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A Quantitative Evaluation of the Permeability of the Blood Brain Barrier of Portacaval Shunted Rats

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The integrity of the blood-brain barrier (BBB) was measured in male Sprague Dawley rats subjected to 16 weeks of portacaval shunting (PCS), the optimal time required for the cerebral changes to develop, by using an *in situ* brain perfusion technique. The penetration of a vascular space marker ^{14}C mannitol, and labelled amino acids ^{3}H-phenylalanine or ^{3}H-glutamate were measured in brain and cerebrospinal fluid (CSF) using an *in situ* brain perfusion technique, over 2 or 20 minutes. The patency of the surgical shunt was confirmed by measurement of significantly increased plasma ammonia (131.5 ± 14.8 $\mu mol.l^{-1}$) and AST (159.5 ± 19.9 $IU.l^{-1}$) concentrations compared to controls 39.9 ± 3.7*, and 82.5 ± 6.6* respectively.

Brain and CSF ^{14}C-mannitol space ($ml.100g^{-1}$), was not increased by PCS where brain space was 1.31 ± 0.27 mL. $100g^{-1}$ compared to control 1.19 ± 0.49 mL. $100g^{-1}$, and CSF was 0.14 ± 0.06 mL. $100g^{-1}$ compared to control 0.15±0.05 (PCS n=10, control n=8). The uptake for ^{3}H-glutamate, which is required for cerebral ammonia detoxification, was also unchanged in both brain and CSF. However, brain uptake of ^{3}H-phenylalanine was significantly reduced from 871 ± 80 $\mu L.min^{-1}.g^{-1}$ to 356 ± 154* $\mu l.min^{-1}.g^{-1}$ (n=4), although there was no change in CSF uptake. These data suggest that there is no generalized breakdown of the blood-brain or blood-CSF barriers during PCS as assessed by mannitol penetration. The reduction in phenylalanine uptake into the brain may help stabilize high cerebral aromatic amino acid levels. *P<0.05, Two-tailed, Student's unpaired t-test.

Key words: Blood-brain barrier, portal-systemic encephalopathy, portacaval anastomosis, amino acids.

INTRODUCTION

It is well established that during portal-systemic encephalopathy (PSE) portal blood enters into the systemic circulation essentially unprocessed and intoxicates the brain. The pathogenesis of the syndrome is still unclear although it has been suggested that "generalized increases" in blood-brain barrier permeability may be responsible for its development. This hypothesis was based upon histological observations of horseradish peroxidase penetration (Laursen and Westergaard, 1977) in the brains of portacaval shunted

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(PCS) rats, a recognized experimental model of PSE (Bergqvist *et al.*, 1997; Cavanagh and Kyu, 1971) although other animal studies have suggested this is not the case (Alexander *et al.*, 1999; Sarna *et al.*,1977; Lo *et al.*, 1987).

The pathogenesis of PSE has also been related to the high plasma and brain levels of ammonia and aromatic amino acids found in PSE (Fischer, 1980; Fischer, 1982; Bugge *et al.*, 1989; Butterworth, 1992). It has been difficult to establish a direct correlation between elevated plasma ammonia concentrations and the degree of PSE (Fischer 1982; Pappas *et al.*, 1982) and attempts to restore consciousness in patients by removing ammonia from plasma using charcoal haemoperfusion have proved ineffective. Many more studies have suggested that selective alterations in the metabolism or turnover of GABA (Maddison *et al.*, 1996; Pomier-Layrargues *et al.*, 1994), catecholamines (Fischer, 1980), 5-hydroxytryptamine (Alexander *et al.*, 1998a; Bergqvist *et al.*, 1996), glutamic acid (Butterworth *et al.*, 1991) and, in particular, aromatic amino acids such as phenylalanine underlie PSE. Although possible mechanisms for such alterations remain largely obscure (Jalan and Hayes, 1997; Riordan and Williams, 1997) it has been suggested that they are a selective effect of compromised liver function, not just systemic diversion of unprocessed portal blood with high amino acid content (Alexander *et al.*, 1998a, Alexander *et al.*, 1999), both of which are common in chronic liver failure (Benjamin *et al.*, 1984).

Uptake of amino acids into brain requires specific transport systems at the BBB, and some workers have found increased activity of the large neutral amino acid carrier (LNAA) (James *et al.*, 1978; Mans *et al.*, 1982), though not all studies agree (Knudsen *et al.*, 1993). Assessment of BBB transport of fast-penetrating molecules such as phenylalanine which uses the LNAA carrier, is complicated in many studies by inability to separate uptake at the BBB from that at the blood-cerebrospinal fluid barrier, and correct for decreased blood flow and haematocrit in PSE models.

The purpose of the present study was to measure the general permeability of the BBB in control and 16 week PCS rats, which are known to display the characteristic cerebral changes associated PSE (Alexander *et al.*, 1991) using a vascular marker ^{14}C mannitol and ^{3}H phenylalanine during *in situ* brain perfusion (Preston and Segal, 1993; Preston *et al.*, 1995). The uptake of ^{3}H-glutamic acid via the separate acidic amino acid transporter X^{-} was also measured since this amino acid is also involved in ammonia detoxification in the brain.

MATERIALS and METHODS

18 male Sprague-Dawley rats (150-200g) were anaesthetized with halothane and were subjected to either control (20 min clamping of the portal vein) or PCS surgical procedures according to our previously described techniques (Alexander *et al.*, 1991; Alexander *et al.*, 1998a). The rats were allowed to recover and fed *ad libitum* for 16 weeks, the optimal time required for the cerebral changes to develop in PCS rats. At 16 weeks, rats were anaesthetized with fentanyl/fluanisone (0.3ml kg-1) and midazolam (2-mg kg-1) I.P. and

following laparotomy, the anastomoses were examined visually and by palpation and found to be patent in every test rat. In addition, blood samples were withdrawn from the inferior vena cava for evaluation of liver function tests. The abdomen was then closed for preparation for a modified *in situ* brain perfusion technique with a Ringer/Albumin plasma substitute which was performed in both groups of rats (Preston and Segal 1990; Preston *et al.*, 1995). All procedures were conducted according to the Animals (Scientific Procedures) Act 1986 and European Committee Council Directive, 1986.

Perfusion technique

In brief, the *in situ* perfusion technique consisted of exposure and cannulation of both carotid arteries, and jugular veins with PVC tubing following heparinisation (100 $U.g^{-1}$). The brain was perfused via both common carotid arteries. During cannulation of each artery, perfusion was always maintained in the contralateral carotid artery, and the artery being cannulated was ligated for less than 30s before perfusion was re-established. The jugular veins were cannulated for outflow of perfusion fluid. The heart was then ligated to prevent systemic perfusion of brain via the vertebral arteries. The perfusion fluid consisted of protein supplemented Ringer solution which contained in $mmol.l^{-1}$: NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2, glucose 10mM and bovine serum albumin 40 $g.l^{-1}$ to maintain colloid osmotic pressure (all from Sigma Chemical Co., UK) and was delivered using a Watson-Marlow peristaltic pump. The Ringer was gassed with 95% O_2 / 5% CO_2 to obtain a pH of 7.4 and pO_2 in excess of 650 mmHg at 37°C by placing the reservoir in a warm water bath and equipment and rat on a heated mat. Perfusion was carried out for 25 min in all rats. Either ^{3}H-phenylalanine or ^{3}H-glutamate (Dupont New England Nuclear Corp. UK) were introduced into each carotid cannula for the final 2 min or 20 min respectively of perfusion time, through a side arm in the circuit connected to a Harvard slow-drive syringe pump (Type 22). ^{14}C-mannitol was present throughout perfusion with the amino acids. The final Ringer concentrations were 0.2 $\mu Ci.ml^{-1}$ (30 μM) ^{14}C-mannitol, 0.05 $\mu Ci.ml^{-1}$ (0.1 μl M) ^{3}H-glutamate, or 0.05 $\mu Ci.ml^{-1}$ (0.1 μM) ^{3}H-phenylalanine. At the end of the perfusion time a CSF sample (50-120μl) was collected by puncture of the cisterna magna, the head removed and brain areas isolated (30-90 mg).

In a separate series of experiments, control rats were perfused for 25 min with Ringer containing 0.25mM NH_4 acetate to simulate elevated plasma ammonia without liver dysfunction or CNS amino acid disturbance. ^{3}H-Phenylalanine and ^{14}C-mannitol were added to this perfusate for the final 2 min as described above, and samples collected in the same way after 2 min perfusion.

Tissue solubiliser, 0.5ml (Soluene-350 Packard, UK) was added to all samples and left overnight. The activities of ^{14}C and ^{3}H in CSF, brain and perfusate were measured using a liquid scintillation ß-counter (Rackbeta, Spectral 1219) following the addition of 3.5 ml scintillant (Uniscint BD, National Diagnostics, UK) to each sample.

Calculations

The brain and CSF ^{14}C-mannitol space, and uptake of ^{3}H-phenylalanine or glutamate relative to ^{14}C-mannitol were calculated from; mannitol space (ml.100g^{-1}) = ($^{14}C.C_{tissue}/^{14}C.C_{pf}$) x100 and amino acid uptake (ml.min^{-1}.g^{-1}) = (^{3}H.C $_{tissue}$/ ^{3}H.C_{pf}) – (^{14}C.C $_{tissue}$/^{14} C.C_{pf}) ÷ t where C_{tissue} is isotope activity in CSF or brain (dpm.g^{-1}); C_{pf} is isotope activity in perfusion fluid (dpm.ml^{-1}); t is time in minutes (modified from Al-Sarraf *et al.*, 1997). Statistical analyses were carried out using Student's unpaired, two-tailed t-test, and one-way ANOVAR, where $P<0.05$ was taken as being a significant difference between groups. All values quoted are mean ± standard error.

Limitations of the perfusion technique

This technique was used since it allows complete control of the flow rate and composition of the cerebral perfusion fluid, and can be extended to time intervals necessary for accurately measuring small changes in mannitol space and BBB penetration of slowly transported markers (Preston *et al.*, 1995). It is not, however, appropriate for short time-course experiments lasting seconds since initial perfusion of all cerebral capillaries to a constant amino acid level cannot be guaranteed during that time. The shortest time-course in this study was 2-minute perfusion with labelled phenylalanine. Because phenylalanine uptake and efflux is fast (Knudsen *et al.*, 1993; del Piño *et al.*, 1995) only the net uptake was calculated, which is the sum of uptake and efflux. Glutamate uptake however, is much slower and at 20 minutes perfusion, uptake remains unidirectional with no detectable backflux (Al-Sarraf *et al.*, 1997).

RESULTS

Liver function tests performed at 16 weeks post PCS are shown in Table 1. All tests, with the exception of alkaline phosphatase, were significantly different to control values indicating that a significant degree of reduced liver function/ chronic liver failure had been induced, and that the surgical shunt was patent. Patency of the shunt was also confirmed by palpation.

In situ brain perfusion

All rats were perfused with erythrocyte-free albumin Ringer as described, with only trace levels of phenylalanine or glutamate. The perfusion flow rate was maintained at 2.10 ± 0.06ml.min^{-1}.g^{-1} brain tissue (n=17) for both control and PCS rats. This is higher than the normal cerebral flow rate in rats (Alexander *et al.*, 1992; Alexander *et al.*, 1999; Preston *et al.*, 1995) since erythrocyte-free perfusate has a low viscosity and therefore higher-than-physiological flow rates are required to maintain normal physiological pressures in fixed-flow preparations (Alexander *et al.*, 1998b), which in the present study were approximately 95mmHg.

Brain and CSF ^{14}C-mannitol space

The brain and CSF mannitol spaces were not significantly different in control and PCS rats (Table 2). The small brain space for mannitol is consistent with the distribution of the marker in the vascular space during these perfusion times (Preston *et al.*, 1995; Ohno *et al.*, 1978). The distribution of mannitol in the CSF represents movement of the marker across the blood-CSF barrier and indicates the degree of 'leak' associated with this barrier, which is

distinct from the BBB and not as tight. Nonetheless, PCS did not affect distribution here, suggesting that the general integrity of both barriers is retained in PCS rats.

Brain and CSF ^{3}H-phenylalanine uptake

^{3}H-phenylalanine uptake was significantly reduced after PCS (Figure 1) by 59% in the brain as a whole. Some regional variation was seen in brain uptake, with the caudate putamen showing the greatest reduction. The average brain uptake was, 871± 80µl.min^{-1}.g^{-1} in controls and 356 ± 154µl.min^{-1}.g^{-1} in PCS rats. However, CSF uptake was only 8-12% of brain uptake, and did not show a significant reduction in PCS rats.

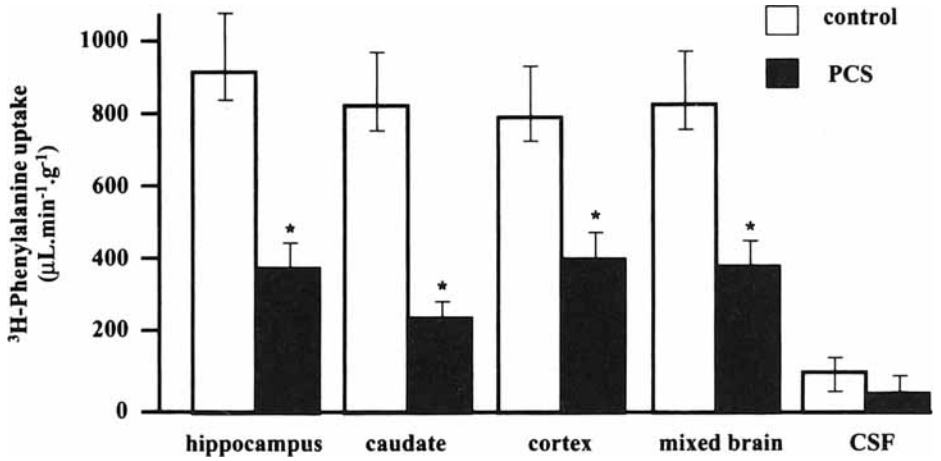

Figure 1. CSF and regional brain ^{3}H-phenylalanine uptake after 2 minutes *in situ* brain perfusion. Values are mean ± SEM for PCS rats (n=4) and controls (n=4). *p<0.05, PCS versus control, calculated by one-way ANOVA.

Brain and CSF ^{3}H-glutamate uptake

The uptake of ^{3}H-glutamate into brain was much less than that for phenylalanine, but was still resolvable from the mannitol space; glutamate space before correction was 5.7±0.9 and 5.2±1.1 ml.100g^{-1} in controls and PCS rats respectively. Neither corrected brain, nor CSF uptake was altered by portacaval shunting, in contrast to phenylalanine (Figure 2).

Effect of NH_4 acetate on ^{3}H-phenylalanine uptake

Since elevated plasma and brain ammonium has been implicated in the changes to amino acid transport at the BBB during PCS and PSE, a separate group of un-operated rats were perfused with 0.25mM NH_4 acetate as described. Neither brain nor CSF uptake of ^{3}H-phenylalanine changed significantly during NH_4 acetate perfusion (Figure 3). Comparison of ^{3}H-phenylalanine uptake after sham-operation (Figure 1) and the non-operated group (Figure 3) indicates that the operation itself had no effect on BBB parameters measured.

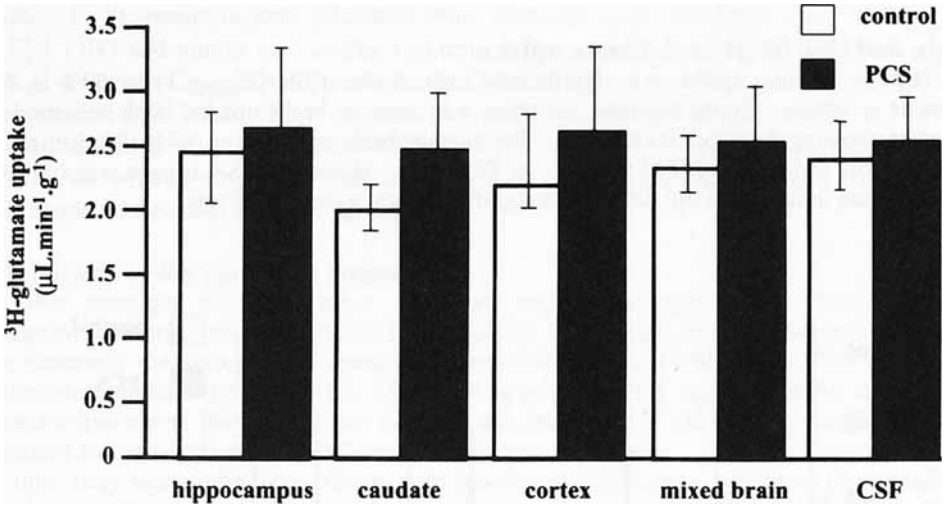

Figure 2. CSF and brain regional ^{3}H-glutamate uptake after 20 minutes *in situ* brain perfusion. PCS is portacaval shunted rats (n=6) control is sham operated rats (n=4). Values are mean ± SEM.

DISCUSSION

The status of the BBB was assessed using *in situ* brain perfusion of ^{14}C-mannitol, and the amino acids ^{3}H-glutamate or ^{3}H-phenylalanine in control and PCS rats exhibiting the cerebral changes associated with PSE (Alexander *et al.*, 1991). Mannitol was chosen to assess generalized structural or permeability changes to the BBB, glutamate to assess the X$^-$ acidic amino acid carrier system and also because this amino acid is required for ammonia detoxification in brain, and phenylalanine to assess the LNAA carrier system L1, which previous studies suggest is upregulated during PSE when plasma and brain phenylalanine levels are elevated (James *et al.*, 1978; Jonung *et al.*, 1985).

The results suggest that there are no generalized increases in BBB or blood-CSF barrier permeability, during experimental PSE based on ^{14}C-mannitol distribution in rats. Mannitol is often used as a permeability probe and vascular space marker since it is not bound to plasma protein, metabolized or transported, and during the time course of these experiments remains within the vascular space (Preston *et al.*, 1995). The mannitol space calculated here is consistent with previous *in vivo* and *in situ* studies (Sisson and Oldendorf 1971; Ohno *et al.*, 1978; Preston *et al.*, 1995) for an intact BBB. The findings of an intact BBB are in contrast to previous studies (Laursen and Westergaard, 1977) which indicated generalized increases in BBB permeability associated with considerable structural, Alzheimer Type II, alterations in astrocytes (Cavanagh and Kyu 1971). The BBB is formed by tight-junctions between cerebral capillary endothelial cells and it has been suggested that

astrocytes contribute to the formation of the tight junctions (Kemelberg and Norenberg 1989). However it is unlikely that these tight junctions are compromised during chronic liver failure. Nonetheless, astrocyte degeneration might be expected to influence CNS homeostasis of some amino acids, especially glutamate, which is taken up by astrocytes and converted to glutamine during ammonia detoxification. However, we saw no change in glutamate uptake during PCS in rats, when brain ammonia levels could reasonably be expected to be high, and labelled glutamate uptake in brain and CSF in control and PCS rats was similar to that previously measured (Al-Sarraf *et al.*, 1995). This phenomenon may not therefore be quantitatively important for amino acid uptake from blood.

A possible explanation for the absence of generalized increases in BBB permeability after 16-week PCS in rats is that the severity of liver failure was insufficient to elicit changes (Alexander *et al.*, 1999). This may be due to regeneration of hepatic portal collateral vessels during the post-operative period to re-establish hepatic portal flow (Groszmann, 1994; Li *et al.*, 1998). This is unlikely, however, because liver function tests (Table 1) suggest that a substantial degree of compromised liver function had been induced compared to control rats which was confirmed by the reduced liver-to-body weight ratios in PCS rats.

Table 1. Liver function tests. Controls are sham operated rats (n=8), PCS are portacaval shunted rats after 16 weeks (n=10). Values are mean ± SEM. P estimated by unpaired Student t-test.

	Control	PCS	P
Liver weight (g)	19.8± 0.7	12.3± 0.9	<0.01
Liver: Body weight ratio	3.5± 0.1	2.5± 0.2	<0.01
Plasma analysis:			
ammonia ($\mu mol.l^{-1}$)	39.9± 3.7	131.5±14.8	<0.001
albumin ($g.l^{-1}$)	42.9± 1.8	36.2± 1.4	<0.01
alkaline phosphatase ($IU.l^{-1}$)	111.9±16.3	89.3±11.8	>0.05
AST ($IU.l^{-1}$)	82.5± 6.6	159.5±19.9	<0.01

The most significant results observed were those for ^{3}H-phenylalanine uptake following PCS when uptake into brain was reduced by half. This is in contrast to some other studies where high phenylalanine uptake into brain has been measured and interpreted as specific upregulation of the LNAA carrier (James *et al.*, 1978; Jonung *et al.*, 1985). However, in the past, studies have been complicated by several factors including altered blood flow, endogenous amino acids and the presence of red cells. PSE is associated with reduced cerebral blood flow (Knudsen *et al.*, 1993) which would result in prolonged blood transit time for phenylalanine, and apparently greater uptake when measurements are based on removal of phenylalanine per unit volume of blood. PSE is also characterized by reduced haematocrit and since red cells can sequester the labelled amino acid being studied, more label may be available during PSE for transport thus giving the appearance of elevated

uptake. During the present study, red cells were excluded in both control and PCS groups, and the flow rate was the same for both groups.

Table 2. ^{14}C-mannitol space (ml.100g^{-1}) after 25 minutes *in situ* perfusion. Control n=8, PCS n=10. Values are mean ± SEM; p estimated using 1-way ANOVA.

	Control	PCS	P
Brain Regions			
Hippocampus	1.34±0.56	1.28±0.25	>0.05
Caudate putamen	0.85±0.39	1.06±0.22	>0.05
Cortex	1.62±0.76	1.58±0.38	>0.05
Mixed brain	1.19±0.49	1.31±0.27	>0.05
Cerebrospinal fluid	0.15±0.05	0.14±0.06	>0.05

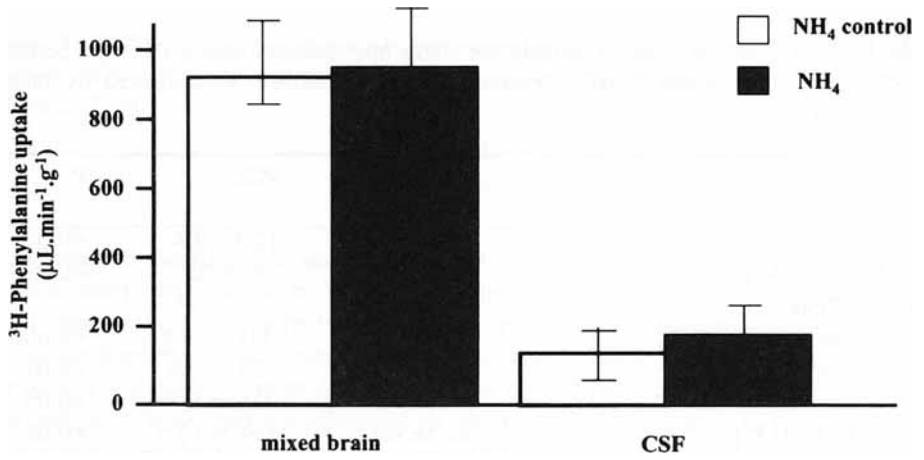

Figure 3. CSF and brain ^{3}H-phenylalanine uptake in the presence (NH_4) or absence (NH_4 control) of 0.2mM ammonium acetate. Values are mean ± SEM for 3 rats in each group.

Previous workers may also have underestimated the importance of rapid removal of labelled amino acid from the brain, in the form of efflux from the brain into blood. As has been pointed out by Knudsen *et al.*, (1993) efflux may fall by as much as 70% during PSE as the brain levels of amino acids increase and compete for efflux. This may be interpreted as increased uptake into brain, when it is more likely due to decreased removal from the brain. This effect can be explained by the distribution of two different types of carrier for phenylalanine at the BBB. The facilitated diffusion transporter, L1, is evenly distributed on

both blood and brain sides of the BBB and is responsible for the net movement of phenylalanine from blood-to-brain, down its concentration gradient (Gjedde, 1988). The Na-dependent B^{0+} transporter, is present only on the brain side of the BBB and can transport neutral amino acids against a concentration gradient from brain to blood (del Piño *et al.*, 1995). Saturation of this carrier is most likely to account for reduced efflux of neutral amino acids in PSE since the Ki for phenylalanine, which is 140μM in rats, (del Piño *et al.*, 1995) is mid-way between normal brain levels of 45μM in rats (Gjedde, 1988) and elevated levels seen in PSE of 224μM, in humans (Knudsen *et al.*, 1993). B^{0+} also interacts with glutamine and elevated brain glutamine, as a consequence of elevated ammonia concentrations in PCS, would add to the saturation and further reduce phenylalanine efflux.

During the present study, plasma phenylalanine was only at the tracer level, so there was no competition from endogenous plasma amino acids. In addition, the perfusate phenylalanine levels were similar for both control and PCS rats because a common perfusate was used. Under these conditions, the concentration gradient for blood-to-brain transport was lower in PCS rats, since the high brain concentrations in PSE would oppose rapid blood-to-brain phenylalanine movement. The high brain levels of phenylalanine in PCS rats previously reported *in vivo* may have been simply a result of the high plasma concentration and blood to brain gradient but need not be due to a specific increase in the activity of the L1 carrier system (James *et al.*, 1978; Jonung *et al.*, 1985).

At the blood-CSF barrier, only the L1 transporter has been identified (Preston and Segal 1990). CSF uptake in the present study was very small compared to brain uptake suggesting that the blood-CSF barrier acts more effectively than the BBB to prevent phenylalanine influx to the CNS. The high aromatic amino acid levels found in CSF during PCS and PSE may therefore be caused by phenylalanine moving from brain to CSF, rather than by direct blood-to-CSF uptake, and thus the CSF could become a 'sink' for brain amino acids helping to stabilize levels.

These data, coupled with unchanged mannitol space and unchanged glutamate transport suggest that PCS does not result in changes to BBB transport systems, but rather that elevated brain levels of aromatic amino acids in particular, are a consequence of normal activity of the NAA transporters and polarization at the luminal and abluminal faces of the BBB, coupled with abnormally high plasma levels of aromatic amino acids. One would not need to propose a more complicated system such as that of James *et al.*, (1978), of excess glutamine efflux from brain in exchange for aromatic amino acid uptake, and indeed no evidence exists for such a transporter, though glutamine transport at the blood-brain barrier has been well characterized (Lee *et al.*, 1998; Lee *et al.*, 1996; del Piño *et al.*, 1995).

The results of this paper, and other recent research, notably that by Knudsen *et al.*, (1993), and modern *in vitro* studies, allow us to speculate on ways to reduce brain aromatic amino acid levels. Possibilities may centre on, reducing plasma aromatic amino acids to prevent initial brain uptake, blocking the L1 carrier to prevent aromatic amino acid uptake into brain and CSF, increasing flow and turnover of CSF to maintain the 'sink' for brain amino acids, reducing plasma phenylalanine to prevent entry into the brain and

monopolization of the B^{0+} transporter, and, finally, stimulating the B^{0+} transporter to increase efflux from brain as has been achieved *in vitro* (Lee *et al.*, 1996).

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