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Dual mechanism of brain injury and novel treatment strategy in maple syrup urine disease

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Maple syrup urine disease (MSUD) is an inherited disorder of branched-chain amino acid metabolism presenting with life-threatening cerebral oedema and dysmyelination in affected individuals. Treatment requires life-long dietary restriction and monitoring of branched-chain amino acids to avoid brain injury. Despite careful management, children commonly suffer metabolic decompensation in the context of catabolic stress associated with non-specific illness. The mechanisms underlying this decompensation and brain injury are poorly understood. Using recently developed mouse models of classic and intermediate maple syrup urine disease, we assessed biochemical, behavioural and neuropathological changes that occurred during encephalopathy in these mice. Here, we show that rapid brain leucine accumulation displaces other essential amino acids resulting in neurotransmitter depletion and disruption of normal brain growth and development. A novel approach of administering norleucine to heterozygous mothers of classic maple syrup urine disease pups reduced branched-chain amino acid accumulation in milk as well as blood and brain of these pups to enhance survival. Similarly, norleucine substantially delayed encephalopathy in intermediate maple syrup urine disease mice placed on a high protein diet that mimics the catabolic stress shown to cause encephalopathy in human maple syrup urine disease. Current findings suggest two converging mechanisms of brain injury in maple syrup urine disease including: (i) neurotransmitter deficiencies and growth restriction associated with branched-chain amino acid accumulation and (ii) energy deprivation through Krebs cycle disruption associated with branched-chain ketoacid accumulation. Both classic and intermediate models appear to be useful to study the mechanism of brain injury and potential treatment strategies for maple syrup urine disease. Norleucine should be further tested as a potential treatment to prevent encephalopathy in children with maple syrup urine disease during catabolic stress.

Introduction

Branched-chain ketoacid dehydrogenase (EC 1.2.4.4) activity is required for catabolism of branched-chain α-ketoacids ketoisocaproate, ketomethylvalarate and ketoisovalarate, which are the transamination products of branched-chain amino acids leucine, isoleucine and valine, respectively. Autosomal recessive inheritance of branched-chain ketoacid dehydrogenase deficiency, known as maple syrup urine disease (MSUD), affects 1 : 100 000 children worldwide (Mackenzie and Woolf, 1959; Chuang and Shih, 2001). However, MSUD affects children of old order Mennonite families at a much higher frequency of 1 : 200 (Morton *et al.*, 2002). MSUD results in accumulation of branched-chain ketoacids and branched-chain amino acids measured in blood, urine and cerebrospinal fluid (Mackenzie and Woolf, 1959). If untreated, affected individuals accumulate substantial branched-chain amino acids, mostly leucine, and suffer encephalopathy within the first week of life as well as cerebral oedema and dysmyelination with chronic brain injury (Crome *et al.*, 1961; Silberman *et al.*, 1961; Chuang and Shih, 2001). In some cases with an intermediate form of MSUD, residual enzyme activity results in a delayed presentation (Chuang and Shih, 2001). The neuropathology associated with MSUD involves vacuolation and oedema of the basal ganglia and brainstem as well as blunted dendritic development in cortex (Crome *et al.*, 1961; Silberman *et al.*, 1961; Kamei *et al.*, 1992; Chuang and Shih, 2001).

Newborn screening for blood alloisoleucine levels >5 µm/l, produced from isoleucine accumulation, is considered diagnostic for MSUD (Schadewaldt *et al.*, 1999). Current therapy for MSUD includes life-long maintenance of a low branched-chain amino acid diet and regular monitoring of plasma branched-chain amino acid levels (Morton *et al.*, 2002). Emphasis is placed on preventing leucinosis, but also avoiding leucine deficiency, which can be equally life threatening. Aggressive treatment of intercurrent illness is used to prevent a catabolic state that can rapidly lead to release of branched-chain amino acids from muscle protein, leucinosis and cerebral oedema (Daniel *et al.*, 1977). Unfortunately, despite careful clinical management, some children with MSUD become encephalopathic during intercurrent illness and require intensive care hospitalization. Additionally, learning disabilities are found at a greater frequency in children and adults with MSUD despite continuous management on special diets (Morton *et al.*, 2002; Schonberger *et al.*, 2004; Simon *et al.*, 2007).

The recent development of mouse models for both classic and intermediate MSUD provided the opportunity to study acute and long-term consequences of metabolic decompensation associated with branched-chain ketoacid dehydrogenase deficiency (Homanics *et al.*, 2006). The cMSUD mouse model was developed by targeting the E2 subunit of the branched-chain ketoacid dehydrogenase complex resulting in complete loss of enzyme activity in homozygous offspring that die within three days of birth (Homanics *et al.*, 2006). The intermediate MSUD model was developed by transgenic addition of human E2 cDNA to heterozygous classic MSUD mice under a liver specific promoter, resulting in 5–6% normal enzyme activity in liver of homozygous offspring (Homanics *et al.*, 2006). Approximately 80% of intermediate MSUD mice survive until weaning, but then die by 8-weeks of age (Homanics *et al.*, 2006). Both models were shown to accumulate branched-chain amino acids in blood and urine. However, brain consequences have not been explored.

In the current study, we used intermediate MSUD mice to show development of neuropathology with striking similarity to human MSUD. Branched-chain amino acid accumulation in this model was associated with neurotransmitter deficiency, behavioural changes and limited survival. Providing intermediate MSUD mice with a choice between normal and branched-chain amino acid-free diet prevented brain injury and dramatically improved survival. We then used classic MSUD mice to identify neuropathological changes and follow the evolution of biochemical derangement that occurred over the first few days of life similar to classic human MSUD. A novel treatment strategy using norleucine was shown to delay encephalopathy and enhance survival in both models. Our current findings indicate that both the intermediate MSUD and classic MSUD models are useful tools to study the mechanism of brain injury and to develop potential interventions, which may prevent encephalopathy and enhance long-term outcomes in children with MSUD.

Methods

Materials

All chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise specified.

Animals

All mice were maintained at Penn State College of Medicine (Department of Comparative Medicine) in accordance with IACUC research guidelines set forth by Pennsylvania State University and the Society for Neuroscience Policy on the use of animals in neuroscience research. The intermediate MSUD mice, age-matched heterozygous and wild-type (WT) controls, both of mixed genetic background (Homanics *et al.*, 2006), were generated from breeding pairs in which both parents were heterozygous for knockout of the endogenous mouse E2 gene and homozygous for both the LAP-tTA and TRE-E2 transgenes (see Homanics *et al.*, 2006 for additional details of mouse production and genetic background). The classic MSUD pups, WT and heterozygous controls were generated by mating heterozygotes as previously described (Homanics *et al.*, 2006). Genotypes of all mice used in these studies were confirmed by PCR of genomic DNA isolated from tail snips using the following

primers: human E2 cDNA Left-GCAGGGTTTGATTGTCCCTA, Right-TCAGCTGACCAGCTCACATT, mouse E2 gene Left-GCCAAGCTCAG GTCCTTATG, Right-GCAGGCGTTGCTAGTGTTTT, PGKneo Left-GGA GAGGCTATTCGGCTATG, Right-CTCGTCCTGCAGTTCATTCA.

Special diets

The diet without branched-chain amino acids was Dyets 510081 (Bethlehem, PA, USA). The normal diet was the Harland Teklad 2018 diet (Indianapolis, IN, USA), which is 18% protein. The high protein diet was Harland Teklad TD.03637 (70% casein) and contained 62% protein by weight. The high protein-norleucine diet was prepared by adding 5% norleucine to the high protein diet. For low branched-chain amino acid diet studies, male and female intermediate MSUD mice were placed on special diets at 3-weeks of age, housed five per cage, observed every 12 h and the mice and diets were weighed daily ($n = 30$ for each study group). All special diet treated animals and corresponding controls were evaluated daily for symptoms as previously described (Zinnanti et al., 2006).

Behavioural evaluation

All animals and corresponding controls were evaluated for motor deficits using a quantitative neurological scale developed previously (Ouary et al., 2000; Zinnanti et al., 2006). Motor abnormalities were scored on the presence and severity of motor symptoms consisting of intermittent dystonia of one hindlimb, score = 1; intermittent dystonia of two hindlimbs, score = 2; permanent dystonia of hindlimbs, score = 3; gait abnormalities consisting mainly of uncoordinated, wobbling gait, score = 2; frequent falls or rolls, score = 2; recumbency, score = 7; recumbency characterized by paralysis with rapid breathing, score = 8; grasp reflex of forepaws, able = 0; unable = 1; and hindpaws able = 0; unable = 1 was assessed. Motor abnormalities were assessed daily and a neurological score was calculated for each animal as the sum of all indices (minimum = 0, normal animal; maximum = 10, animal showing near death recumbency).

Cage hang test was performed by placing the mouse on a wire mesh cage lid and inverting the lid. The time that the mouse could hang upside down without falling was recorded over three trials.

Histology

Mice were anesthetized with 100 mg/kg pentobarbital (i.p.), perfused with lactated Ringers (Baxter Deerfield, IL) followed by 4% paraformaldehyde for 15 min. Brains were post-fixed in 4% paraformaldehyde for 48 h, and paraffin embedded. Haematoxylin and eosin stained slides were prepared from paraffin embedded brains. Sequential 10-µm thick coronal brain slices were made within 0.5 mm of the Bregma line for striatal sections and between Bregma −5.5 and −6.25 for brain stem sections (Franklin and Paxinos, 1997).

Immunohistochemistry and confocal microscopy

Glial fibrillary acidic protein (GFAP) and neurofilament (N52) were detected using deparaffinized 10-µm thick coronal brains slices. Tissue was blocked with normal serum and incubated with polyclonal α-GFAP (1 : 500) (Dako, Carpenteria, CA, USA) and monoclonal α-N52 (1 : 200) (Sigma) at the same time. All incubations were in PBS with normal serum overnight at 4°C. The slides were washed separately three times each in PBS. Polyclonal GFAP was detected

with goat anti-rabbit coupled to Cy^3 and N52 was detected with goat anti-mouse coupled to Cy^2 (all secondary antibodies diluted 1 : 2000; Jackson ImmunoResearch, West Grove, PA, USA). All slides were counterstained with 4′,6′-Diamidino-2-phenylindole (DAPI) 0.1 µg/ml in PBS for 5 min. Confocal microscopy was performed using a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems Wetzlar, Germany).

Biochemical analysis

All mice were sacrificed by focused beam microwave irradiation to preserve brain metabolite status as previously described (O'Callaghan and Sriram, 2004). Plasma, livers and brains were collected and frozen to −80°C. Samples were extracted with 8% perchloric acid (PCA) and neutralized with potassium hydroxide as previously described (Williamson and Corkey, 1979). All neutralized perchloric acid extracts were kept at −80°C until analysis.

Amino acids

Plasma, brain and liver amino acids were derivatized with phenyl isothiocyanate (PITC) and separated and quantified by reverse-phase high-performance liquid chromatography as previously described (Hariharan et al., 1993) using a C_{18} 'Econosphere' column (150 × 4.6 mm ID, 3 µm) from Alltech (Deerfield, IL) with norvaline (100 M) as an internal standard.

Branched-chain ketoacids, pyruvate and α-ketoglutarate were measured by HPLC as previously described (Loi et al., 2005). Brain extracts were acidified and derivitized using O-phenylenediamine, separated by HPLC using a CB_{18B} 'Econosphere' column (150 × 4.6 mm ID, 3 m) from Alltech (Deerfield, IL, USA) and detected with fluorescence by excitation and 350 nm and absorbance at 410 nm.

ATP, lactate and phosphocreatine

Brain extracts were assayed for ATP, phosphocreatine and lactate enzymatically (Williamson and Corkey, 1979). ATP was measured by following the formation of NADPH spectrophotometrically using hexokinase coupled with glucose-6-phosphate dehydrogenase in the presence of glucose and NADP+. Phosphocreatine was measured by adding creatine kinase to the above reaction and following the additional formation of NADPH. Lactate was measured by following the formation of NADH using lactate dehydrogenase in the presence of NAD+. Protein concentrations were measured by the method of Lowry (Lowry et al., 1951).

MRI

MRI was performed on a 7.0 T Bruker system using a 2-mm birdcage coil ($n = 6$ mice per diet group). Mice were anesthetized with isoflurane (1–1.5%) and imaged with a transverse relaxation time constant (T_2)-weighted multi-echo spin echo sequence (five 0.5-mm thick slices, TR/TE = 3000/10.6–148.4 ms, 14 echoes, 117 × 117 µm resolution, two averages). T_2 values were calculated on a pixel-by-pixel basis from the corresponding exponential fits using CCHIPS software (Schmithorst et al., 2001).

Statistics

Normally distributed data sets were analysed by ANOVA with Fisher LSD post hoc test. Kruskal–Wallis one-way analysis of variance on ranks was performed with Student–Neuman–Keuls post hoc test on samples that were not normally distributed. Sigma Stat software

(Jandel Scientific, San Rafael, CA, USA) was used for analysis. All $P < 0.05$ were considered statistically significant.

Results

Behavioural changes and neuropathology in intermediate MSUD mice

Since most intermediate MSUD (iMSUD) mice were shown to survive past weaning (Homanics *et al.*, 2006), we first used this model to identify behavioural changes and neuropathology. Behavioural changes were evident in iMSUD mice at 3-weeks of age including hypoactivity, hindlimb dystonia and uncoordinated gait (Table 1). In surviving iMSUD mice, behavioural changes became more severe over the next few weeks and included frequent falls and intermittent recumbency. There were no behavioural changes in heterozygous or WT littermate controls. To identify neuropathological changes in iMSUD mice, we performed MRIs beginning at 4-weeks of age. MRI of 4- and 6-week-old iMSUD mice revealed moderate and severe neuropathological changes in the striatum, thalamus and corpus callosum as indicated by increased T_2 signals (Fig. 1).

Histological sections of iMSUD mouse brains at 6-weeks of age show pathologic changes localized to the striatum, thalamus and brainstem (Fig. 2). Vacuolated white matter tracks were present in the striatum and brainstem as well as diffuse vacuolation of grey matter structures (Fig. 2B and D). Higher power confocal images of similar sections reveal disruption of neuronal tracts in the striatum with reactive astrocytes (Fig. 2F). Confocal images of the brainstem of iMSUD mice reveal depleted neurons and neural networks with reactive astrocytosis (Fig. 2H). These changes are strikingly similar to neuropathology reports for human MSUD (Crome *et al.*, 1961; Silberman *et al.*, 1961) and show a similar pattern of neuronal disruption likely indicating the brain regions most dependent on normal branched-chain ketoacid dehydrogenase function.

Branched-chain amino acid accumulation was associated with growth restriction and reduced survival in iMSUD mice

The iMSUD mice maintained on a normal diet survived a maximum of 8 weeks (Fig. 3A) with most of these mice dying between 5 and 8 weeks as previously reported (Homanics *et al.*, 2006). There was no reduced survival of WT or heterozygous littermates of iMSUD mice (Fig. 3A). Body weights of iMSUD mice were 25 and 30% lower than age-matched heterozygous littermate controls at 3- and 4-weeks of age, respectively (Fig. 3B). Examination of individual organs (brain, kidney and liver) revealed a disproportionate growth restriction in the brain of 70% in 4-week-old iMSUD mice compared with age-matched controls (Fig. 3B). These changes were accompanied by substantial branched-chain amino acid accumulation in blood, brain and liver (Fig. 3C) with

Table 1 Average neurologic scores in surviving iMSUD mice on a normal diet

Neurologic score	3–4 weeks of age ($n = 16$) (%)	5–6 weeks of age ($n = 12$) (%)	7–8 weeks of age ($n = 4$) (%)
3–4	70	30	0
5–6	30	50	50
7–10	0	20	50

See behavioural evaluation in Methods section for neurologic score determinations.
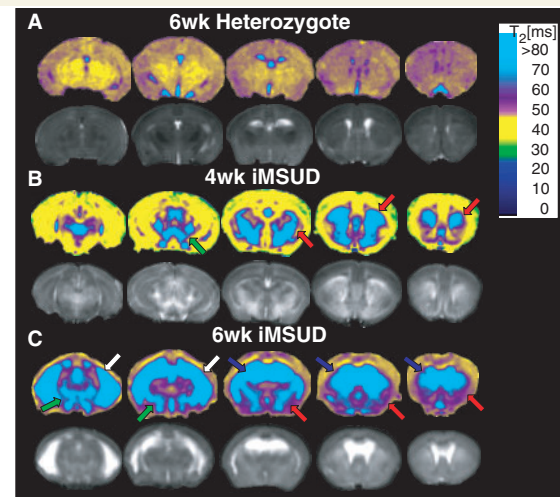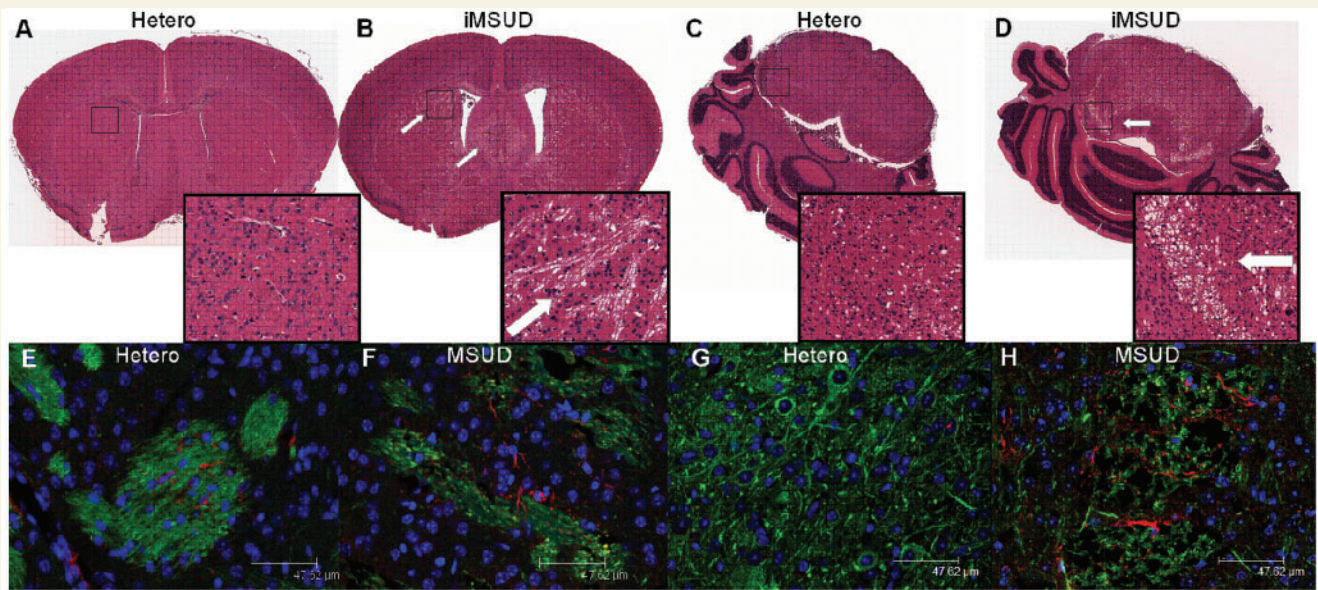

Figure 1 Neuropathology in iMSUD mice detected by MRI. (**A**) T_2 maps (top) and T_2 weighted brain images (bottom) of a heterozygote control at 6wk of age (6 weeks) from left to right, caudal to rostral. (**B**) Similar images from an iMSUD mouse at 4-weeks of age show increased T_2 signals in the striatum (red arrows) and thalamus (green arrows). (**C**) At 6-weeks of age an iMSUD mouse shows enlarged ventricles (blue arrows), increased T_2 signals in the striatum (red arrows), thalamus (green arrows) and fluid collection along the white matter tracts of the corpus callosum (white arrows). [Colour bar indicates T_2 values (right side)].

the greatest accumulation of leucine compared with valine and isoleucine in plasma, similar to reports of leucinosis in human MSUD (Morton *et al.*, 2002). Alloisoleucine, considered diagnostic for MSUD, was >60-fold increased in blood (126 ± 48 versus $2 \pm 1 \mu mol/l$) and >5-fold increased in liver (375 ± 92 versus $65 \pm 20 \mu mol/kg$ wet wt) of iMSUD mice compared with heterozygous controls, respectively (Fig. 3C). Alloisoleucine was not detected in brain tissue of iMSUD mice or controls.

Branched-chain amino acid accumulation in human MSUD is associated with alterations of other amino acids (Wajner *et al.*, 2000; Morton *et al.*, 2002), therefore we measured large neutral, small neutral and basic amino acids in blood, brain and liver from iMSUD mice. Similar to human MSUD, large neutral amino acids, threonine and tyrosine, were significantly reduced in blood along with small neutral amino acid, alanine (Fig. 3D) (Wajner *et al.*, 2000; Morton *et al.*, 2002). However, unlike human data,

Figure 2 Neuropathology in iMSUD mice by light and confocal microscopy. Coronal sections from a heterozygote control (**A** and **C**) show the normal appearance of cortex and striatum (**A**) as well as cerebellum and brainstem (**C**). Similar sections from an iMSUD mouse at 6-weeks of age show striatal vacuolation (**B**) and disruption of white matter patches in the striatum (inset B, white arrow). Additionally, white matter structures in the brainstem were shown to be disrupted in iMSUD mice (**D** and inset, white arrow). Confocal images of similar sections as in **A–D** show disruption of neuronal processes (green) in the striatum (**F**) and the brainstem (**H**) with astrocytosis of iMSUD mouse compared with heterozygotes control (**E** and **G**, respectively). (AND: H&E, inset 25×; E–H: N52-green, GFAP-red and DAPI-blue).

tryptophan was significantly increased in blood of iMSUD mice compared with controls (Fig. 3D) (Wajner *et al*., 2000). In brain, threonine, tryptophan and tyrosine were all reduced while levels of lysine and alanine were increased in iMSUD mice compared with WT and heterozygous controls (Fig. 3D). These data are consistent with previous studies using leucine loading in rats (Araujo *et al*., 2001) and cerebral spinal fluid samples of MSUD patients (Wajner *et al*., 2000). In contrast, threonine, tryptophan, tyrosine and lysine were all increased in liver while alanine levels were reduced in iMSUD mice (Fig. 3D). These data are consistent with brain specific growth restriction and suggest that accumulating branched-chain amino acids compete with essential large neutral amino acida for brain access through the large neutral amino acid transporter (LAT1) (Killian and Chikhale, 2001). Alternatively, the increased amino acid levels in liver may indicate an enhanced catabolic state (Daniel *et al*., 1977).

Depletion of essential large neutral amino acida in iMSUD mouse brains may affect neurotransmitter levels, especially dopamine, which is dependent on available tyrosine. Therefore, we investigated the possibility of neurotransmitter deficiencies in iMSUD mice (Fig. 3E). Depletion of neurotransmitters included decreased glutamate and aspartate levels by >40%, GABA by ∼20% and dopamine by >60% (Fig. 3E). Reduced dopamine levels are consistent with depleted precursor, tyrosine, in blood and brain of iMSUD mice and blood of MSUD patients (Morton *et al*., 2002). Depletion of glutamate, GABA and aspartate is consistent with previous findings in newborn MSUD calves (Dodd *et al*., 1992) and may indicate impaired glutamate cycling or disrupted energy metabolism secondary to branched-chain ketoacid

accumulation as previously suggested (Patel *et al*., 1973, 1974; Yudkoff *et al*., 2005).

Rapid postnatal branched-chain amino acid accumulation limits neuronal development and survival of classic MSUD mice

The limited survival of classic MSUD (cMSUD) mice suggested that similar pathological changes found in iMSUD mice might evolve more rapidly in cMSUD mice. Therefore, we examined blood, brain and liver from infant cMSUD mice over the first few days of life for development of neuropathology that correlated with metabolic decompensation (Figs 4 and 5). Comparison of confocal images from motor cortex of cMSUD and heterozygous mice shows disruption of neurofilaments and reactive astrocytosis in cMSUD mice at 3 days of age (Fig. 4A and B). These changes are consistent with reports of abnormal and decreased dendritic development in human MSUD (Kamei *et al*., 1992). Survival of cMSUD mice is limited to 3 days of life (Fig. 4C) as previously reported and there were no significant differences in body or brain weights between newborn cMSUD mice and littermate controls (Homanics *et al*., 2006). All three branched-chain amino acids were significantly higher in blood, brain and liver of newborn cMSUD mice compared with heterozygous controls (Fig. 4D). However, leucine levels rose exponentially in blood and brain of cMSUD mice over postnatal day 1 and 2, while liver levels increased linearly (Fig. 4D). Similar to human MSUD, alloisoleucine
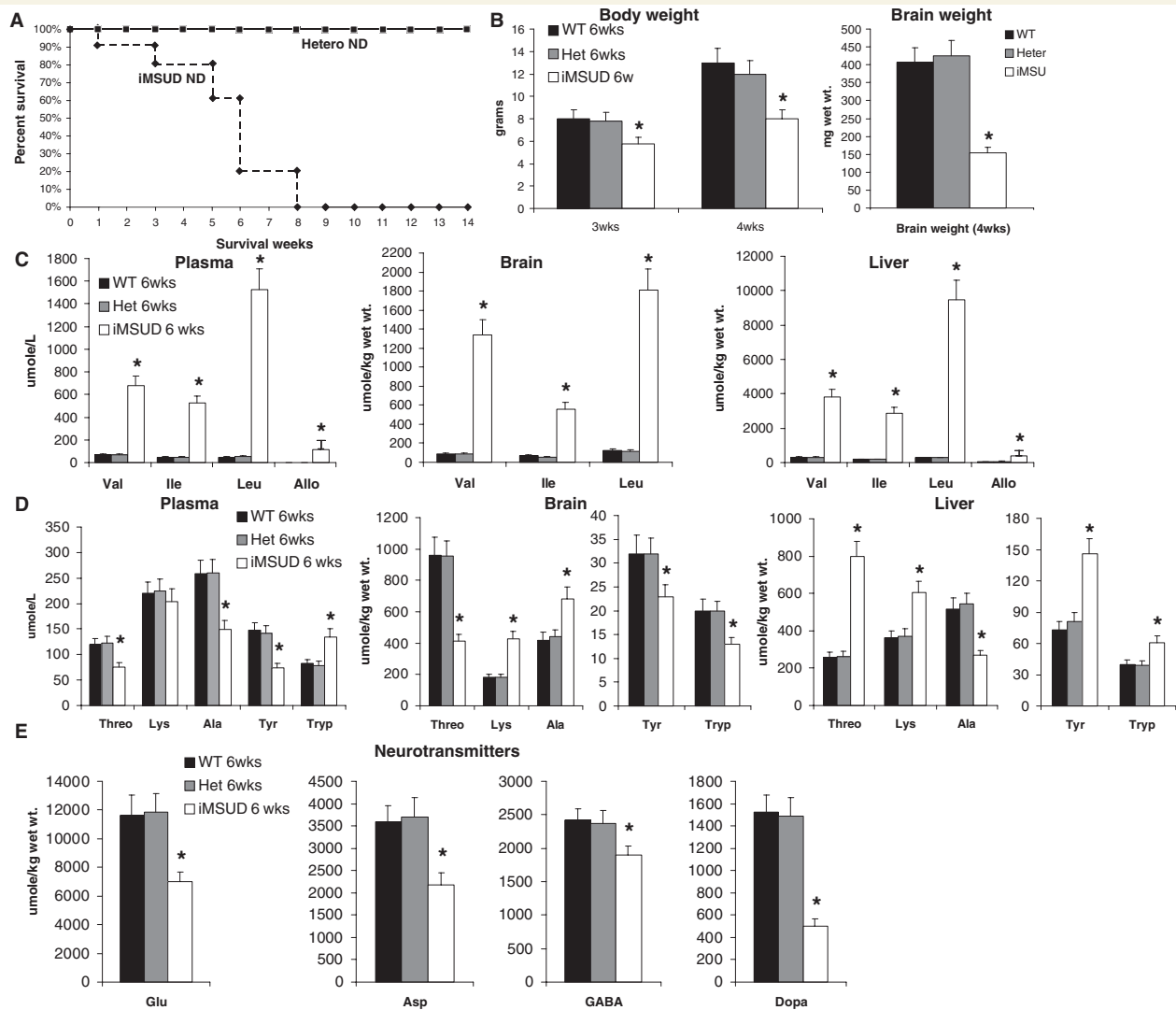
Figure 3 Limited survival, body and brain weights associated with biochemical derangement in iMSUD mice. (**A**) Survival of iMSUD mice is limited compared with heterozygous controls both on a normal diet (ND) (*n* = 20 per group). (**B**) Body weight of iMSUD mice at 3- and 4-weeks of age compared with WT and heterozygous controls. Brain weight of iMSUD mice at 4-weeks of age was about one-third of WT and heterozygous age-matched controls. (**C**) Plasma, brain and liver branched-chain amino acids of iMSUD compared with WT and heterozygous controls. (**D**) Large neutral, basic and small neutral amino acid changes in plasma, brain and liver associated with increased branched-chain amino acids in iMSUD mice. (**E**) Whole brain neurotransmitter changes associated with branched-chain amino acids accumulation in iMSUD mice compared with WT and heterozygous controls all on a normal diet at 6-weeks of age. (Mean ± SEM, *P < 0.01, *n* = 6 each group).

blood levels were indistinguishable from heterozygous controls (2 ± 1 µmol/l) until the third day of life (8 ± 2 µmol/l) (Fig. 4D). Accordingly, liver alloisoleucine levels were similar to control, but more than doubled on the third day of life in cMSUD mice only (342 ± 48 µmol/kg wet wt) (Fig. 4D).

Comparable with iMSUD mice, leucine accumulation in cMSUD mice was associated with changes in other large neutral, basic and small neutral amino acids as well as neurotransmitter depletion (Fig. 5). Comparing blood from newborn cMSUD mice and heterozygous controls, threonine, tryptophan and alanine levels were similar, but then decreased significantly over the first few days of life (Fig. 5). Blood lysine levels were unchanged in

cMSUD mice compared with controls, but tyrosine levels were reduced and remained lower than controls through postnatal day 2 (Fig. 5). These changes are similar to those found in human MSUD (Wajner et al., 2000) and further indicate differences exacerbated by leucine accumulation.

Brain threonine, tryptophan, lysine and alanine levels were similar comparing newborn cMSUD with heterozygous controls, although brain tyrosine levels were lower in cMSUD mice (Fig. 5). Both threonine and tyrosine were progressively decreased in the brain of cMSUD mice while these levels were shown to increase in heterozygous mice over the same period (Fig. 5). Alanine levels increased in cMSUD brains compared with controls over the first few days
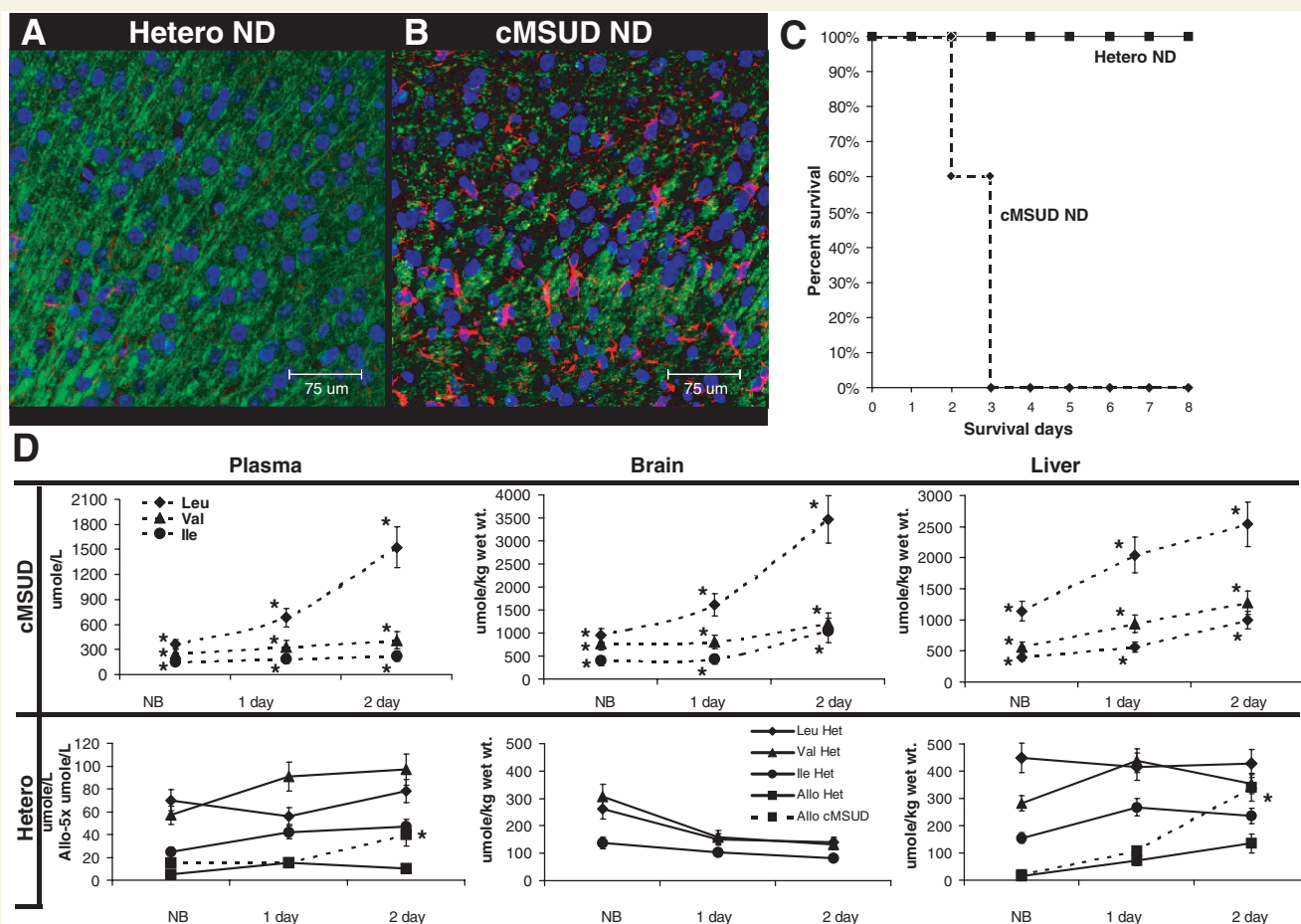
Figure 4 Neuropathology and limited survival associated with postnatal branched-chain amino acid accumulation in cMSUD mice. Confocal images of motor cortex from a heterozygous control (**A**) and cMSUD mouse (**B**) both at 3 days of age (**A** and **B**: N52-green, GFAP-red and DAPI-blue). (**C**) Survival of cMSUD mice is limited to postnatal day 2 or 3 compared with normal survival of heterozygous controls both on a normal diet (ND) (cMSUD $n = 20$, Hetero $n = 36$). (**D**) Plasma, brain and liver branched-chain amino acids were substantially increased in newborn (NB), 1- and 2-day-old cMSUD mice compared with heterozygous (Het) controls (Note different scale for cMSUD and Hetero, and 5x scale for alloisoleucine plasma levels, Mean ± SEM, *$P < 0.01$, $n = 6$ each group).

of life. Glutamate, glutamine, aspartate, GABA and dopamine levels were similar in newborn cMSUD mice and heterozygous controls, but each declined by postnatal day 2 in cMSUD mice (Fig. 5). These changes are similar to those found in 6-week-old iMSUD mice and newborn MSUD calves (Dodd *et al.*, 1992), although to a greater magnitude in a shorter period of time for cMSUD mice. The development of these changes during an exponential rise in plasma and brain leucine levels suggests a causative relationship and provides further evidence for competition at the blood–brain barrier between leucine and other essential large neutral amino acids.
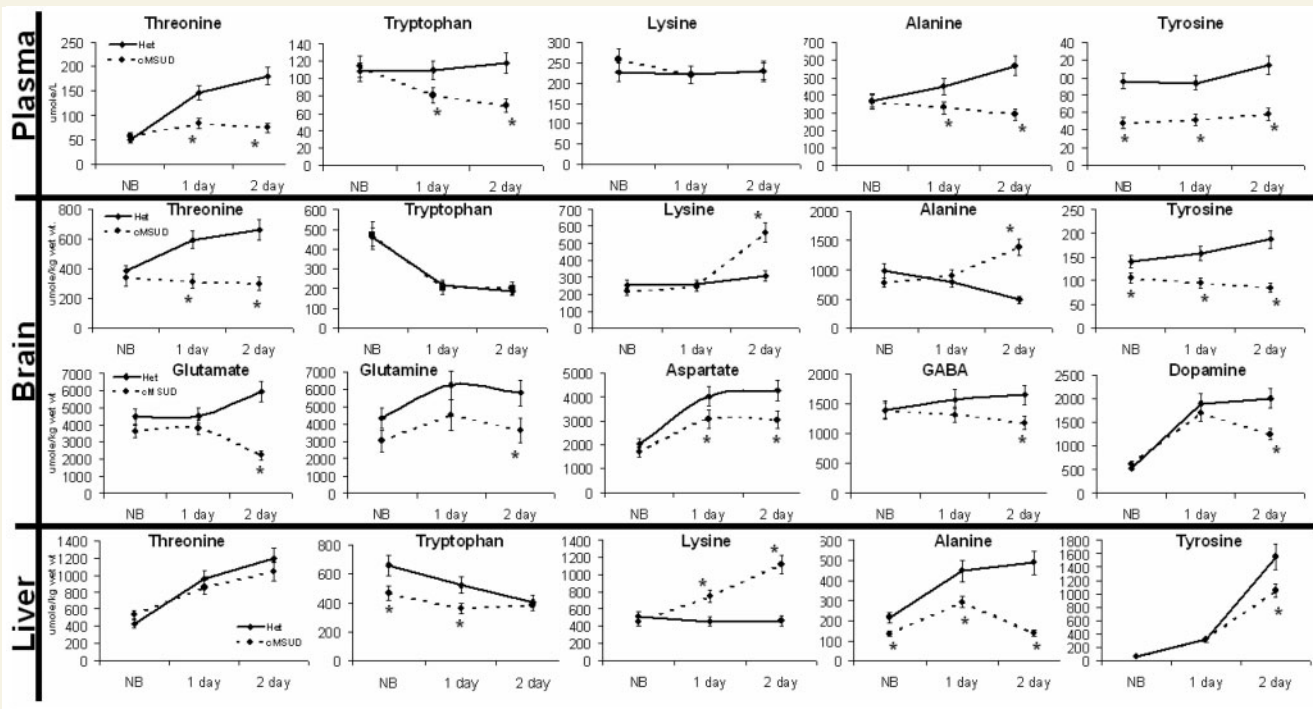
In newborn cMSUD liver, threonine, lysine and tyrosine levels were similar to controls, but tryptophan and alanine were lower (Fig. 5). Over the first few days of life, tryptophan levels increased to match controls, lysine increased above control levels and alanine and tyrosine levels were both reduced in cMSUD liver compared with controls (Fig. 5). Increased brain and liver lysine levels with decreased plasma alanine suggests increased ketone body utilization and reduced gluconeogenesis as previously shown in

other models (Daniel *et al.*, 1977; Zinnanti *et al.*, 2007). These changes likely represent events that contribute to biochemical decompensation associated with brain injury in MSUD.

Low branched-chain amino acid diet improves survival and prevents brain injury in iMSUD mice

Standard treatment for maple syrup urine disease is life-long low branched-chain amino acid diet (Chuang and Shih, 2001; Morton *et al.*, 2002). Survival of some iMSUD mice to weaning (3-weeks of age) provided the opportunity to test the effect of a low branched-chain amino acid diet for this MSUD mouse model. At 3-weeks of age iMSUD mice were provided equal access to both normal and branched-chain amino acid-free diet. This method takes advantage of the rodent's ability to discriminate between diets as previously reported (She *et al.*, 2007). The iMSUD mice

Figure 5 Plasma, brain and liver amino acid changes over the first few days of life in cMSUD mice. Plasma threonine, tryptophan, lysine, alanine and tyrosine in newborn (NB), postnatal day 1 and day 2 of cMSUD compared with heterozygous control mice (Mean ± SEM, *P<0.01, n = 6 each group). Brain threonine, tryptophan, lysine, alanine, tyrosine, glutamate, glutamine, aspartate, GABA and dopamine in NB, postnatal day 1 and 2 of cMSUD mice compared with heterozygous controls (Mean ± SEM, *P<0.01, n = 6 each group). Liver threonine, tryptophan, lysine, alanine and tyrosine levels in cMSUD and heterozygous control mice over the first few days of life (Mean ± SEM, *P<0.01, n = 6 each group).

consumed an average of 3.6 g of diet daily (73% branched-chain amino acid-free diet and 27% normal diet, n = 20). Consumption of this low branched-chain amino acid diet resulted in reduced plasma and brain branched-chain amino acid accumulation, increased survival and neuronal protection (Fig. 6). Eighty percent of iMSUD mice survived for >1 year on the low branched-chain amino acid diet. We performed MRI on iMSUD mice and heterozygous controls that were exposed to the low branched-chain amino acid diet for 8-weeks (Fig. 6A and B). The iMSUD mice maintained on the low branched-chain amino acid diet had slightly increased T_2 signals throughout the brain compared with controls indicating increased water content. Similar changes have been reported in non-encephalopathic adolescent and adult patients with MSUD (Schonberger *et al.*, 2004). There was no evidence of behavioural changes such as those found in iMSUD mice on a normal diet (Fig. 1). Furthermore, there was no difference in brain weights compared with heterozygous controls.
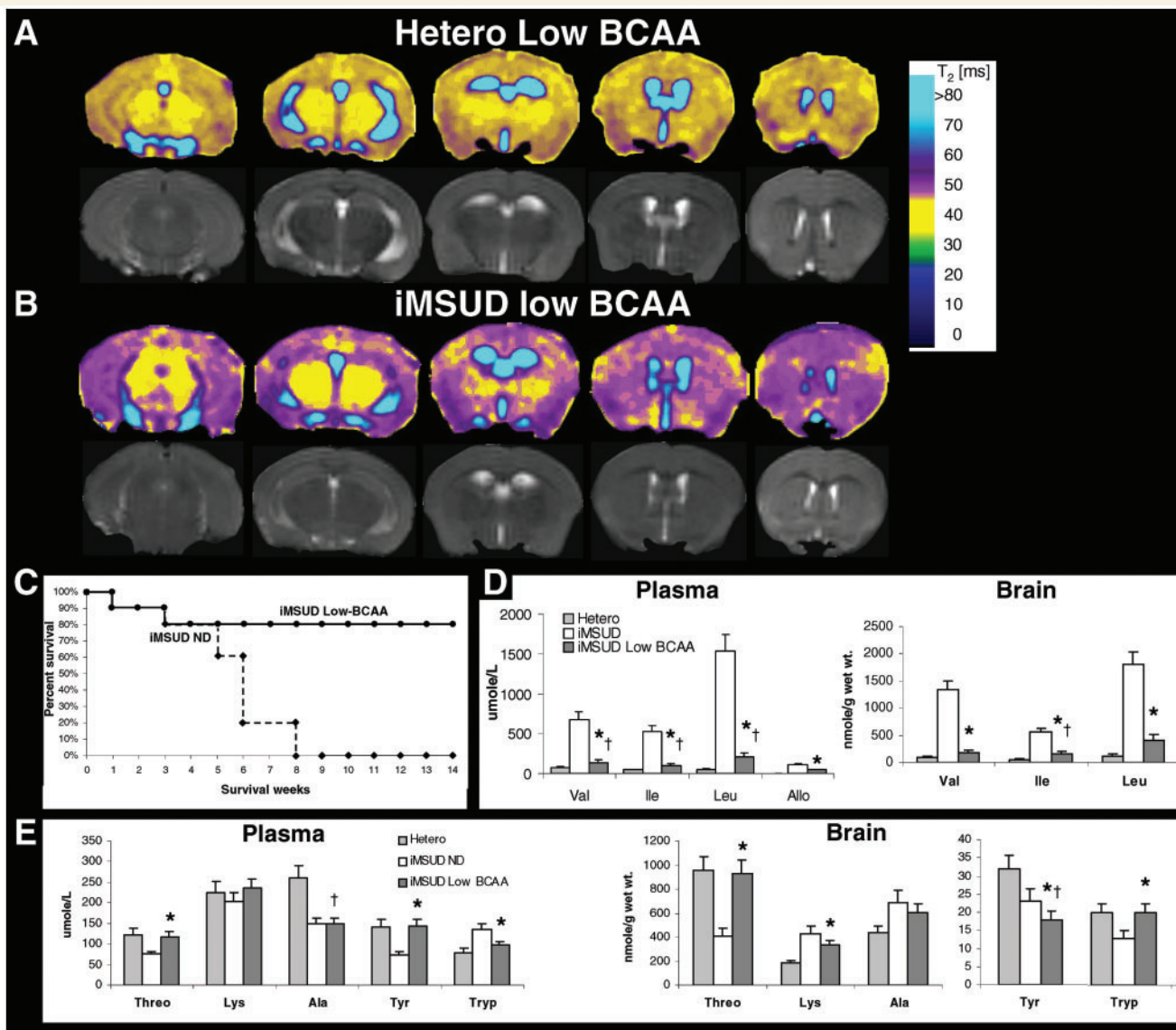
The low branched-chain amino acid diet reduced plasma leucine levels to within 2-fold of control values and reduced brain leucine to control levels in iMSUD mice (Fig. 6D). Plasma alloisoleucine levels were reduced >50% in iMSUD mice on the low branched-chain amino acid diet, but remained about 20-fold higher than control levels (44 ± 15 µmol/l) (Fig. 6D). Other large neutral, basic and small neutral amino acids were regulated to within

control values in plasma and brain, except for plasma alanine that remained low and brain tyrosine that was further reduced (Fig. 6E). These data demonstrate the protective effect of a low branched-chain amino acid diet to reduce brain leucine accumulation through dietary manipulation. Additionally, the reduced brain branched-chain amino acid levels correlate with increased availability of other essential large neutral amino acids and normal brain development.

High protein challenge reveals energy deprivation in MSUD encephalopathy

The response of iMSUD mice to a low branched-chain amino acid diet provided the opportunity to challenge these mice with a high protein diet, which may mimic the catabolic state considered to cause the most severe episodes of biochemical intoxication in patients with MSUD (Morton *et al.*, 2002). Heterozygous controls and iMSUD mice were maintained on a low branched-chain amino acid diet for 3 weeks. Half of each group was placed on the high protein diet and all were monitored for behavioural and biochemical changes. Heterozygous mice exposed to the high protein diet and iMSUD mice maintained on a low branched-chain amino acid diet did not show any behavioural changes (Fig. 7A and B). After 48 h of high protein diet exposure, iMSUD mice developed widening of hind leg stance, unsteady gait
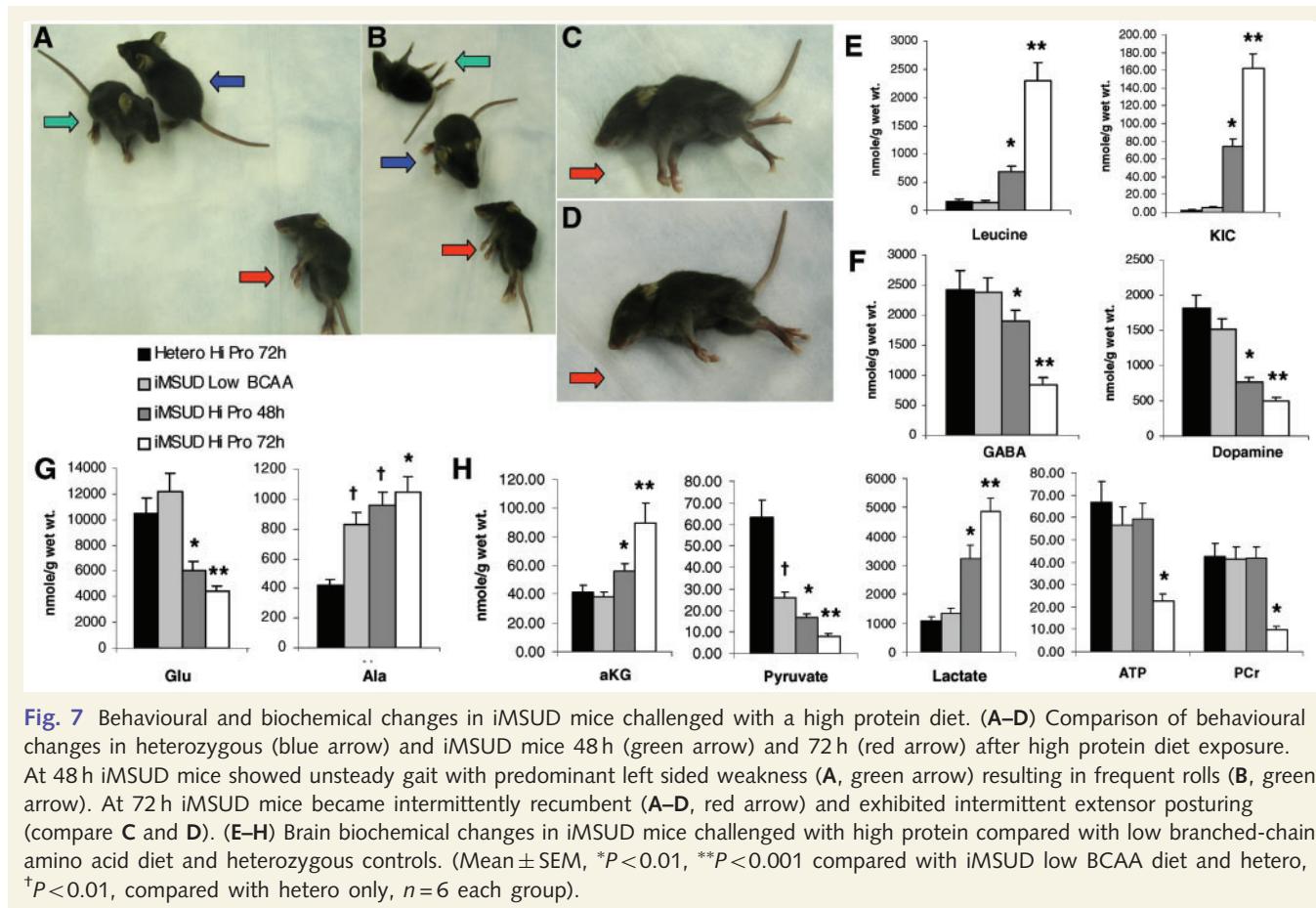
Figure 6 Low branched-chain amino acid diet protects brain and increases survival of iMSUD mice. (**A**) T_2 maps (top) and T_2-weighted brain images (bottom) of a heterozygous control at 8-weeks of age from left to right, caudal to rostral. (**B**) Similar images from an iMSUD mouse at 8-weeks of age show only slight increase in T_2 signals throughout the brain. [Colour bar indicates T_2 values (right side)] (**C**) Survival of iMSUD mice on a low branched-chain amino acid diet compared to normal diet ($n = 40$ per group). (**D**) Plasma and brain branched-chain amino acids in iMSUD mice and heterozygous controls allowed to select a low branched-chain amino acid diet compared with iMSUD normal diet controls at 6-weeks of age. (**E**) Plasma and brain large neutral, basic and small neutral amino acids in iMSUD mice and heterozygous controls allowed to select a low branched-chain amino acid diet compared with iMSUD normal diet controls at 6-weeks of age (Mean ± SEM, $^*P < 0.01$ compared with iMSUD ND, $^\dagger P < 0.01$ compared with hetero, $n = 6$ each group).

and left or right sided weakness with frequent rolls or falls to the weak side (Fig. 7A and B). These changes were similar to those observed in iMSUD mice on a normal diet between 3 and 6 weeks of age (Table 1). Within 72 h of protein diet exposure, iMSUD mice developed intermittent recumbency and extensor posturing, which was exacerbated by stimulating the animal with mild tail pressure (Fig. 7C and D). These behavioural changes are similar to reports of severe dystonia in children with MSUD during acute decompensation (Crome *et al*., 1961; Silberman *et al*., 1961;

Morton *et al*., 2002; Jan *et al*., 2003) and may represent an animal model of opisthotonus.

Progressive biochemical decompensation accompanied behavioural changes between 48 and 72 h in iMSUD mice exposed to high protein. At 48 h, brain leucine levels increased more than 4-fold and α-ketoisocaproate (αKIC) increased more than 10-fold compared with low branched-chain amino acid diet and heterozygous controls (Fig. 7E). At 72 h, high protein exposure raised cerebral leucine levels another 3-fold as αKIC levels doubled.

Fig. 7 Behavioural and biochemical changes in iMSUD mice challenged with a high protein diet. (**A–D**) Comparison of behavioural changes in heterozygous (blue arrow) and iMSUD mice 48 h (green arrow) and 72 h (red arrow) after high protein diet exposure. At 48 h iMSUD mice showed unsteady gait with predominant left sided weakness (**A**, green arrow) resulting in frequent rolls (**B**, green arrow). At 72 h iMSUD mice became intermittently recumbent (**A–D**, red arrow) and exhibited intermittent extensor posturing (compare **C** and **D**). (**E–H**) Brain biochemical changes in iMSUD mice challenged with high protein compared with low branched-chain amino acid diet and heterozygous controls. (Mean ± SEM, $^*P < 0.01$, $^{**}P < 0.001$ compared with iMSUD low BCAA diet and hetero, $^\dagger P < 0.01$, compared with hetero only, $n = 6$ each group).

Plasma alloisoleucine levels did not change significantly with high protein exposure (not shown). At 48 h, GABA levels were depleted by ~20% and dopamine levels were reduced by 50% in the brain of iMSUD mice exposed to high protein (Fig. 7F). After 72 h, both GABA and dopamine levels were reduced to one-third of control levels. These changes are consistent with appearance of motor impairment involving initiation and control and further correlate with striatal damage found in these mice and children with MSUD (Hauber, 1998; Morton *et al*., 2002). Additionally, glutamate levels were reduced by ~50% at 48 h and further reduced at 72 h of high protein exposure (Fig. 7G) while glutamine levels remained unchanged during this period (not shown). Alanine levels were increased by about 12% at 48 h and another 11% at 72 h in the brain of iMSUD mice on the high protein diet (Fig. 7G).

Glutamate depletion with alanine accumulation may indicate impaired energy metabolism (Regunathan and Sundaresan, 1984). To evaluate the status of energy metabolism in the brain, we measured α-ketoglutarate (αKG), pyruvate, lactate, ATP and phosphocreatine levels. In iMSUD mice maintained on a low branched-chain amino acid diet, pyruvate levels were >50% reduced compared with heterozygous controls, although αKG, lactate, ATP and phosphocreatine levels were similar in both groups (Fig. 7H). After 48 h of high protein diet exposure, αKG was elevated by 25% and pyruvate was further decreased as lactate more than doubled (Fig. 7H). However, ATP and

phosphocreatine levels were unchanged at 48 h in iMSUD mice on the high protein diet compared with controls (Fig. 7H). At 72 h, αKG increased 50% >48-h levels, pyruvate was further depleted and lactate increased to more than 3-fold control levels (Fig. 7H). At this time, ATP and phosphocreatine levels were substantially depleted compared with 48-h levels and controls. These brain biochemical changes are consistent with the development of severe behavioural deficits at 72 h.

Norleucine prevents αKIC accumulation and delays encephalopathy in iMSUD mice

Atypical amino acid, norleucine, has been shown to effectively compete with leucine at the blood–brain barrier for brain access (Tews *et al*., 1991). Therefore, we tested the ability of norleucine to reduce branched-chain amino acid accumulation and improve survival in iMSUD mice on a high protein diet. Supplementing the high protein diet with 5% norleucine resulted in survival of 80% of iMSUD mice for up to 5 days, 40% up to 7 days and 20% longer than 8 days compared with 2–3 day survival on the high protein diet alone (Fig. 8A). Norleucine supplementation delayed the onset of encephalopathy in iMSUD mice based on average neurologic scores for each group (Fig. 8B). The cage hang test was highly sensitive for treated versus untreated
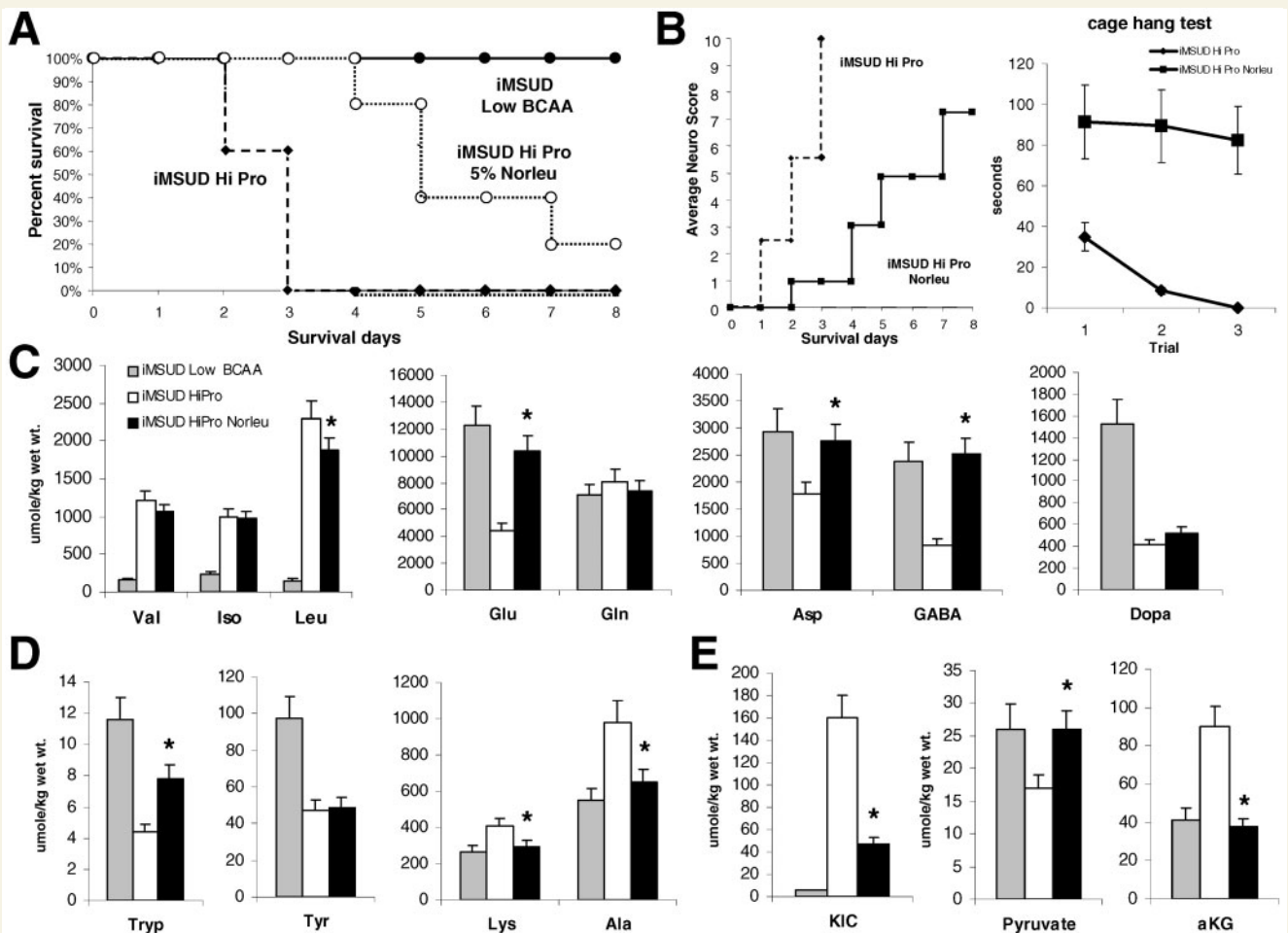
Figure 8 Norleucine improved survival, behaviour and neurochemistry of iMSUD mice challenged with a high protein diet. (**A**) Survival of adult iMSUD mice challenged with a high protein diet (Hi Pro), or challenged with a high protein diet containing 5% norleucine (Hi Pro 5% Norleu) ($n = 20$ per group). (**B**) Average neurological score of iMSUD mice challenged with the high protein diet compared with and without 5% norleucine ($n = 5$ per group). Cage hang test compared the time (seconds) over three trials for iMSUD mice to hang upside down after 48 h of high protein diet with and without 5% norleucine ($n = 5$ per group). (**C**) Branched-chain amino acid, glutamine and neurotransmitter levels. (**D**) Large neutral, basic and small neutral amino acid levels. (**E**) αKIC, pyruvate and αKG levels in brain of iMSUD mice challenged with a high protein diet with and without 5% norleucine compared with low branched-chain amino acid diet controls. (Mean ± SEM, *$P < 0.01$, compared with iMSUD high protein without treatment, $n = 6$ each group).

groups (Fig. 8B). These data indicate a protective effect of norleucine to delay encephalopathy.

To evaluate the protective effect of norleucine, we examined brain biochemical changes with and without treatment in high protein challenged iMSUD mice for 2 days compared with low branched-chain amino acid diet controls (Fig. 8C–E). Norleucine treatment reduced brain leucine levels by >20%, although there was no difference in valine or isoleucine compared with untreated controls (Fig. 8C). Glutamate, aspartate and GABA were all protected by norleucine treatment, although dopamine was still depleted compared with untreated iMSUD mice challenged with high protein (Fig. 8C). Of the large neutral amino acids, tryptophan was partially protected by norleucine treatment, but tyrosine was not, consistent with depleted dopamine levels. Both lysine and alanine were lower with norleucine treatment (Fig. 8D). Taken together these data suggest that norleucine may be more

effective in protecting energy metabolism than in preventing brain branched-chain amino acid accumulation. Consistent with this hypothesis, αKIC levels were substantially reduced with norleucine treatment and pyruvate and αKG were restored to near normal levels compared with high protein diet alone (Fig. 8E). Reduced levels of αKIC and protected levels of glutamate, aspartate and GABA suggest that norleucine may inhibit transamination of leucine (formation of αKIC) in the brain.

Norleucine treatment improves survival of suckling cMSUD mice

The rapid decompensation of newborn cMSUD mice over the first few days of life poses a significant challenge to testing potential treatments. If a treatment can be effective in this model, it is more
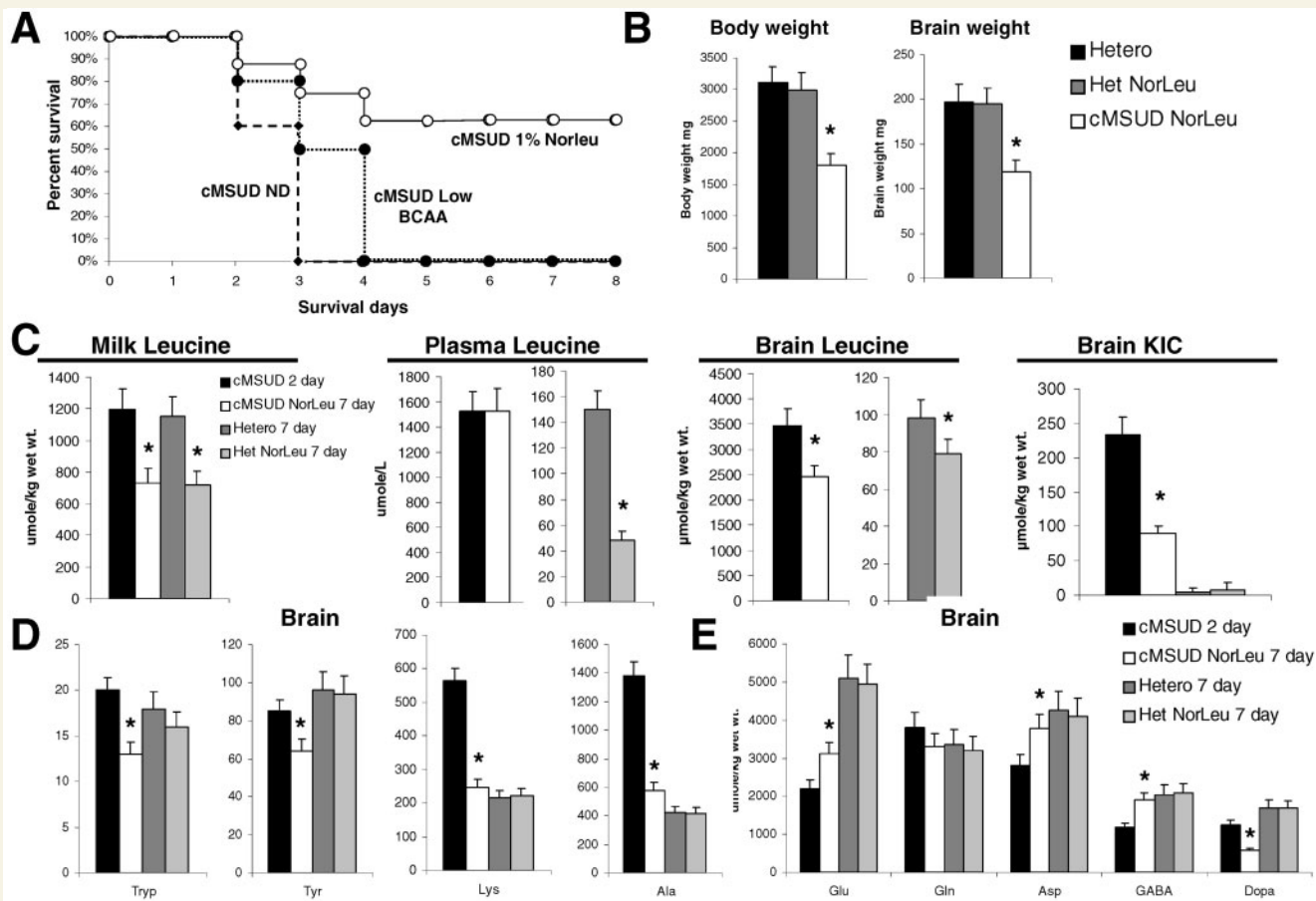
Figure 9 Norleucine treatment improved survival and neurochemistry in infant cMSUD mice. (**A**) Survival of suckling cMSUD mice from mothers on a normal diet (ND), low branched-chain amino acid diet or with 1% norleucine added to daily water intake (n = 20 per group). (**B**) Body and brain weight of cMSUD mice after 1 week of norleucine treatment through mothers compared with heterozygous controls with and without norleucine treatment. (**C**) Leucine content of milk taken from the stomach of cMSUD or heterozygous mice with and without norleucine treatment of the mothers. Plasma and brain leucine concentrations and brain αKIC in cMSUD and heterozygous control mice with and without norleucine treatment of mothers. (**D**) Large neutral, basic and small neutral amino acid levels in brain of cMSUD and heterozygous mice with and without norleucine treatment. (**E**) Brain glutamine and neurotransmitter levels in cMSUD and heterozygous mice with and without norleucine treatment. (Mean ± SEM, $^*P < 0.01$, compared with cMSUD without treatment, n = 6 each group).

likely to be effective in human MSUD. Since these infant mice are dependent on suckling, we hypothesized that treatment may be possible through the heterozygous mothers. We first tested this possibility by placing the pregnant mothers on a low branched-chain amino acid diet one week before giving birth. Compared with normal diet controls, this provided minimal improvement as 50% of cMSUD offspring survived one additional day (Fig. 9A).

Based on the effect of norleucine to compete with branched-chain amino acid transport (Tews et al., 1991), we hypothesized that norleucine supplementation of heterozygous mothers may provide a low branched-chain amino acid milk and improve survival of suckling cMSUD mice. Supplementation of pregnant heterozygous mothers with 1% norleucine, added to daily water intake, allowed 60% of infant cMSUD mice to survive up to 8 days compared with 2–3 day survival in untreated controls (Fig. 9A). Both body and brain weight of cMSUD mice from treated mothers were ~30% reduced compared with heterozygous controls with and without norleucine treatment (Fig. 9B).

These data indicate a substantial improvement in survival with norleucine treatment. Although growth restriction is still present, similar to iMSUD mice on a normal diet, disproportionate brain growth restriction is eliminated suggesting better availability of essential large neutral amino acids in the brain of cMSUD mice.

Since cMSUD mice normally survive only 2–3 days, we used biochemical findings in 2-day-old untreated cMSUD mice to compare with norleucine treated mice at postnatal day 7. Milk taken from the stomach of infant cMSUD mice and heterozygous littermates from either norleucine treated or untreated mothers showed a 40% reduction in leucine content with treatment (Fig. 9C). Plasma leucine levels were reduced by 70% in heterozygous littermates but not cMSUD pups from norleucine treated mothers compared with untreated controls (Fig. 9C). Although there was no difference, the similar levels likely represent delayed accumulation in the 5-day-older treated group. Accordingly, there was no significant difference in plasma alloisoleucine levels with norleucine treatment (not shown). Brain leucine levels were

reduced by 30% in cMSUD pups and 20% in heterozygous pups from treated mothers compared with untreated controls (Fig. 9C), indicating the effectiveness of norleucine to control brain leucine levels. Additionally, αKIC was reduced by 60% in the brain of cMSUD mice from treated mothers (Fig. 9C). These changes are consistent with data from iMSUD mice and support the role of norleucine to inhibit transamination of leucine to αKIC in addition to reducing brain leucine accumulation.

Norleucine was present in plasma and brain of cMSUD mice and heterozygous controls from mothers treated with norleucine, indicating that norleucine crosses into the milk, blood and brain of the suckling mice (not shown). Tryptophan and tyrosine were lower in the brain of cMSUD but not heterozygous pups from norleucine treated mothers compared with untreated controls (Fig. 9D). Brain lysine and alanine were both reduced in cMSUD pups with norleucine treatment (Fig. 9D), which may reflect protection of energy metabolism in the brain similar to changes found in iMSUD mice (Fig. 8). Glutamate, aspartate and GABA levels were increased in cMSUD pups from treated mothers compared with untreated controls (Fig. 9E). However, dopamine levels were further reduced with norleucine treatment consistent with the reduced brain tyrosine levels (Fig. 9E). The protective effect of norleucine in preserving brain glutamate, aspartate and GABA levels correlates with reduced αKIC levels.

Discussion

In the current study, we correlate biochemical, behavioural and neuropathologic changes in recently developed MSUD mouse models in order to provide mechanistic insight and test therapeutic interventions for MSUD encephalopathy. Examinations of iMSUD mice showed a mouse model that develops neuropathology similar to human MSUD (Crome et al., 1961; Silberman et al., 1961), large accumulation of branched-chain amino acids, depletion of other essential large neutral amino acids, and brain specific growth restriction. Both iMSUD and cMSUD mice showed neurotransmitter depletion including dopamine and glutamate, as well as a correlation between the level of depletion with the rise in brain leucine. Placing the iMSUD mice on a high protein diet showed that initiation of behavioural symptoms was concomitant with neurotransmitter depletion and more severe symptoms were associated with energy depletion secondary to Krebs cycle disruption. The use of norleucine revealed a potential acute-treatment for MSUD that effectively delayed encephalopathy in cMSUD pups and iMSUD mice challenged with a high protein.

Current findings indicate a model of brain injury in MSUD that involves two converging mechanisms: (i) amino acid competition at the blood–brain barrier secondary to branched-chain amino acid accumulation; and (ii) disruption of energy metabolism secondary to branched-chain ketoacid accumulation (Fig. 10). On the one hand, accumulation of branched-chain amino acids, especially leucine, likely inhibits transport of other essential large neutral amino acids (threonine, tryptophan and tyrosine) that share the same transporter for brain access. This competition results in the observed depletion of essential large neutral amino acids in the brain with subsequent consequences for both protein and neurotransmitter synthesis similar to previous studies of dietary amino acid manipulation in rats (McKean et al., 1968; Tews et al., 1991; Le Masurier et al., 2006). Impaired protein synthesis secondary to unbalanced essential amino acids results in brain specific growth restriction in iMSUD mice and disruption of dendritic development in cMSUD pups. Additionally, inhibition of neurotransmitter synthesis (dopamine) is associated with early behavioural symptoms such as intermittent dystonia and gait abnormalities within 48 h of protein diet exposure.

The proposed second converging mechanism in MSUD encephalopathy involves energy deprivation secondary to branched-chain ketoacid (αKIC) accumulation. Severe brain damage and death in both cMSUD and iMSUD mice correlated with accumulation of αKIC levels above 100 μmol in the brain. Similarly, enhanced survival of infant cMSUD and adult iMSUD mice with norleucine treatment was associated with reduced levels ($<$100 μmol) of αKIC. Energy deprivation likely results when αKIC accumulation inhibits Krebs cycle activity as previously demonstrated in vitro (Patel et al., 1973; Patel, 1974; Sgaravatti et al., 2003). αKIC toxicity is likely responsible for cerebral oedema as ATP depletion leads to Na^+/K^+ ATPase failure and cell swelling. Increased αKG and αKIC levels found in the brain of iMSUD mice on the high protein diet are consistent with α-ketoglutarate dehydrogenase inhibition secondary to αKIC accumulation as previously shown (Patel, 1974). Additionally, decreases in glutamate and aspartate are consistent with disruption of glutamate cycling secondary to αKIC accumulation as previously postulated (Yudkoff et al., 2005). These changes are consistent with increased lactate levels and depletion of pyruvate and ATP as shown in Fig. 10. These data underscore the importance of branched-chain ketoacid control and also suggests that branched-chain ketoacids are produced in the brain from available branched-chain amino acids rather than transported in secondary to accumulation in the liver.

Similar biochemical changes were found between iMSUD and cMSUD mice. However, these changes evolved more rapidly over the first few days of life in cMSUD mice, which highlights the delicate nature of MSUD in the newborn requiring prompt treatment. Leucine levels were shown to accumulate exponentially, doubling each day, in the blood and brain of newborn cMSUD mice. These findings suggest enhanced utilization of leucine in the normal immature brain, consistent with previous studies that showed increased transport of branched-chain amino acids across the blood–brain barrier in suckling versus adult rats (Banos et al., 1978). The immature brain readily uses alternate energy substrates such as ketone bodies (Cremer, 1982), which may be supplied as ketogenic amino acids (i.e. leucine and lysine). Additionally, ketogenic amino acids have been shown to provide a substantial proportion of ketone bodies used for myelin synthesis (Dhopeshwarkar and Subramanian, 1979). The pathway for ketone body production from leucine is blocked in MSUD and may account, in part, for disrupted myelination (Crome et al., 1961; Silberman et al., 1961). Accordingly, we observed an increase in brain lysine levels, which may represent compensation for ketogenic substrates similar to previously observed accumulation of brain leucine when lysine breakdown is disrupted (Zinnanti et al., 2006).
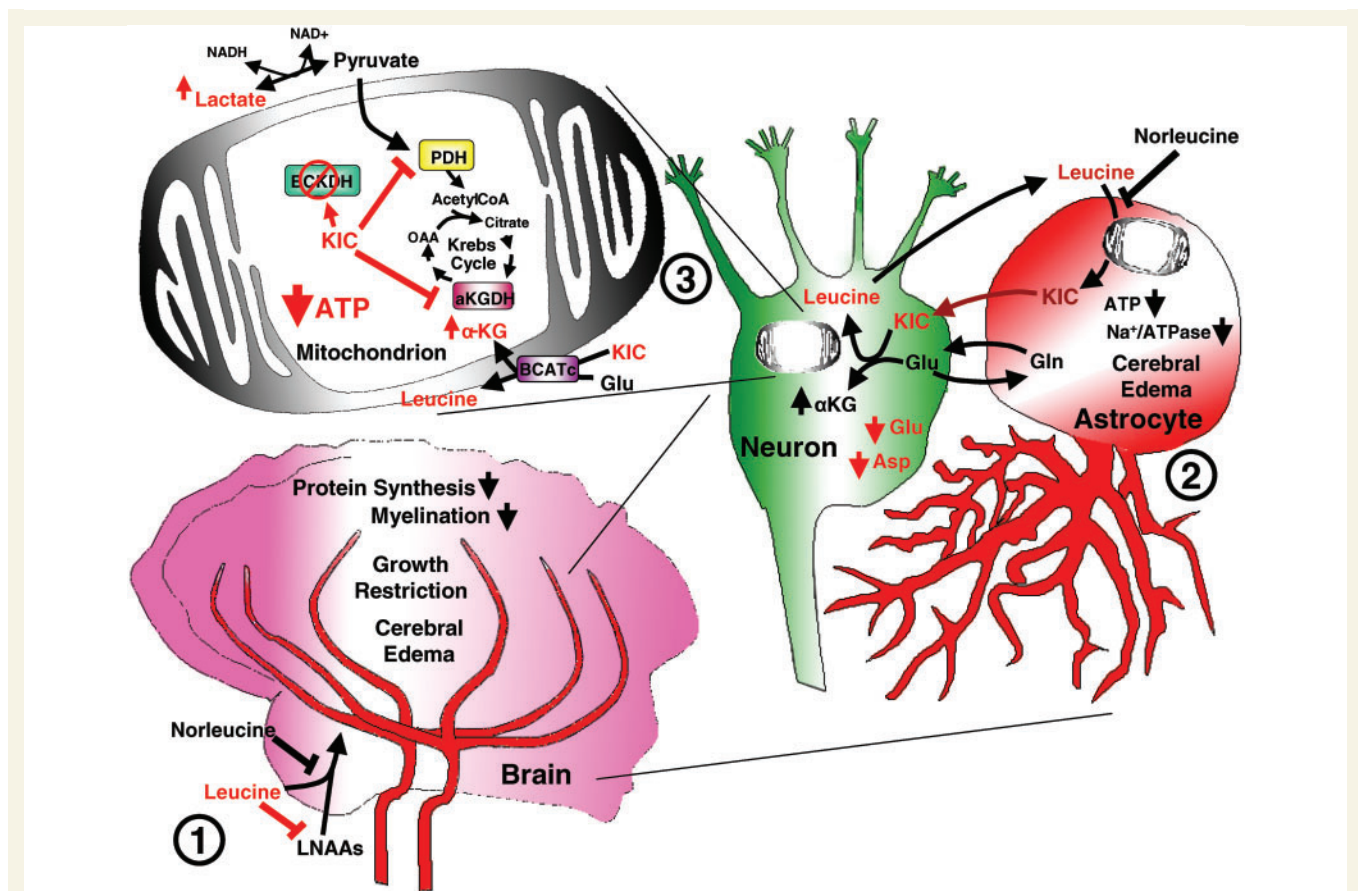
Figure 10 Proposed model of brain injury and treatment effect in MSUD. (**1**) Loss of branched-chain ketoacid dehydrogenase function results in accumulation of branched-chain amino acids (mostly leucine) in blood and tissues including muscle, liver and brain. Rapid accumulation of leucine competes with other essential large neutral amino acids using the same transporter (LAT1) for brain access. Reduced levels of these essential amino acids inhibit protein synthesis and deplete dopamine and serotonin by limiting available precursors. Furthermore, loss of branched-chian ketoacid dehydrogenase impairs use of leucine for myelin synthesis, combined with impaired protein synthesis leads to dysmyelination. (**2**) Accumulation of branched-chain ketoacid (αKIC) within astrocytes and neurons may drive reverse transamination toward leucine resulting in increased αKG/glutamate ratios. (**3**) An increase in αKG/glutamate ratios may inhibit the malate/aspartate shuttle resulting in increased NADH/NAD+ ratios preventing conversion of lactate to pyruvate. Alternatively, at the mitochondrial level, accumulation of αKIC has been previously shown to inhibit oxidative metabolism through inhibition of pyruvate dehydrogenase (PDH) and α-ketogutarate dehydrogenase (αKGDH) resulting in Krebs cycle dysfunction. Energy failure results in loss of Na+/ATPase function (**2**) leading to cell swelling and cerebral oedema. Norleucine attenuates leucine transport across the blood–brain barrier (**1**) and may also limit transamination to αKIC at the astrocyte level (**2**) and mitochondrial leucine transport (**3**).

Neuropathological changes identified in iMSUD mice are consistent with previous reports of MSUD patients with inadequate dietary control or repeated catabolic crises (Morton *et al*., 2002; Simon *et al*., 2007). Similar to human MSUD, the iMSUD mice were responsive to a low branched-chain amino acid diet. Neurochemical analysis of these mice are consistent with MR spectroscopy findings in human MSUD that suggested dietary intervention could reduce brain branched-chain amino acid accumulation to near normal levels (Jan *et al*., 2003). However, adult iMSUD mice maintained on the low branched-chain amino acid diet continue to have increased T_2 signals on MRI, which is similar to previous reports in adolescents and adults with MSUD and suggests lingering myelin disruption (Schonberger *et al*., 2004). Further study of this model may provide insight into the cause of these residual changes and suggest ways to prevent them.

Challenging the iMSUD mice with a high protein diet induced a catabolic crisis, which allowed the correlation of early neurochemical changes with appearance of symptoms. Using this strategy, we showed that whole brain dopamine, glutamate and GABA depletion are concomitant with onset of initial behaviour deficits. These findings correlate with neuropathological and behavioural changes during encephalopathy in human MSUD, that indicate susceptibility of motor control centers (Crome *et al*., 1961; Silberman *et al*., 1961; Hauber, 1998; Morton *et al*., 2002). Furthermore, this model shows the first direct experimental evidence that dopamine levels are depleted in the brain during MSUD encephalopathy, which has been previously suggested based on clinical observations and reduced blood tyrosine levels in human MSUD (Morton *et al*., 2002). This finding also suggests that other catecholamines and serotonin are likely compromised

secondary to tyrosine and tryptophan depletion. Therefore, tyrosine and tryptophan supplementation is expected to benefit long-term outcomes in MSUD patients and possibly lessen or prevent learning disabilities.

In the current study, whole brain homogenates were used for biochemical analysis to correlate and compare changes in the cellular amino acid pool directly with effects on Krebs cycle function and neurotransmitter synthesis. Although, associations can be made from these studies about whole brain metabolism, these findings also provide targets for future studies in CSF, which can then be compared with human MSUD for possible translational value.

Despite careful management of MSUD patients, non-specific illness can trigger catabolic crisis leading to life threatening cerebral oedema, which can be extremely difficult to manage clinically (Morton et al., 2002). Norleucine may provide an acute strategy to control αKIC levels and prevent cerebral oedema during catabolic crisis. In the current study, norleucine attenuated brain leucine accumulation, but had a proportionally greater affect to control αKIC levels. These data suggest that norleucine may inhibit branched-chain transaminase in addition to competing with leucine for brain access. In astrocytes, branched-chain transaminase functions in the glutamate cycle by transferring amines from branched-chain amino acids to αKG to produce glutamate and the associated branched-chain ketoacid (Yudkoff et al., 2005) (Fig. 10). The opposite reaction takes place in neurons to complete the glutamate cycle. In the case of MSUD, αKIC accumulation in neurons may drive formation of leucine and αKG over glutamate. A high αKG/glutamate ratio can disrupt mitochondrial glutamate, aspartate, malate and αKG transport resulting in Krebs cycle failure and impaired glutamate cycling (Yudkoff et al., 2005). This hypothesis is consistent with our current findings including increased αKG, decreased glutamate and aspartate with lactate accumulation and ATP depletion with αKIC above 100 μM. Additionally, control of αKIC levels with norleucine treatment prevented these changes and strongly supports the hypothesis that αKIC disrupts Krebs cycle function and glutamate cycling (Yudkoff et al., 2005).

The current findings provide insight into the mechanism of brain injury and potential treatment strategy for MSUD. Both iMSUD and cMSUD mouse models were shown to be invaluable for the study of MSUD and may also be used to study cerebral glutamate and dopamine metabolism. Further studies in these mouse models can help define the role of tyrosine and tryptophan supplementation in MSUD. Use of atypical amino acids to limit toxic accumulation may provide effective treatments for other neurometabolic disorders.

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