrocytic labelling (9/18 brains examined), in the vicinity of the hippocampus and within the white matter (images not shown). The subependymal and the ependymal tissue were immunopositive (Figure 3F).

The molecular layer of the cerebellar cortex was diffusely immunopositive, with stronger labelling of the cell bodies of many Purkinje cells (Figure 4A-C). Weak labelling was observed within the granule cell layer (Figure 4C). The white matter was unlabelled apart from some immunopositive capillaries (Figure 4D-F) and small glial cells (probably oligodendrocytes). Strong, exclusively neuronal labelling was observed within the dentate and fastigial nuclei (Figure 5A-C). In the deep grey matter,

1 the neuropil was weakly labelled within the putamen, globus pallidus and
2 hypothalamus. As previously noted with respect to hBCATc, neurons of the
3 hypothalamus, raphe nuclei, nucleus basalis of Meynert, pontine nuclei and locus
4 coeruleus were immunopositive for BCKD-E1α (Figure 5D-F).

5

6 **Distribution of GDH in the human brain**

7 GDH immunoreactivity was expressed in astrocytes throughout the human brain
8 (Figure 6A), with labelling of the astrocytic foot processes adjacent to the vasculature,
9 in addition to the vasculature itself (Figure 6B-C). Whilst GDH and GFAP were co-
10 localised, some astrocytic processes labelled for GDH but not GFAP. This might be
11 expected, as GFAP is located primarily in the larger processes and not those
12 enveloping synapses where you would find GDH (Kosaka & Hama, 1986). Most
13 labelling for GDH was stippled/granular within the cytoplasm of the cell, in keeping
14 with the mitochondrial restriction demonstrated in previous work (Spanaki *et al*., 2014).
15 Not all astrocytes labelled for GDH but labelling was detected in all sections and areas
16 studied.

17 Within the hippocampus, GDH labelling was entirely astrocytic (Figure 7A-C). There
18 was also labelling of astrocytes in the neocortex and white matter (Figure 7D-E), but
19 labelling in the white matter was less dense than that in the cortex. The subpial
20 feltwork of astrocytic processes was strongly immunopositive for GDH (Figure 7F) in
21 all brain regions, not just in the cerebrum. Neurons were largely unlabelled (16/18
22 brains examined), apart from occasional pyramidal neurons in the vicinity of blood
23 vessels (Figure 8A). The labelling of perivascular astrocytic processes was evident
24 throughout the cerebral cortex and cerebellum (Figure 8B); in places, the labelling also
25 involved the adjacent endothelium (Figure 8C).

26 Within the cerebellum (Figure 9A), there was punctate labelling in the processes of
27 cells within the Purkinje cell layer, most likely to be Bergmann astrocytes (Figure 9B-
28 C). The Purkinje cells themselves were unlabelled (12/18 brains examined) or weakly
29 immunopositive (Figure 9B-C, small arrows). Within the granule cell layer, rosette like
30 structures (glomeruli) were immunopositive. Within the deep cerebellar nuclei, there

was strong granular immunopositivity in small glial cells in contrast to the largely immunonegative neurons (Figure 9D-E).

In addition to the granular/stippled cytoplasmic labelling of astrocytes in the cerebral cortex and deep grey matter structures, there was GDH immunopositivity of some glial nuclei within these regions (most notably in the putamen) (Figure 9F, arrows). Whilst none of the brains was from a donor with dementia, in one brain GDH labelling occurred in conjunction with what appeared to be an amyloid plaque.

Summary of distribution of BCKD-E1α and GDH in the human brain

In summary, labelling of BCKD-E1α was associated with the neuronal cells and the vasculature in all brain regions studied, reminiscent of the distribution of hBCATc and hBCATm proteins (Hull *et al*., 2012). However, the labelling of blood vessels was weaker and less consistent than that of neurons. GDH was detected in all brain regions studied and was mainly within astrocytes. There was strong labelling of GDH in regions containing glutamatergic neurons – likely associated with neurotransmission within this region. Furthermore, close association of GDH positive astrocytic processes with the vasculature and in the deep nuclei of the cerebellum suggest additional roles for this protein.

Discussion

The BCKD complex and the GDH isoenzymes play a key role in brain glutamate metabolism. However, to date most of the information we have of their distribution within the brain is limited to data from small animal studies. Previous work from this group has already demonstrated key differences between the expression of BCATm within the human brain and that in lower mammals such as rats (Cole *et al*., 2012; Hull *et al*., 2012). Current findings indicate that within the human brain, BCKD-E1α is neuronal, supporting its proposed role in neuronal BCAA metabolism. GDH was primarily localised to astrocyte populations of the human brain, as in rodents (Subbalakshmi & Murthy, 1985; Zaganas *et al*., 2001; Zaganas *et al*., 2012). However, we have shown that, BCKD-E1α and GDH can also be detected within the vasculature of the human brain. These findings add complexity to our interpretation of the roles of these proteins in BCAA and glutamate metabolism.

Our understanding of the contribution of BCAAs to glutamate production is based on *ex vivo* and *in vivo* animal model [^{15}N] studies, in which BCAAs (particularly leucine) contributed approximately 25% of the nitrogen for glutamate synthesis (Yudkoff *et al*., 1983; Kanamori *et al*., 1998). We have previously demonstrated that hBCAT was strongly expressed within the human brain (Hull *et al*., 2012). The hBCATc enzyme was localised to neurons, whereas hBCATm expression was associated with the vasculature. We proposed that, in the human brain, hBCATc metabolises BCAAs for the transamination of α-ketoglutarate to glutamate, with hBCATm re-transaminating glutamate to cycle back into BCAAs. The hBCATc-mediated transfer of NH_3 for production of glutamate would require further metabolism of the BCKAs to prevent their re-transamination. In muscle, it is thought that the expression of minimally active BCKD results in up to 80% re-transamination of BCKAs, the remainder of which are predominantly exported rather than completely metabolised to Krebs cycle intermediates (Matthews *et al*., 1981; Nissen & Haymond, 1981; Suryawan *et al*., 1998).

As in the rat study of Cole *et al*., (2012), the majority of BCKD-E1α labelled neurons were either glutamatergic (e.g. hippocampal granule cells), GABAergic (e.g. Purkinje cells) or cholinergic (e.g. motor neurons). Reamination of BCKAs is well defined in many organs (Harper *et al*., 1984; Staten *et al*., 1984); however, our work also raises

1 the potential for complete BCAA metabolism within neurons expressing BCKD. This
2 is supported by previous documentation of very low levels (<1 mM) of BCKAs within
3 the human brain (Keen *et al*., 1993; Matsuo *et al*., 1993), reflecting the formation of
4 glutamate from BCAAs and the complete oxidation of BCKAs to the Kreb's cycle
5 substrate acetyl-CoA, and the Kreb's cycle intermediate succinyl-CoA. The labelling
6 of BCKD-E1α varied in different parts of the cerebrum but was particularly strong within
7 the hippocampus. The strong labelling of BCKD-E1α in the CA4 region and weaker
8 labelling towards the CA1 region mirrors the distribution of hBCATc (Hull *et al*., 2012).
9 BCKD-E1α was primarily restricted to the somatic region of nerve cells, suggesting
10 that the cell body is the primary site of BCKA oxidation. In the human brain, BCKD-
11 E1α expression was also noted within the vasculature, unlike in the rodent model. The
12 metabolism of BCAAs within the vasculature may be similar to that in muscle, i.e.
13 incomplete metabolism to BCKAs, which either re-transaminate glutamate or are
14 released for further metabolism by other cells (e.g. neurons). However, the expression
15 of hBCATm, BCKD and GDH within vascular cells raises the possibility that complete
16 metabolism of BCAAs occur, which could generate ATP for the active transport
17 required in this cell type. This would be in keeping with the reported active metabolon
18 described by Islam *et al*., (2007 & 2010), in which hBCATm, the BCKD complex and
19 GDH1 work to metabolise BCAAs completely and restore the Kreb's cycle substrate
20 acetyl-CoA, and the Kreb's cycle intermediate succinyl-CoA.

21 The absence of BCKD and hBCAT labelling within astrocytes indicates that BCAA
22 oxidation is neuronal rather than astrocytic, and that this oxidation is largely complete.
23 These findings do not rule out the possibility of translocation of BCKAs to neurons but
24 make it unlikely that those BCKAs are produced by astrocytes. Skeletal muscle is
25 thought to be the predominant source of BCKAs metabolised in the liver, due to the
26 relatively reduced expression of hBCAT within this organ (Suryawan *et al*., 1998).
27 Suryawan *et al*., (1998) also noted that the BCKD in the human brain is predominantly
28 active, with 59% of the expressed protein maintained in the active state. Translocation
29 of BCKAs from the bloodstream would depend on their transport across the blood-
30 brain-barrier (BBB). The transport of ketoacids (e.g. α-ketoglutarate) across the BBB
31 occurs via the organic anion transporter family – and it is possible the BCKAs are
32 transported in this manner (Riedmaier *et al*., 2012).

1 Work by our group showed hBCATc expressed in magnocellular neurosecretory cells,
2 the substantia nigra, the nucleus basalis of Meynert and the raphe nuclei (Hull *et al*.,
3 2012). The expression of BCKD closely mirrors that of hBCATc within these cell types,
4 supporting a role for hBCATc in cells that secrete a range of neurotransmitters and
5 peptides. The action of leucine as a potent secretagogue has already been reported
6 for the hormone insulin, where ketoisocaproate but not leucine, required
7 transamination to stimulate insulin secretion (Xu *et al*., 2001; Zhou *et al*., 2010).
8 Regulation of leucine levels is likely to be important in regulating neurosecretory
9 function, and the contribution of BCAA metabolism to this regulation warrants further
10 investigation.

11 Biochemical studies on mouse, rat and human tissue have detected GDH
12 activity/immunoreactivity in both neurons and glia (Hohnholt *et al*., 2017;
13 Subbalakshmi & Murthy, 1985; Zaganas *et al*., 2001; Zaganas *et al*., 2012). Rothe *et*
14 *al*., (1994) estimated that GDH activity in neurons is approximately 15% of that in
15 astrocytes and was crucial for neurons to metabolise glutamine-derived carbon during
16 glucose deprivation (Hohnholt *et al*., 2017). Our work confirms the expression of GDH
17 within many astrocytes, accounting for the predominance of glial activity; the detection
18 within neurons was more selective and the immunolabelling weaker. However,
19 Burbaeva *et al*., (2002) purified three isoforms of GDH protein from human brain
20 tissue, and it is possible that additional GDH reactions are occurring within the brain
21 in glia or neurons through the activity of a different GDH isoform.

22 Scant consideration has been given within the literature to GDH activity within the
23 vasculature. The study of Helms *et al*., (2012) was one of the first to consider the
24 potential for glutamate metabolism within endothelial cells, corroborated by our study.
25 The expression of both hBCATm and GDH suggests that the vasculature has the
26 potential to metabolise BCAAs fully for energy, or to metabolise glutamate for recycling
27 of α-ketoglutarate and BCAAs. In health, the brain is protected from variations in
28 plasma glutamate by restriction of entry of glutamate across the BBB. However,
29 glutamate efflux does occur and is affected by blood glutamate level (Hosoya *et al*.,
30 1999; Gottlieb *et al*., 2003; Zlotnik *et al*., 2008; Teichberg *et al*., 2009; Uchida *et al*.,
31 2011; Campos *et al*., 2012; Zlotnik *et al*., 2012). The ability of the vasculature to
32 metabolise glutamate may occur as a supplementary mechanism to the glutamate-

glutamine cycle, whereby the hBCATm-BCKD-GDH metabolon works to metabolise BCAAs fully except in circumstances of excess glutamate (i.e. glutamate toxicity). Increased expression of hBCAT was previously observed in Alzheimer's disease, perhaps as a response to an increase in glutamate (Ashby *et al*., 2015; Hull *et al*., 2015a). Conversely, if the metabolon exists only on the abluminal surface, it would function solely for the disposal of glutamate.

The direction of GDH metabolism will be determined by substrate concentration and the expression of other key metabolic proteins. The predominant action of GDH within the human brain is for the production of α-ketoglutarate from glutamate, allowing glutamate to contribute carbon to the Kreb's cycle within the brain (McKenna *et al*., 2016; Nissen *et al*., 2015). This is likely to be of particular importance within astrocytic processes surrounding the vasculature, where ATP generation through glutamate metabolism may provide the energy required for active transport across the BBB (Figure 10). Additionally, ammonia (NH_3 - lipid soluble) produced in this reaction may then leave the brain; this process, together with glutamine export, constitutes a major mechanism by which the brain disposes of excess ammonia (Hawkins *et al*., 2002). In astrocytes, the ammonia may also be incorporated into the structure of glutamate to form glutamine, through the work of the glutamine synthetase enzyme. This incorporation into glutamine is unlikely to be a major process at the astrocyte-vasculature boundary but is part of the glutamate-glutamine cycle in the astrocyte cell body (Figure 10).

The subcellular location of GDH is predominantly mitochondrial; however, we also found GDH immunopositivity within the nucleus of some cells. Lai *et al*., (1986) previously observed GDH activity in both the mitochondrial and nuclear fractions of rat brain homogenate and our observations indicate that GDH is present within a subset of nuclei in the human brain as well. Nuclear GDH was observed in a small proportion of glial cells, mainly within the putamen. This nuclear presence raises interesting questions about additional functions of GDH, of which two have been proposed. Rajas *et al*., (1996) noted that membrane-bound GDH in pig liver had microtubule-binding activity and suggested that GDH was involved in microtubule-dependent lysosome formation. Purohit *et al*., (2013) reported that GDH acts as a histone H3-specific

1 protease in chicken liver, a process that relates directly to the regulation of gene
2 expression.

3 Another proposed function of GDH is in the regulation of autophagy, for which the
4 relationship between GDH and leucine is important. In a HeLa knockdown of GDH1,
5 leucine failed to inhibit autophagy whilst inhibiting mTORC1 activity (Lorin *et al*., 2013).
6 The authors proposed that GDH1 regulates autophagy (at least in part) through the
7 modulation of mTOR but may also contribute to the production of ROS scavengers
8 and Krebs cycle intermediates (further inhibiting autophagy). We previously found
9 hBCAT to be absent from astrocytes in the human brain, in contrast to findings in the
10 rat (Hull *et al*., 2012; Hull *et al*., 2015b). It is of note that astrocytes in the human brain
11 lack the capacity to metabolise BCAAs, including leucine, a key activator of GDH. The
12 expression of glutamine-leucine counter-transporters on the surface of astrocytes
13 (Bak *et al*., 2006), raises the possibility that astrocytes can serve as a partial leucine
14 sink. This would maintain high intracellular astrocyte levels of leucine to (1) supply
15 neurons with leucine for glutamate production and (2) support astrocytic GDH activity
16 for the continuous metabolism of glutamate for the production of free ammonia (to
17 either be used in the production of glutamine or exported out of the brain) and α-
18 ketoglutarate (to be used for the production of ATP).

19

20 In summary, this work builds upon previous work from this group (Hull *et al*., 2012)
21 and provides further insight to the metabolism of BCAAs within the human brain and
22 their association with the glutamate-glutamine cycle. We propose that under normal
23 physiological conditions, neurons are the main site of BCAA metabolism, where
24 glutamate is replenished through hBCATc transamination and BCKD activity controls
25 re-transamination of the BCKAs. Metabolism of the BCAAs may also occur within the
26 vasculature, likely contributing to the buffering of glutamate levels within the brain. We
27 propose that the vasculature may also be a site at which the hBCATm-BCKD-GDH
28 metabolon described by Islam *et al*., (2007 & 2010) exists *in vivo*. Without the
29 expression of hBCAT or the BCKD complex in astrocytes (Hull *et al*., 2012), it is
30 unlikely that BCAAs are metabolised within this cell type. Our findings have
31 implications for the understanding of neurotransmitter metabolism within the human

1 brain, and consequently of neurological diseases in which there is dysregulation of
2 glutamate or BCAA homeostasis.

3

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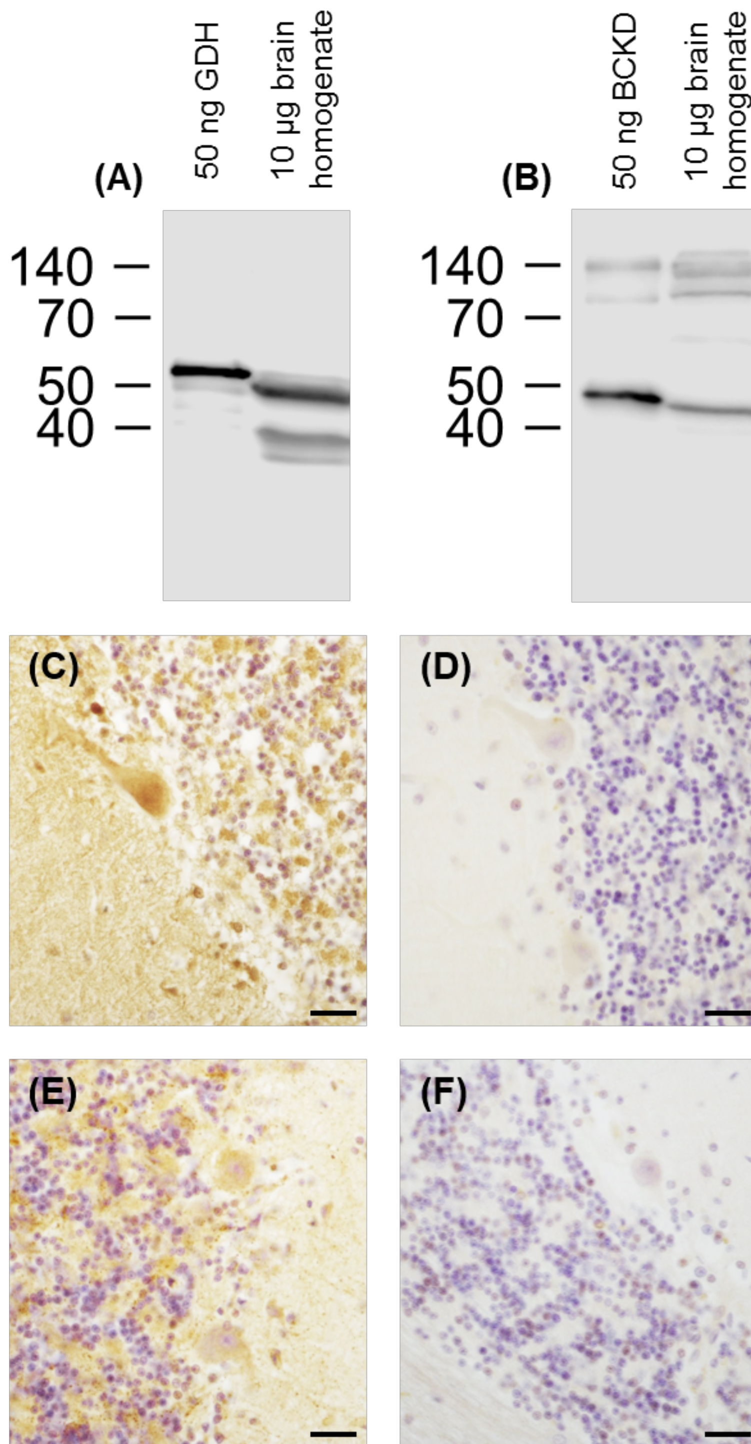
1 **Figures**



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3 **Figure 1. Specificity of the GDH and BCKD-E1α antibodies**. Western blot analysis
4 of pure protein (50 ng) and human brain homogenate (10 μg). A: Western blot analysis
5 using anti-GDH. B: Western blot analysis using anti-BCKD (E1α subunit). C:
6 Immunohistochemistry BCKD staining of the cerebellum. D: Antigen incubation of
7 serial section of panel C, at 200X molar excess showing complete removal of

1 immunoreactivity. E: Immunohistochemistry GDH staining of the cerebellum. F:
2 Antigen incubation of serial section of panel E, at 200X molar excess showing
3 complete removal of immunoreactivity. Scale: C, D, E and F, 25 μm, 40x magnification.

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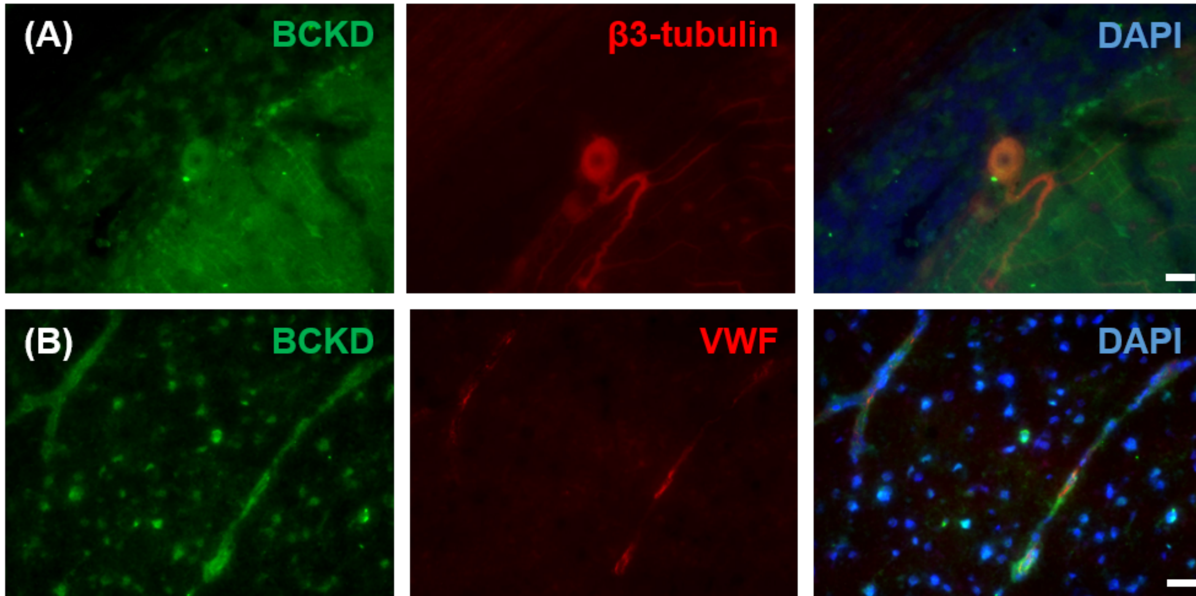

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6 **Figure 2. Co-localisation of BCKD-E1α to cell type within the cerebellum.** A: (From
7 left to right). Green immunofluorescence labelling BCKD-E1α, red immunofluorescence
8 labelling β3 tubulin (a marker of neuronal cells) and finally a merge showing BCKD-E1α
9 is present in neurons. B: (From left to right). Green immunofluorescence labelling
10 BCKD-E1α, red immunofluorescence labelling VWF (a marker of vessels) and finally a
11 merge showing BCKD-E1α is present in vessels. Scale bar: A and B, 25 μm, 40x
12 magnification.

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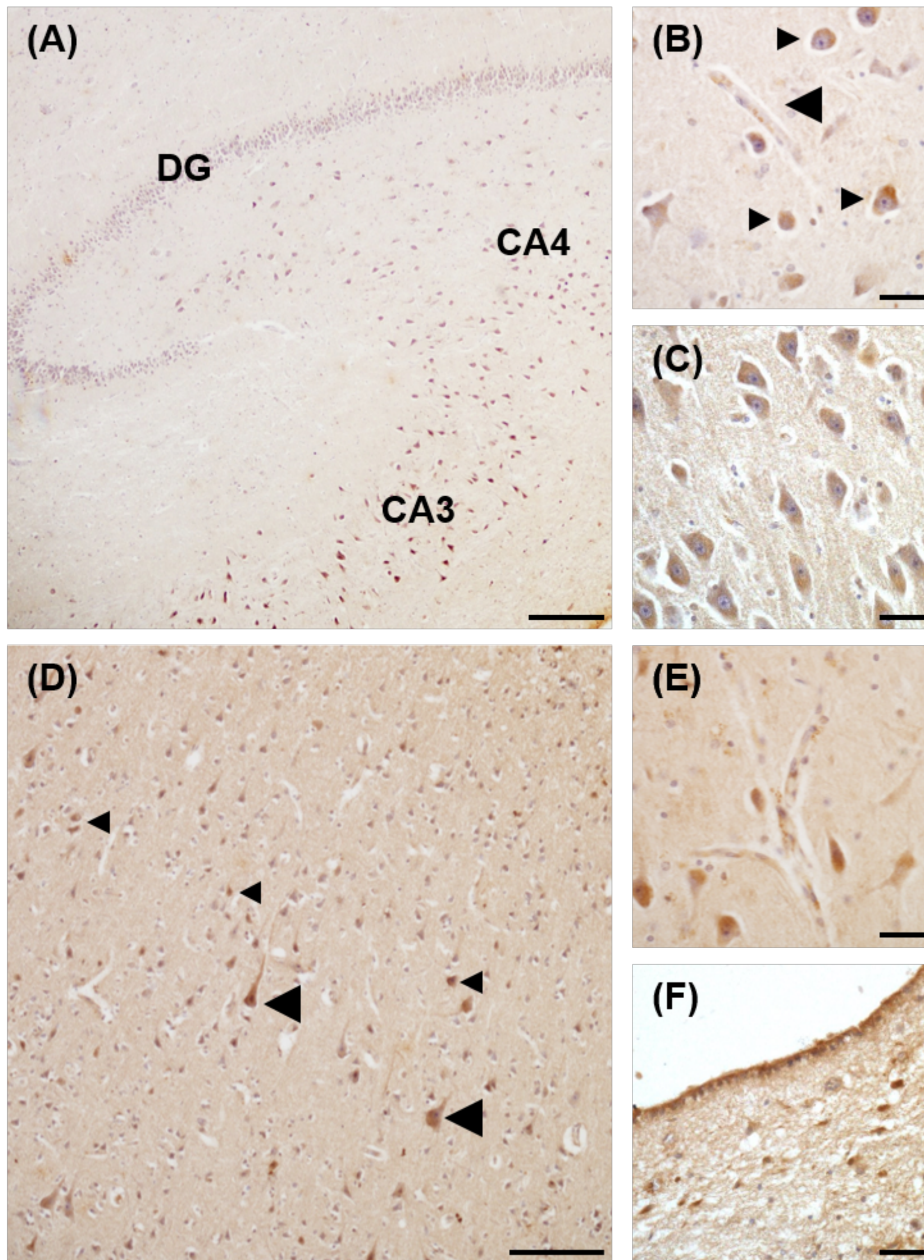

2 **Figure 3. Staining of BCKD-E1α in the frontal and temporal lobe**. A: The
3 hippocampal formation showing immunopositive neurons within the cornu ammonis
4 area 4 (CA4) and CA3 regions. B: Hippocampal region CA4 showing positive neuronal
5 cells (small arrows) and a positive vessel (large arrow). C: Hippocampal region CA2
6 showing positive neuronal cells. D: Small immunoreactive neurons (small arrows) and
7 large pyramidal neurons (large arrows) within the temporal cortex. E: Small
8 immunoreactive neurons and a small vessel within the temporal cortex. F: Ependymal
9 tissue showing immunopositive cells within the frontal cortex. Scale bar: A, 200 µm,
10 4x magnification; B, C, E and F, 25 µm, 40x magnification; D, 100 µm, 10x
11 magnification.

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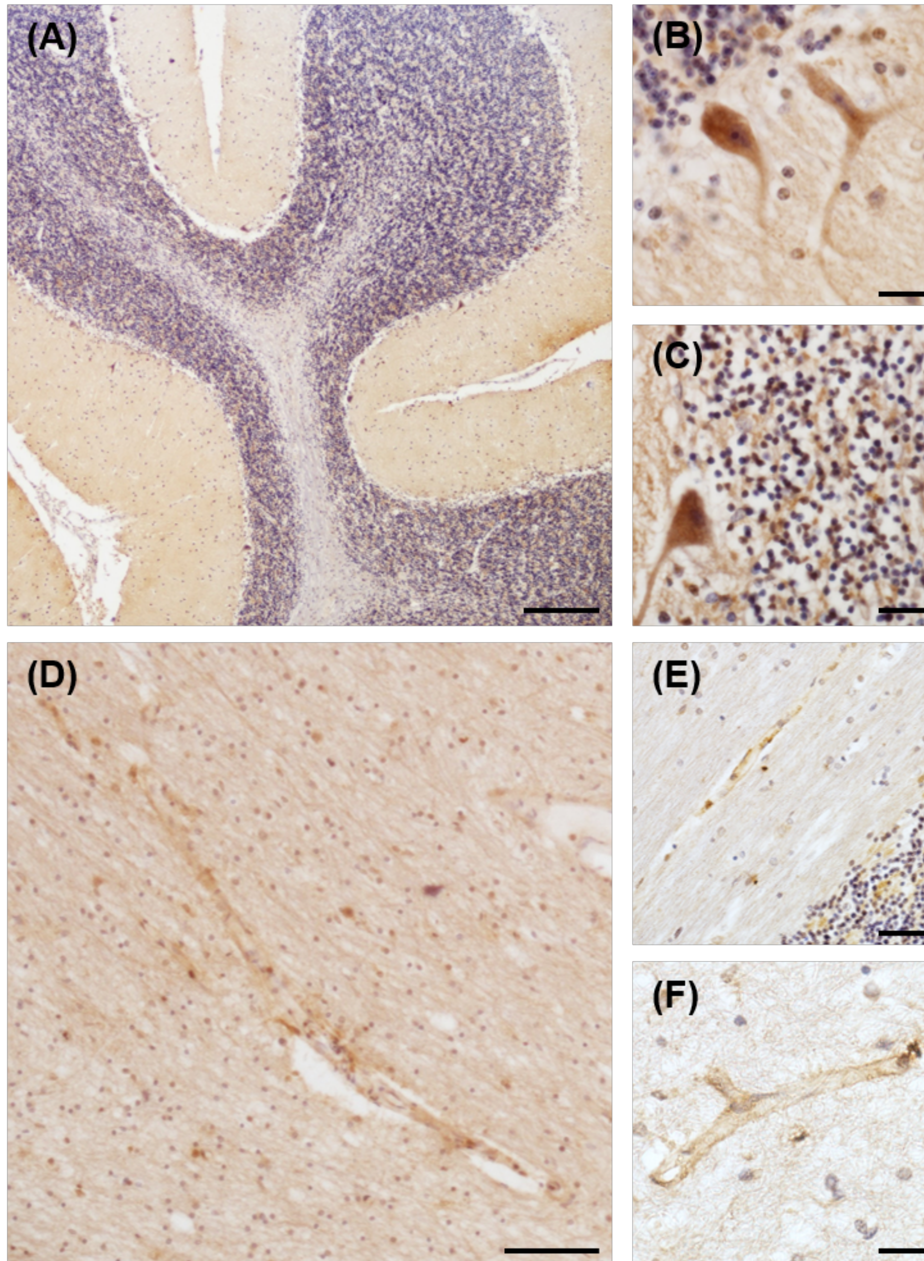

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3 **Figure 4. Staining of BCKD-E1α in the cerebellum**. A: The cerebellum structure
4 showing immunopositive neurons within the molecular layer. B-C: Immunopositive
5 Purkinje neurons within the molecular layer of the cerebellum with weak labelling of
6 the granule cell layer. D-F: The white matter of the cerebellum showing
7 immunoreactive capillaries. Scale bar: A, 200 µm, 4x magnification; B, C, E and F, 25
8 µm, 40x magnification; D, 100 µm, 10x magnification.

9

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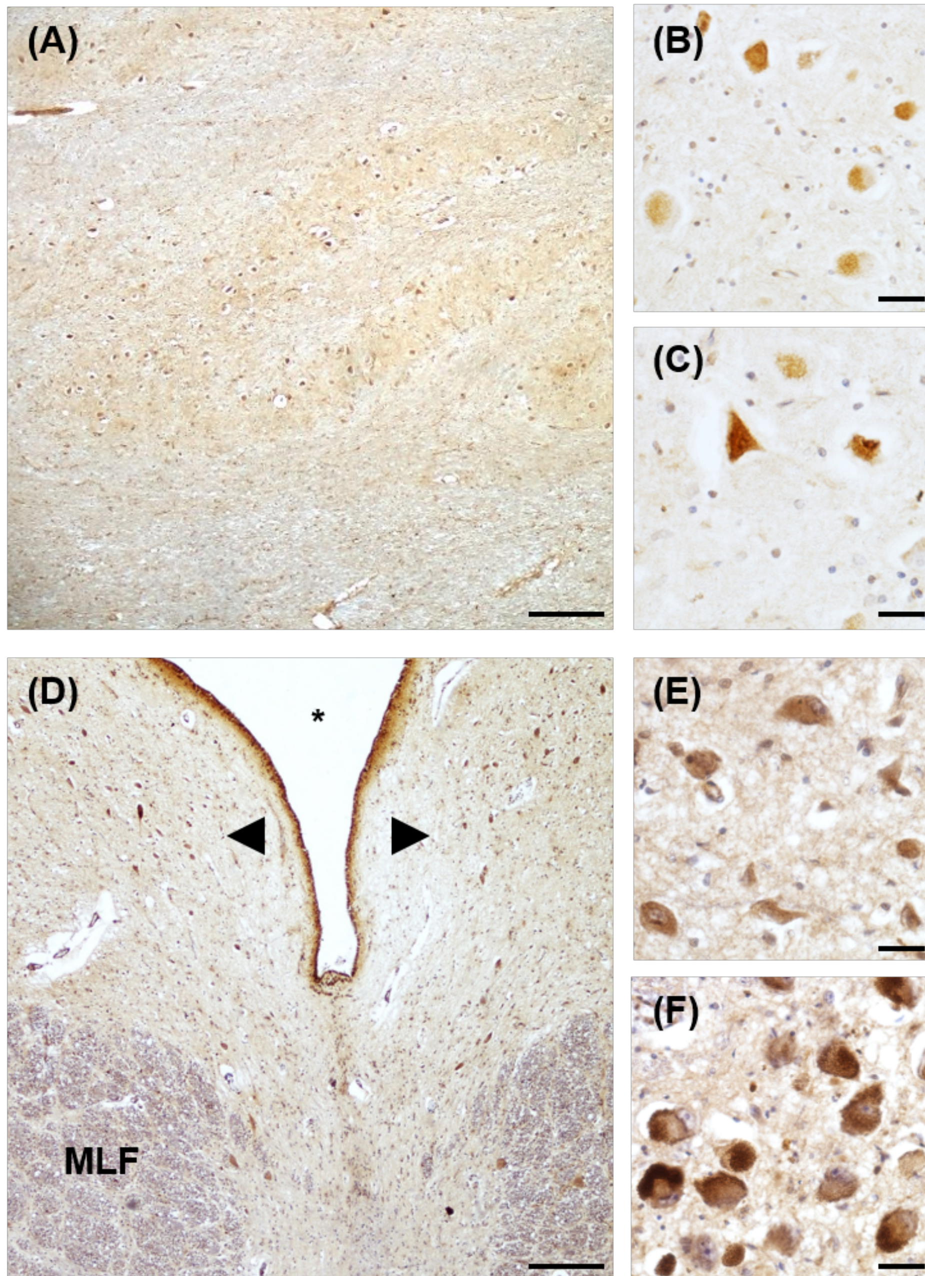

2 **Figure 5. Staining of BCKD-E1α in the cerebellum and brainstem**. A: The dentate
3 nucleus within the cerebellum. B-C: Increased magnification of the dentate nucleus
4 showing staining of small neurons. D: The Raphe nuclei (large arrows) showing
5 immunopositive neurons adjacent to the 4th ventricle (*) and medial longitudinal
6 fasciculus (MLF). E: Immunopositive neurons within the nucleus basalis of Meynert.
7 F: The locus coeruleus showing immunopositive neurons. Scale bar: A and D, 200
8 μM, 4x magnification; B, C, E and F, 25 μm, 40x magnification.

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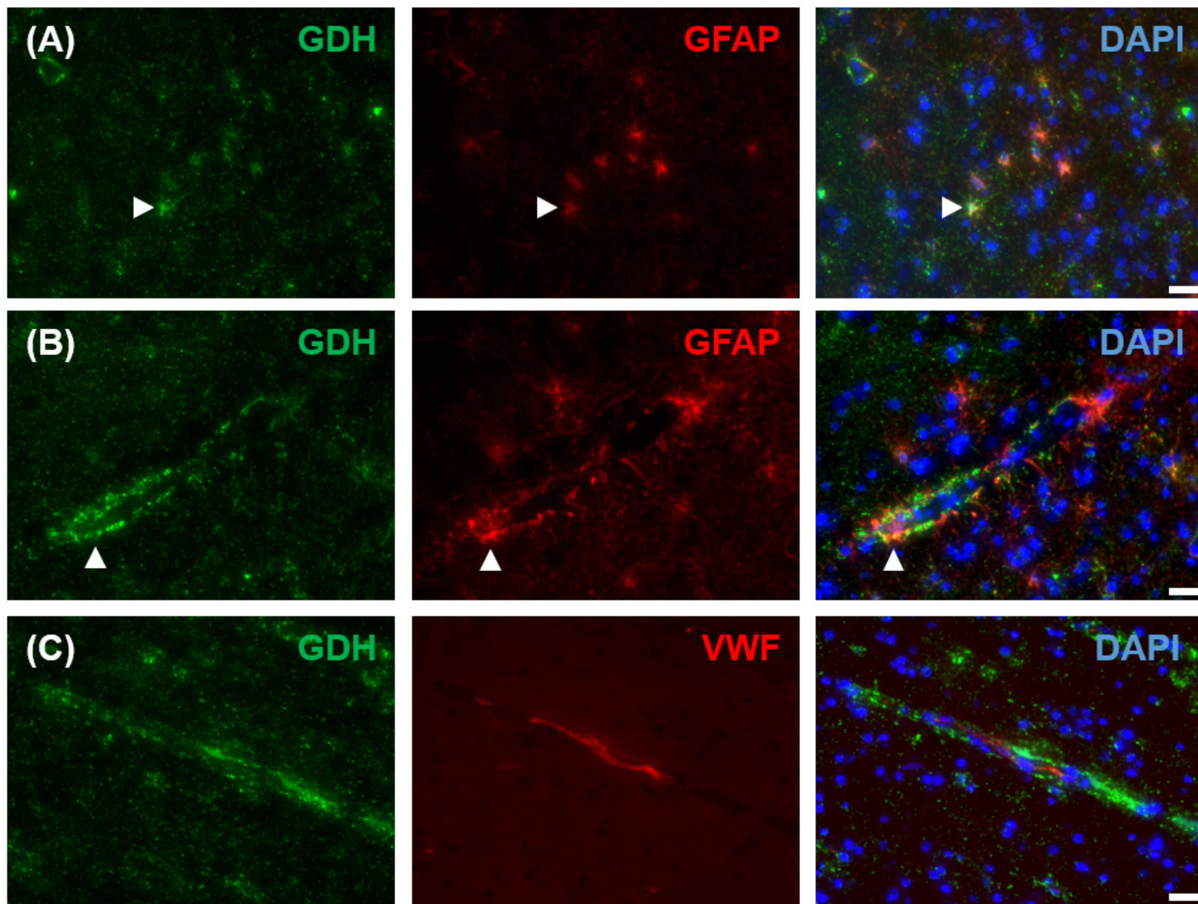

2 **Figure 6. Co-localisation of GDH to cell type within the cerebellum**. A: (From left
3 to right). Green immunofluorescence labelling GDH, red immunofluorescence labelling
4 GFAP (a marker of astrocytes) and finally a merge showing GDH is detected in
5 astrocytes (small arrows). B: (From left to right). Green immunofluorescence labelling
6 GDH, red immunofluorescence labelling GFAP (a marker of astrocytes) and finally a
7 merge showing GDH is present in astrocytes surrounding vessels (small arrows). C:
8 (From left to right). Green immunofluorescence labelling GDH, red
9 immunofluorescence labelling VWF (a marker of vessels) and finally a merge showing
10 GDH is present within certain vessels. Scale bar: A, B and C, 25 μm, 4x magnification.

11

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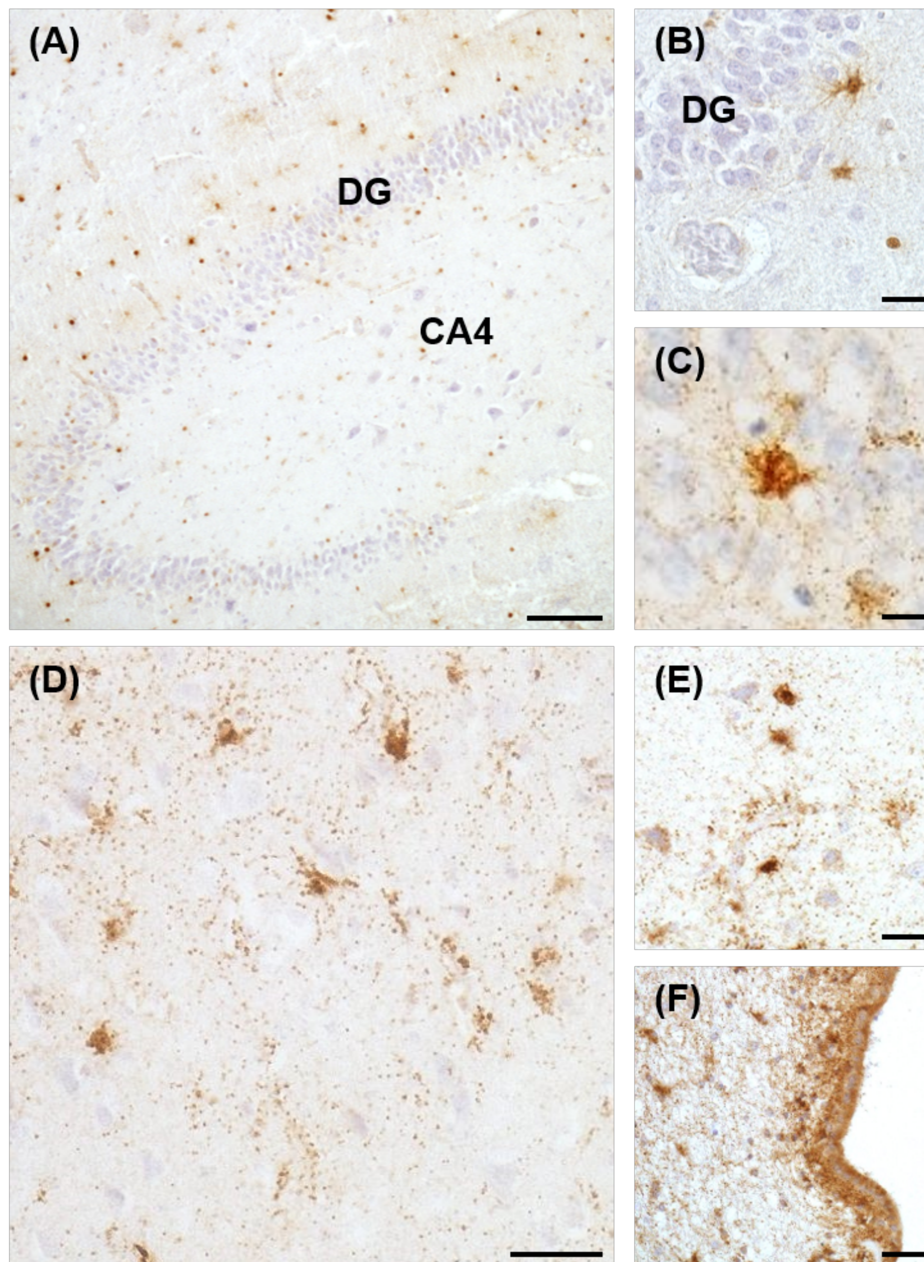

2 **Figure 7. Staining of GDH in the frontal and temporal lobe**. A: The hippocampal
3 formation showing supportive immunopositive astrocytes within the molecular layer.
4 B: Increased magnification of the granule cell layer of the dentate gyrus (DG) showing
5 small immunopositive astrocytes. C: Increased magnification of astrocyte staining
6 showing both cell body and process labelling. D: Immunoreactive astrocytes within
7 the temporal white matter. E: Increased magnification of immunoreactive astrocytes.
8 F: Ependymal and subependymal tissue showing immunopositive cells within the
9 frontal cortex. Scale bar: A, 200 μm, 4x magnification; B, C, E and F, 25 μm, 40x
10 magnification; D, 50 μm, 40x magnification.

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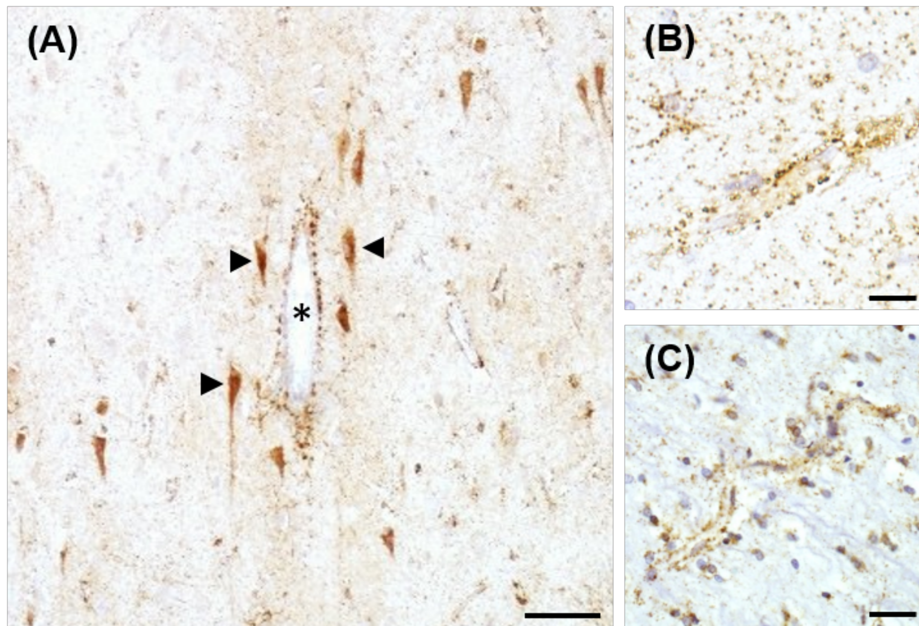
2 

3 **Figure 8. Staining of GDH in the temporal lobe and cerebellum**. A: Temporal
4 cortex with labelling of large pyramidal neurons (small arrows) adjacent to an
5 immunopositive vessel (*). B: Cerebellar white matter showing perivascular astrocytic
6 labelling. C: Cerebellar white matter showing vessel staining. Scale bar: A, 100 µm,
7 10x magnification; B and C, 25 µm, 40x magnification.

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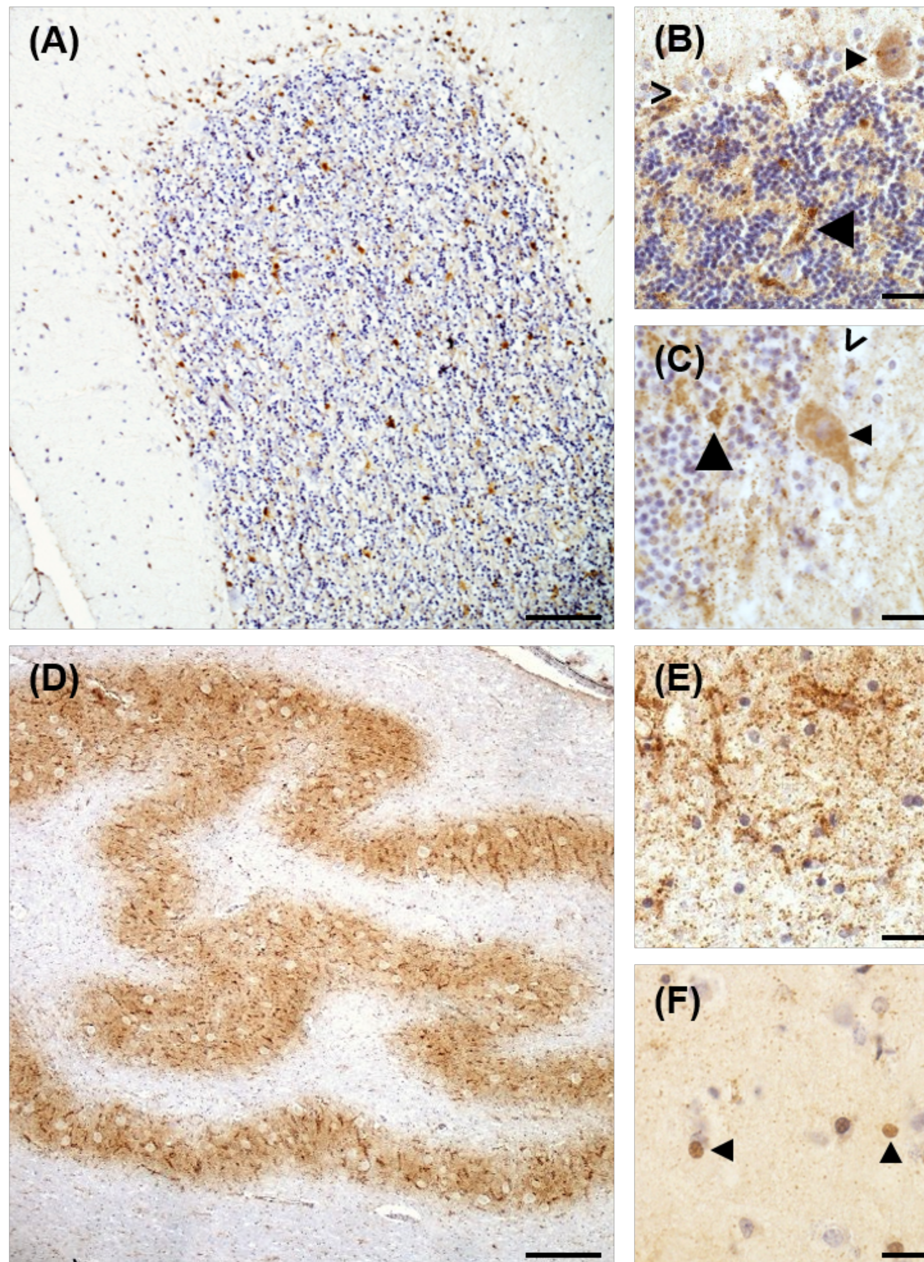

Figure 9. Staining of GDH in the cerebellum and putamen. A: The cerebellum formation showing immunopositive cells within the molecular and nuclear layers. B-C: Granule cell layer with immunopositive purkinje cell (small arrow) and labelling within the nuclear layer (large arrow) likely representing a vessel or astrocytic processes. Also present are immunopositive Bergman astrocytes at the level of the golgi cell layer (arrow head). D: Dentate nuclei immunopositive for GDH. E: Increased magnification of the dentate nuclei demonstrating negative neuronal staining with immunopositive astrocytes and astrocytic processes. F: Nuclear presence of GDH (small arrow) within the putamen. Scale bar: A and D, 200 μm, 4x magnification; B, C, E and F, 25 μm, 40x magnification.

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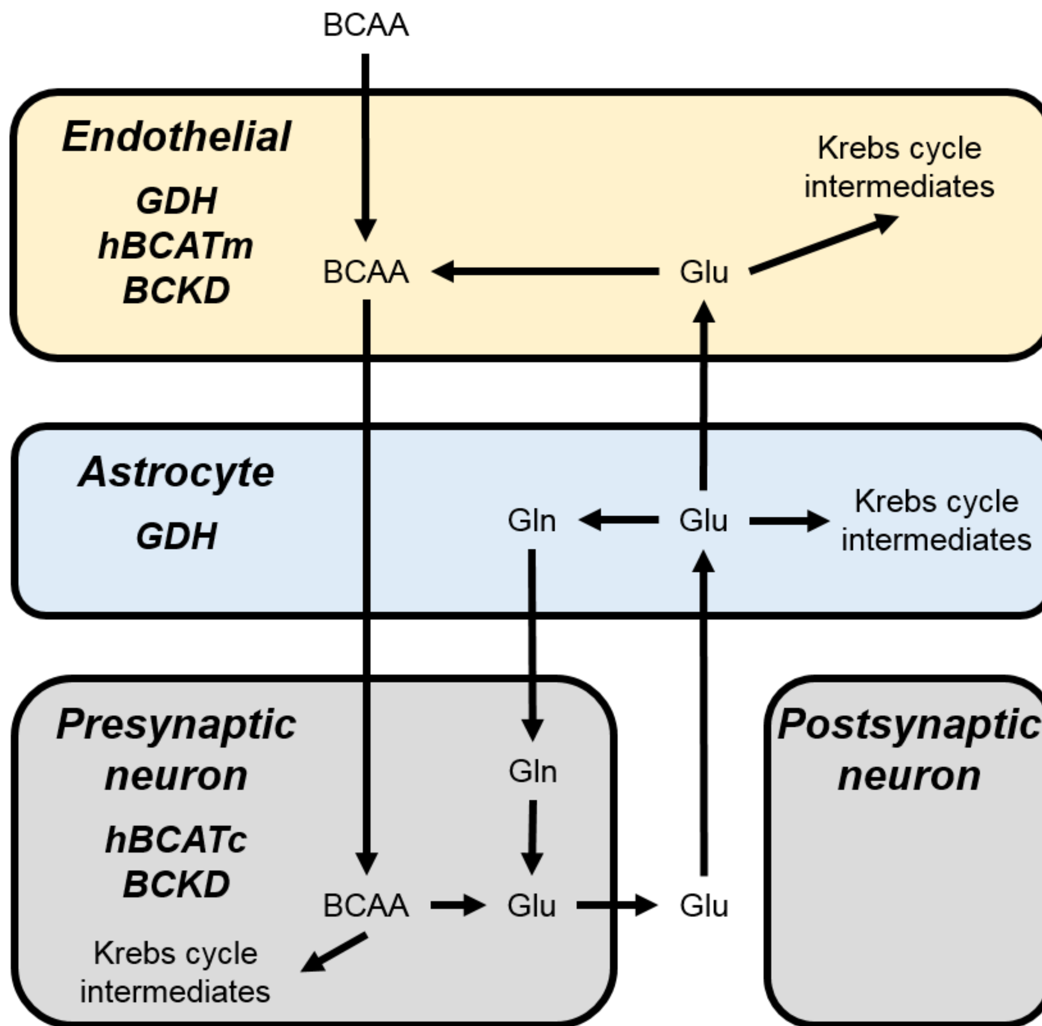

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3 **Figure 10. The glutamate-glutamine cycle and the supporting role of the hBCAT,**
4 **BCKD and GDH proteins**. Glutamate is released from neuronal cells during
5 excitatory neurotransmission. Post-excitation, glutamate is taken up via excitatory
6 amino acid transporters on astrocytes. In astrocytes much of the glutamate undergoes
7 amidation to glutamine by glutamine synthetase. Glutamine is then released for
8 uptake by neuronal cells, to replenish the glutamate pool through the action of
9 glutaminase. However, much glutamate can be lost through oxidation via glutamate
10 dehydrogenase, or generation of glutathione/purines in astrocytes. The glutamate-
11 glutamine cycle must work with other anapleurotic pathways to regenerate the
12 neuronal pool of glutamate. BCAA metabolism is thought to participate in nitrogen
13 shuttling in the *de novo* synthesis of glutamate. The BCAAs, particularly leucine, can
14 pass easily through the blood brain barrier through an L system transporter. Uptake

1 by neuronal cells results in transamination by hBCATc with α-ketoglutarate forming
2 glutamate, contributing to the neuronal pool. The BCKAs are completely metabolised
3 via BCKD to produce Kreb's cycle intermediates and avoid re-transamination of
4 glutamate. In addition to astrocytes, the endothelial cells of the vasculature play a key
5 role in removing excess glutamate and regenerating neuronal pools of glutamate. As
6 levels of hBCATm are strongly expressed and GDH is also present in many vessels,
7 we propose that glutamate is either transaminated to the BCAAs or metabolised by
8 GDH. The BCAAs can then re-enter the pool for glutamate generation, and the
9 ammonia (NH_3) generated by GDH activity can be exported from the brain.
10 Abbreviations: BCAA, branched chain amino acid; BCKD, branched chain keto acid
11 dehydrogenase complex; GDH, glutamate dehydrogenase; Gln, glutamine; Glu,
12 glutamate; hBCAT, human branched chain aminotransferase.

13

14 **Table 1. Clinical outcome for patients used in this study.**

	Age	Gender	PM (delay)	Brain weight (g)	Hypertension	Braak staging	Ca of d
Patient 1	62	M	4	1230	Yes	0	Ventricu
Patient 2	80	F	39	750	No	0	Colon
Patient 3	71	M	25	1245	No	1	Ventricu
Patient 4	82	F	35	1150	No	3	Myocardia
Patient 5	64	M	16	1475	No	0	Abdominal ac
Patient 6	78	M	12	1200	No	2	Myocardia
Patient 7	80	M	67	1460	Yes	3	Pneu
Patient 8	70	M	50	1380	No	2	Carcinc
Patient 9	72	F	24	1200	No	0	Unk
Patient 10	76	M	23	1450	No	2	Pneu
Patient 11	73	M	35	1350	Yes	3	Heart
Patient 12	80	M	48	1279	Yes (T)	0	Pneu
Patient 13	94	F	21	1015	Yes	2	Adenoc
Patient 14	87	M	24	1364	Yes	2	Acute re
Patient 15	94	M	40	1260	Yes	2	Debility
Patient 16	85	M	31	1337	Yes (T)	2	Ischaemic h
Patient 17	77	M	42	1351	Yes (T)	1	Pneu
Patient 18	87	F	47	1262	No	3	Septicaemia

F, Female; M, Male; PM, Post-mortem; (T) Requiring treatment. Cause of death was determined

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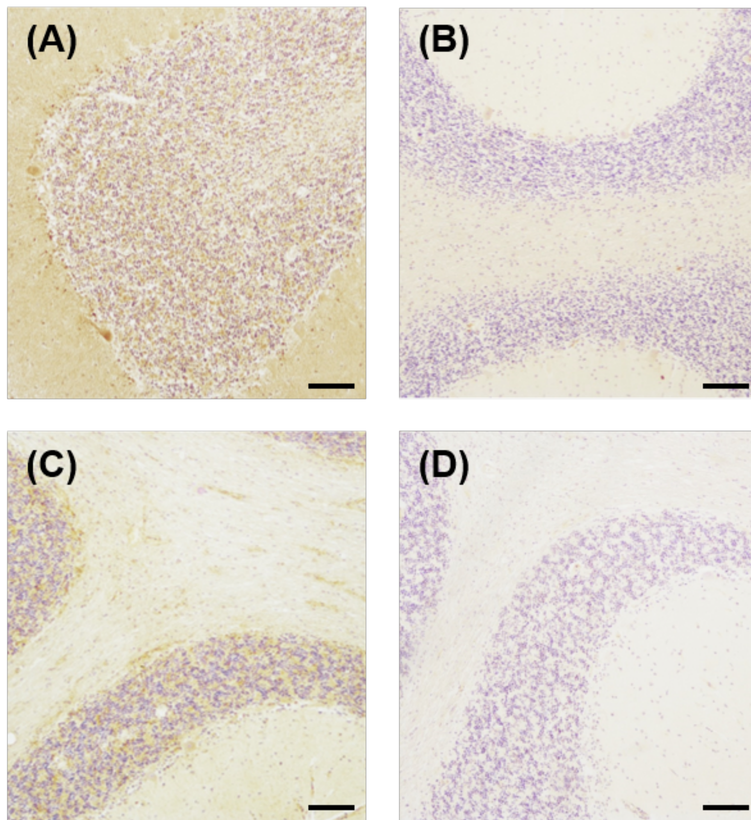

2 **Supplementary figure 1. Specificity of the GDH and BCKD-E1α antibodies**.
3 Immunohistochemical analysis of human brain sections, including antigen incubations.
4 A: Immunohistochemistry BCKD staining of the cerebellum. B: Antigen incubation of
5 serial section of panel A, at 200X molar excess showing complete removal of
6 immunoreactivity. C: Immunohistochemistry GDH staining of the cerebellum. D:
7 Antigen incubation of serial section of panel C, at 200X molar excess showing
8 complete removal of immunoreactivity. Scale: C, D, E and F, 50 μm, 10x magnification.

9

10 **Supplementary table 1. Clinical outcome for patients used in this study.**

	Age	Gender	PM (delay)	Brain weight (g)	Hypertension	Treated hypertension	SVD Frontal
Patient 1	62	M	4	1230	Yes	No	-
Patient 2	80	F	39	750	No	No	-
Patient 3	71	M	25	1245	No	No	-
Patient 4	82	F	35	1150	No	No	-
Patient 5	64	M	16	1475	No	No	-
Patient 6	78	M	12	1200	No	No	-
Patient 7	80	M	67	1460	Yes	No	2
Patient 8	70	M	50	1380	No	No	-
Patient 9	72	F	24	1200	No	No	2
Patient 10	76	M	23	1450	No	No	1
Patient 11	73	M	35	1350	Yes	No	0
Patient 12	80	M	48	1279	Yes	Yes	2
Patient 13	94	F	21	1015	Yes	No	2
Patient 14	87	M	24	1364	Yes	No	2
Patient 15	94	M	40	1260	Yes	No	1
Patient 16	85	M	31	1337	Yes	Yes	2
Patient 17	77	M	42	1351	Yes	Yes	1
Patient 18	87	F	47	1262	No	No	2

F, Female; M, Male; PM, Post-mortem; SVD, Small vessel disease. Cause of death was determine

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