Metab Brain Dis (2006) 21:1–10
DOI 10.1007/s11011-006-9006-5

ORIGINAL PAPER

Amino Acid Challenge in Patients with Cirrhosis and Control Subjects: Ammonia, Plasma Amino Acid and EEG Changes

Hanan Al Mardini · Andrew Douglass · Christopher Record

Received: 1 December 2004 / Accepted: 1 August 2005 / Published online: 4 May 2006

Abstract *Background/aims*: The pathogenesis of hepatic encephalopathy (HE) is controversial. We have therefore studied the effect of induced hyperammonaemia in man. *Patients and methods*: 108 g of an amino acid mixture was given orally to 18 cirrhotics and 11 control subjects and changes in blood ammonia, EEG and plasma amino acids were observed. *Results*: Basal (39 ± 6 versus 14 ± 2 μmol/l) and 120-min post amino acid (77 ± 10 versus 27 ± 4) blood ammonia concentrations in cirrhotics were significantly increased compared to healthy controls ($p < 0.001$). Associated with these changes there was a significant increase in the ratio of slow-to-fast wave activity indicating EEG slowing ($+0.41 \pm 0.16$; $N = 13$ versus -0.05 ± 0.08; $N = 8$; $p = 0.036$). As expected in cirrhotics, basal valine and leucine concentrations were decreased while phenylalanine, tyrosine and methionine were significantly increased. Although the basal molar ratio of branched chain amino acids to the aromatic amino acids phenylalanine and tyrosine was significantly decreased in cirrhotics (1.5 ± 0.2 versus 3.2 ± 0.2; $p < 0.0001$), after the challenge when EEG changes were apparent in cirrhotics, the ratio significantly increased ($p < 0.005$) in both groups to 2.7 ± 0.3 versus 4.1 ± 0.3 ($p = 0.002$). In the combined groups, there were significant correlations between EEG ratio change and the 120-min blood ammonia concentration ($r = 0.498$; $p = 0.022$). *Conclusion*: The alterations in plasma amino acid patterns do not support a specific role for any of the amino acid groups in the pathogenesis of hepatic encephalopathy. They are however more in keeping with the direct or indirect role of ammonia.

Keywords Amino acids · Ammonia · Electroencephalography · Hepatic encephalopathy

H. A. Mardini · A. Douglass · C. Record (✉)
Department of Medicine, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK
e-mail: chrisrecord@rvincl.fsnet.co.uk

C. Record
Present address: Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, NE1 4LP, UK

Introduction

The assessment of primary treatments for hepatic encephalopathy (HE) in humans is difficult because of the necessity to treat precipitating factors (Cordoba and Blei, 1997; Riodan and Williams, 1997). With the recognition that minimal (sub-clinical) encephalopathy can impair patients' quality of life (Groeneweg *et al*., 1998), there is an increasing necessity to explore therapeutic options. We have therefore attempted to develop a model of the condition where assessment of treatment of the encephalopathy can be dissociated from the treatment of precipitating factors. In a previous study, patients awaiting liver transplantation were challenged with 20 g of oral glutamine (Oppong *et al*., 1997) but the test was limited by the lack of any change in EEG frequency distribution. Nevertheless, the test was used to show beneficial effects of the therapeutic agents L-ornithine-L-aspartate (Rees *et al*., 2000) and lactitol (Masini *et al*., 1999). Upper gastrointestinal bleeding is a frequent precipitant of HE (Riodan and Williams, 1997) and we have shown that the mimicking of such an event by the ingestion of 54 g of an amino acid mixture of comparable composition to adult haemoglobin leads to the characteristic slowing of EEG frequency distribution seen in the primary condition (Douglass *et al*., 2001). Haemoglobin differs from other proteins in that it is deficient in the branched chain amino acid isoleucine and thus, the increase in blood amino acid concentrations after intestinal digestion of haemoglobin will be associated with a relative depletion of isoleucine. We hypothesised that this might influence the cerebral uptake of aromatic amino acids, and thus have important neurochemical consequences. The aim of this study was therefore to examine the effect of a 108 g isoleucine-deficient mixed amino acid challenge upon plasma amino acid concentrations in patients with cirrhosis and any associated changes in blood ammonia and EEG frequency distribution.

Methods

Patient selection

All patients were clinically stable and had cirrhosis identified by biopsy or on clinical and ultrasonographic grounds. None were on protein-restricted diets, were taking medication for the prevention of HE or were known to be abusing alcohol. None were taking sedative medication or were obviously wasted. Four patients had had a transjugular intrahepatic portasystemic shunt (TIPS) inserted for at least 12 months. Child Pugh scoring (Pugh *et al*., 1973) was performed on all patients. There were no adverse effects and all outpatients returned home in the afternoon after the test when peak abnormalities had shown an improvement. Controls with functional disorders and normal liver function were selected from gastroenterology outpatients. The Joint Ethics Committee of Newcastle and North Tyneside Health Authority, University of Newcastle and University of Northumbria at Newcastle approved the study and written informed consent was obtained from all patients and control subjects.

Analysed EEG recordings were undertaken with a cerebral function analyser monitor (CFAM), a microprocessor-based EEG machine that provides analysis of frequency distribution and amplitude trends of weighted EEG signals (Prior and Maynard, 1986). It is frequently used for EEG monitoring during surgery and intensive care. Silver chloride electrodes are attached with electrode gel to the vertex and parietal area on each side. The latter was chosen as it is relatively unaffected by scalp muscle activity. Data was stored on a data recorder and transferred to a PC for analysis. Ratios of slow and fast wave activity [calculated as $(\%\theta + \%\delta)/(\%\alpha + \%\beta)$ were chosen to express any detected changes, as these have been

Table 1 Composition (g) of the administered amino acid mixture

L-Glycine	8
L-Alanine	14
L-Serine	6
L-Threonine	6
L-Proline	6
L-Valine	12
L-Leucine	14
L-Phenylalanine	6
L-Tyrosine	2.5
L-Aspartic acid	6
L-Asparagine	4
L-Glutamic acid	4
L-Arginine	2.5
L-Histidine	8
L-Lysine	9
Total	108

noted to be sensitive to drowsiness and altered consciousness (Pugh *et al*., 1973). The values quoted (recorded at 30 min intervals) are the basal and 120-min ratio or the ratio at peak abnormality, i.e. the maximal change.

Amino acid mixture

One hundred and eight grams of an amino acid mixture (Table 1) deficient in the branched chain amino acid isoleucine, and thus of comparable composition to the adult haemoglobin present in approximately 800 ml blood was dispersed in approximately 100 ml of diluent (Keltrol A 1%) and flavoured. The mixture was made in the hospital pharmacy from commercially available constituents.

Amino acid challenge

These were performed in the morning after an overnight fast. Blood samples were taken from a venous cannula in an ice-cold EDTA tube for immediate measurement of whole blood ammonia (Ammonia Checker II, Kyoto Daiichi Kaguku Co. Ltd.) and plasma amino acids. Ten minutes after blood sampling, 5 min of CFAM data was recorded with eyes open, the latter to ensure that the subject was awake. The amino acid mixture was then administered as a bolus drink. CFAM data was recorded and blood ammonia checked every 30 min until the end of the experiment at 180 min. Plasma amino acid concentrations were repeated at 120 min. All subjects and patients rested throughout the test. The oral amino acid mixture was well tolerated and no overt neuropsychiatric changes became evident. No patients vomited the mixture.

Data analysis

All data was processed using SPSS statistics package. The results are presented as means $\pm$ SEM, and p-values quoted are all two-tailed. p-values were calculated using the non-parametric Mann–Whitney U-test and Wilcoxon signed rank tests while correlations were performed using the non-parametric Spearman coefficient.

Results

Eighteen patients (15 males) and 11 (9 males) controls were recruited for the study but amino acid values were only available in 16 of the cirrhotics. The mean age of the patients was 49 (25–70) years and 55 (31–70) years for controls. The aetiology of the cirrhosis was cryptogenic in two and alcohol in 16 patients, one of whom also had hepatitis C. Five patients were Child's grade B, seven Child's grade C while six were grade A.

Basal blood ammonia concentrations were significantly increased in cirrhotics (39 ± 6 versus 14 ± 2 μmol/l; $p < 0.001$) while after the amino acid challenge values rose significantly ($p < 0.004$) to 77 ± 10 versus 27 ± 4; $p < 0.001$ at 120 min. EEG data was available in 13 cirrhotics (five Child C) and eight controls. Basal EEG ratios were similar in the two groups (0.91 ± 0.14 versus 0.63 ± 09; NS) but after the challenge, the ratio significantly increased in cirrhotics to a peak value of 1.32 ± 0.28 versus 0.58 ± 0.07 in controls ($p < 0.004$) while the change in ratio between 0 and 120 min was significantly different in the two groups ($+0.41 \pm 0.16$ versus -0.05 ± 0.08; $p = 0.036$).

Amino acid changes

In cirrhotics, basal valine and leucine concentrations were decreased while phenylalanine, tyrosine, methionine, serine, asparagine, glycine, citrulline, histidine and arginine were significantly increased (Table 2; Fig. 1). The remaining amino acids were similar in the two groups, as was basal total amino acid nitrogen (3.18 ± 0.08 versus 2.86 ± 0.13 mmol/l). One hundred and twenty minutes after the challenge in cirrhotics phenylalanine, tyrosine and methionine continued to be increased compared to controls while glutamate and tryptophan were decreased (Table 2; Fig. 2). The concentrations of the amino acids (Table 2) increased after the challenge except for isoleucine which decreased from a basal value of 44 ± 3 to 6 ± 2 μmol/l in cirrhotics and 50 ± 4 to 11 ± 4 μmol/l in controls ($p < 0.00001$), methionine which decreased from 69 ± 20 to 56 ± 19 in cirrhotics and from 20 ± 2 to 13 ± 2 μmol/l in controls ($p < 0.0001$) and tryptophan which decreased in the patient group only ($p < 0.02$). None of these were present in the challenge mixture. Despite being absent from the challenge mixture, ornithine increased slightly but significantly from 80 ± 4 to 117 ± 6 and 69 ± 4 to 107 ± 7 μmol/l in cirrhotics and controls, respectively ($p < 0.0001$), presumably due to synthesis from arginine (Table 2). Total amino acid nitrogen increased in both groups to the same extent (8.12 ± 0.40 versus 7.89 ± 0.50 mmol/l). The basal molar ratio of branched chain amino acids to aromatic amino acids was significantly decreased in cirrhotics (1.5 ± 0.2 versus 3.1 ± 0.2; $p < 0.0001$) while after the challenge when EEG changes were apparent in cirrhotics the ratio significantly increased ($p < 0.005$) in both groups to 2.7 ± 0.3 versus 4.1 ± 0.3; $p = 0.002$ (Fig. 3). There were significant correlations between peak EEG abnormality ($n = 21$; $r = 0.496$; $p = 0.022$) and EEG ratio change ($n = 21$; $r = 0.498$; $p = 0.022$) with the 120-min blood ammonia concentration (Fig. 4) (but not the 120-min ammonia increment) in the combined group of patients and controls. In the smaller group of patients alone this correlation did not reach significance ($n = 13$; $r = 0.456$; $p = 0.117$). Despite the post-challenge fall in methionine concentration in the combined patient and control group, the EEG ratio change correlated with the 120-min methionine concentration ($n = 19$; $r = 0.544$; $p = 0.016$). There was also a highly significant correlation between 120-min methionine and 120-min tyrosine ($p < 0.001$) and phenylalanine ($p < 0.008$) concentrations. There was no correlation between EEG ratio change and 120-min phenylalanine ($n = 19$; $r = 0.047$; $p = 0.85$), tyrosine ($n = 19$; $r = 0.381$; $p = 0.107$) or ornithine ($n = 19$; $r = 0.353$; $p = 0.138$)

Table 2 Amino acid concentrations (μmol/l) in 16 patients with cirrhosis and 11 control subjects before and 120 min after a mixed amino acid challenge (mean ± SEM)

Amino acid	Time 0		p	Time 120		p
	Patients	Controls		Patients	Controls	
Taurine	66 ± 6	69 ± 4	NS	80 ± 7	78 ± 6	NS
Aspartate	8 ± 0.1	8 ± 0.1	NS	21 ± 2	27 ± 4	NS
Threonine	145 ± 11	121 ± 14	NS	508 ± 31	498 ± 56	NS
Serine	135 ± 8	100 ± 8	0.009	509 ± 33	454 ± 58	NS
Asparagine	57 ± 5	33 ± 5	0.002	205 ± 23	161 ± 17	NS
Glutamate	79 ± 26	110 ± 36	NS	99 ± 26	169 ± 27	0.012
Glutamine	693 ± 40	628 ± 42	NS	1059 ± 55	1004 ± 62	NS
Proline	224 ± 12	209 ± 15	NS	587 ± 34	606 ± 39	NS
Glycine	256 ± 17	205 ± 11	NS	914 ± 69	757 ± 81	NS
Alanine	357 ± 24	390 ± 29	NS	1175 ± 83	1139 ± 97	NS
Citrulline	44 ± 5	28 ± 3	0.023	82 ± 10	74 ± 11	NS
Valine	128 ± 10	175 ± 16	0.018	752 ± 63	928 ± 64	NS
Cystine	50 ± 3	42 ± 5	NS	54 ± 4	45 ± 6	NS
Methionine	69 ± 19	20 ± 2	0.001	56 ± 19	13 ± 2	0.002
Isoleucine	44 ± 3	50 ± 4	NS	6 ± 2	11 ± 3	NS
Leucine	83 ± 5	108 ± 11	0.023	386 ± 48	419 ± 41	NS
Tyrosine	126 ± 14	56 ± 7	0.001	201 ± 14	144 ± 15	0.012
Phenylalanine	90 ± 9	51 ± 4	0.001	266 ± 21	191 ± 15	0.018
Tryptophan	61 ± 5	49 ± 4	NS	54 ± 5	72 ± 38	0.048
Ornithine	80 ± 4	69 ± 4	NS	117 ± 6	106 ± 6	NS
Lysine	185 ± 8	182 ± 12	NS	460 ± 29	516 ± 35	NS
Histidine	94 ± 3	82 ± 3	0.026	348 ± 31	313 ± 31	NS
Arginine	106 ± 9	78 ± 11	0.034	184 ± 14	161 ± 17	NS

concentration or the molar ratio of branched chain to aromatic amino acids ($n = 19$; $r = 0.339$; $p = 0.156$).

Discussion

The aetiology of HE is unknown but for many years ammonia has featured strongly as a candidate neurotoxin. Several studies (Amodio *et al.*, 1998; Groeneweg *et al.*, 1998; Oppong *et al.*, 1997; Quero *et al.*, 1996) have demonstrated that increased basal ammonia levels in stable cirrhotics are associated with abnormal daily functioning, neuropyschomotor performances and simple reaction times. In acute liver failure however, high ammonia concentrations have been shown to be associated with the development of cerebral oedema (Clemmesen *et al.*, 1999), while recently arterial, venous and ammonia partial pressure have all been shown to correlate with the severity of hepatic encephalopathy (Nicolao *et al.*, 2003; Ong *et al.*, 2003). Ammonia levels are a reflection of its production, and its disposal. Traditionally the principal sources of ammonia are thought to be from protein oxidation, glutaminases in the small intestine and urea-splitting bacteria in the colon and, while this may be so during post-absorptive conditions, it has recently been shown that renal ammoniagenesis is at least as important as intestinal production after simulated and actual gastrointestinal bleeding (Olde Damink *et al.*, 2003). The main disposal pathways are via the liver (through the urea and glutamine cycles) and through altered renal and muscle

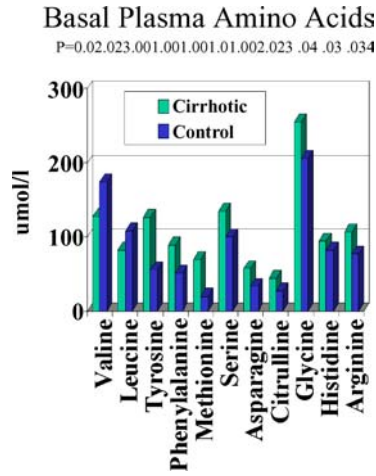

Fig. 1 Basal plasma amino acids in cirrhotics (16) and control subjects (11)

metabolism and renal excretion (Deutz *et al*., 1996; Lockwood *et al*., 1979; Olde Damink *et al*., 2002, 2003; Oppong *et al*., 1997;) and it has now been shown that volume expansion can decrease plasma ammonia probably by increased renal excretion and decreased renal ammoniagenesis (Jalan and Kapoor, 2003). Portal and superior mesenteric concentrations of ammonia are higher than systemic (Olde Damink *et al*., 2002, 2003) and after oral glutamine challenge in cirrhotics, the increase in portal ammonia was three-fold greater than in venous blood (Rees *et al*., 2000). Thus, advanced liver disease, with increased porto-systemic shunting and decreased hepatic reserve may be expected to modulate ammonia levels.

After the amino acid challenge, blood ammonia levels rose significantly, which is consistent with the changes demonstrated after large oral protein loads and upper gastrointestinal haemorrhage (Kromhout *et al*., 1980; Olde Damink *et al*., 1997, 2003). Unlike upper gastrointestinal bleeding, the amino acid challenge, while of similar composition to haemoglobin,

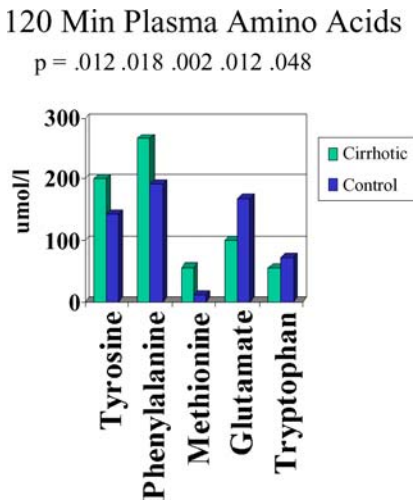

Fig. 2 Plasma amino acid concentrations in cirrhotics (16) and control subjects (11) 120 min after a 108 g mixed amino acid challenge

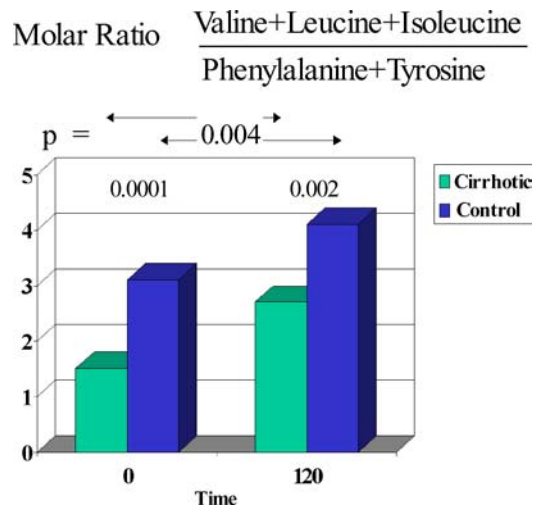

Fig. 3 Molar ratio of valine + leucine + isoleucine/ phenylalanine + tyrosine in cirrhotics (16) and controls (11) before and 120 min after a 108 g mixed amino acid challenge

did not precipitate episodic HE. A considerably larger dose of amino acid equivalent to a greater volume of blood may be required for clinically apparent HE to develop. Nevertheless, EEG ratios increased following the challenge consistent with the development of minimal (subclinical) encephalopathy. Amino acid concentrations in the blood of patients with HE are important because plasma concentration can influence uptake across the blood–brain barrier where some can act either as neurotransmitters or their precursors. Because of the evidence for the importance of 5-hydroxytryptamine in HE (Al Mardini *et al*., 1993), in a previous study we administered the precursor amino acid tryptophan as a single agent to patients with cirrhosis (Douglass *et al*., 2003). Despite a 10-fold elevation in plasma concentration, no psychometric or electroencephalographic abnormalities were evident. Tryptophan is only a minor constituent of haemoglobin and for this study it was therefore omitted from the amino acid mixture used.

It has been known since the 1970s that there is an elevation in aromatic and a decrease in branched chain amino acids in patients with cirrhosis. This has been the rationale for therapy with branched chain amino acid solutions. The molar ratio of branch chain to aromatic groups however correlated with the severity of liver disease rather than HE (Morgan *et al*., 1978). The present study does not provide any support for the roles of either the branched chain or aromatic groups among neurochemical mechanisms (Jalan *et al*., 1996; Record, 1991) recognised in the pathogenesis of HE. On the contrary the correlation between ammonia and EEG abnormalities is more in keeping with a direct or indirect effect of this agent. In the rat brain, ammonia is typically two-fold higher than in blood and after porto-caval shunt, this difference is increased (Cooper, 1990). In patients with severe liver disease and minimal HE, there is an increase in cerebral extraction of ammonia (Lockwood *et al*., 1991). It is now known that ammonia modulates glutamatergic excitation (Hermenegildo *et al*., 2000) and GABA-ergic and benzodiazepine-induced neuroinhibition (Basile and Jones, 1997) while disturbed glutamatergic transmission (Butterworth, 1997; Oppong *et al*., 1995) in both acute and chronic liver failure is likely to be mediated by ammonia. Other potential explanations include decreased regional cerebral perfusion as recently demonstrated following similar challenges (Jalan *et al*., 2003) and altered amino acid uptake across the blood–brain barrier (Rao *et al*., 1995). It has recently been suggested that cerebral oedema is a unifying cause of HE in both acute and chronic liver disease (Mullen, 2003) and Balata *et al*. (2003) have

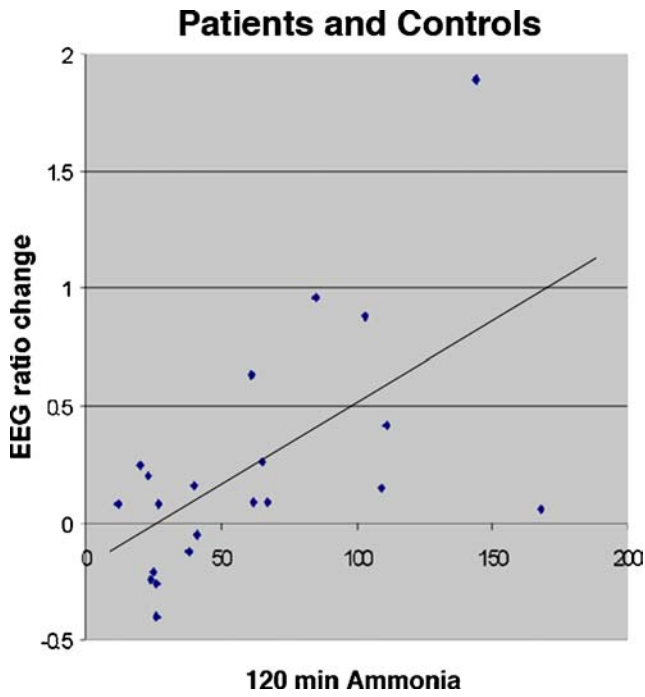

Fig. 4 Correlation between the change in EEG ratio and blood ammonia concentration 120 min after a mixed amino acid challenge in cirrhotics (13) and control subjects (8) (EEG change = 0.01 × ammonia − 0.19; $r = 0.498$; $p = 0.022$)

shown alterations in magnetisation transfer in cirrhotics during hyperammonaemia after mixed amino acid challenge in support of this hypothesis. Others however have suggested that glutamine challenge leading to greater elevation in blood ammonia than induced with mixed amino acids does not cause psychometric abnormalities and other factors may be pathogenetic accomplices (Riggio *et al*., 2003). Our previous studies (Al Mardini *et al*., 1988) and the correlation in the present study between 120-min methionine concentration and EEG ratio change in the combined patient and control groups supports this suggestion.

It has been postulated that haemoglobin may be an important precipitant of hyperammonaemia, and thus HE because it lacks the branched chain amino acid isoleucine (Olde Damink *et al*., 1999). An alternative explanation is that haemoglobin lacks ornithine an important constituent of the urea cycle (Meijer *et al*., 1990). The importance of ornithine for control of urea cycle activity is controversial, as the activity of the enzyme carbamoyl-phosphate synthase is rate limiting (Meijer *et al*., 1990). This enzyme is principally activated by *N*-acetylglutamate but ornithine may also be important (Meijer *et al*., 1990). Thus, intravenous amino acid mixtures lacking arginine (an ornithine precursor) are associated with hyperammonaemia and encephalopathy, an effect which can be prevented by simultaneous administration of arginine (Fahey, 1957). In the rat, ornithine and arginine ameliorate hyperammonaemia and encephalopathy due to intraperitoneal ammonium chloride and decrease orotic acid excretion after repeated doses of subcutaneous glycine (Zieve *et al*., 1986). After protein and glutamine challenge, L-ornithine L-aspartate decreases blood ammonia concentrations compared to placebo (Rees *et al*., 2000; Staedt *et al*., 1993). The amino acid mixture used in the present study also lacked ornithine and contained only 1.25 g of arginine. Whether further

orthinine or arginine supplementation will ameliorate the changes observed in the present model requires investigation. In conclusion, we suggest that the neurochemical consequences of an isoleucine-deficient mixed amino acid load are mediated via the cerebral effects of ammonia rather than through any alteration in cerebral uptake of any of the aromatic amino acids administered.

Acknowledgments This research was supported by a grant from the late Dame Catherine Cookson. Mr. J. Gilroy produced the amino acid mixture, Dr. S.T. Kometa and Mr. A. Davidson assisted with the statistical and EEG analyses, respectively.

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