Journal of Neurochemistry
Lippincott–Raven Publishers, Philadelphia

Glutamine Uptake at the Blood–Brain Barrier Is Mediated by N-System Transport

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Abstract: The mechanism of unidirectional transport of glutamine from blood to brain in pentobarbital-anesthetized rats was examined using in situ perfusion. Amino acid uptake into brain across the blood–brain barrier (BBB) is classically thought to be via the Na-independent large neutral (L-system), acidic and basic amino acid transporters. In the presence of physiological concentrations of amino acids in the perfusate, which should saturate the known amino acid transporters at the BBB, the cortical transfer constant (K_i) for L-[^{14}C]glutamine was 11.6 ± 1.1 μl/g/min. The addition of either 10 m*M* 2-amino-2-norbornanecarboxylic acid or 10 m*M* 2-amino-2-norbornanecarboxylic acid and 5 m*M* cysteine had no effect on the cortical K_i for L-[^{14}C]glutamine, indicating that glutamine transport under these conditions does not occur by the L-, A-, or ASC-systems. Decreasing perfusate Na from 140 to 2.4 m*M* by Tris substitution reduced the cortical K_i for L-[^{14}C]glutamine by 62% ($p \leq 0.001$). The Na-dependent uptake has the characteristics of N-system transport. It was inhibited by L-histidine and L-glutamine, both N-system substrates, and it was pH sensitive and moderately tolerant of Li substitution for Na. This putative N-system transporter at the luminal membrane of the BBB plays an important role in mediating brain glutamine uptake. **Key Words:** Blood–brain barrier—Transport—Glutamine—N-system—In situ perfusion.
J. Neurochem. **71,** 2565–2573 (1998).

Despite the fact that glutamine is the most abundant amino acid in plasma and CSF and is found at high concentrations in the brain as a whole, its transport at the blood–brain and blood–CSF barriers has not been fully clarified. Studying isolated rat choroid plexus, we have found that glutamine uptake is mediated by two systems, the Na-independent L-system amino acid transporter and a Na-dependent system that also transports histidine (Keep and Xiang, 1995; Xiang et al., 1998). The latter resembles the N-system transporter found in liver (Kilberg et al., 1993) and it has not previously been described at the blood–brain barrier (BBB). This system may be of particular importance in controlling the levels of CSF and brain glutamine and histidine, as L-system uptake is subject to competition by many other neutral amino acids (Pardridge, 1986; Smith et al., 1987).

At the cerebral endothelium, the site of the BBB, much of the initial evidence on mechanisms of amino acid transport at the luminal membrane came from the pioneering experiments of Oldendorf and Szabo (1976), who demonstrated uptake of amino acids into brain via three systems, the large neutral amino acid carrier (the leucine-preferring L-system), a basic amino acid carrier, and an acidic amino acid carrier. The latter acts as a transporter of glutamate but is of low capacity. There is clear evidence that the transport of amino acids at the luminal membrane of the brain capillary endothelial cell is not through the A- (alanine-preferring; Na-dependent) system (Ennis et al., 1994; Benrabh and Lefauconnier, 1995). Conflicting evidence exists for amino acid transport through the ASC-system (alanine–serine–cysteine preferring; Na-dependent) at the luminal membrane (Sershen and Lajtha, 1979; Wade and Brady, 1981; Tovar et al., 1988).

This study examines whether the N-system is present in the luminal membrane of the BBB and characterizes some of its basic properties, using in situ perfusion. In particular, we examined the following questions: Is glutamine transport at the BBB Na-dependent; is it tolerant of Li substitution for Na; is it inhibitable by histidine; and is it pH sensitive? These are all properties of the N-system transporter at the rat choroid plexus (Keep and Xiang, 1995; Xiang et al., 1998) and liver (Kilberg et al., 1980). In as much as glutamine has significant affinity for the Na-dependent ASC-system and the N-system, as well as the L-system

Received May 21, 1998; revised manuscript received July 15, 1998; accepted July 15, 1998.

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Abbreviations used: BBB, blood–brain barrier; BCH, 2-amino-2-norbornanecarboxylic acid; CBF, cerebral blood flow; IMP, isopropyliodoamphetamine; *pCBF*, perfusate cerebral blood flow; *pCPV*, perfusate cerebral plasma volume; *PS*, permeability–surface area; RBCs, red blood cells.

transporters, we used both 2-amino-2-norbornanecarboxylic acid (BCH) and cysteine to probe for transport of glutamine by the L- and ASC-systems, respectively. In addition, we examined the pH sensitivity of glutamine uptake at the BBB in vivo, but we did not measure Na dependence or inhibition by histidine because of the inability to control these parameters in vivo. Some of these results were presented in a preliminary communication (Keep et al., 1997).

MATERIALS AND METHODS

Transport of L-[^{14}C]glutamine across the BBB was measured by using a modification of a previously described technique for in situ perfusion of the rat brain with a perfusate containing sheep red blood cells (RBCs) (Zloković et al., 1986; Ennis et al., 1994, 1996), but in this instance using bilateral perfusion. In brief, male Sprague–Dawley rats, weighing 300–450 g, were anesthetized with pentobarbital (65 mg/kg i.p.). The right and left common carotid and pterygopalatine arteries were isolated and ligated. Catheters of PE-50 tubing, filled with heparinized saline, were placed in the left femoral artery and in the left and right external carotid arteries. Rats were heparinized (1.2 U/kg of body weight) and samples for blood gases obtained from the left femoral artery. The left and right and common carotid arteries were clamped and a bilateral perfusion initiated through the external carotid arteries at a rate of 2.5 ml/min. The left and right external carotid artery cannulas were used for retrograde perfusion of sheep RBCs in saline at a temperature of 37°C. The chest cavity was opened and the ventricles cut to prevent recirculation. All of the animals in the present study had an intact EEG during the time course of a perfusion. A hematocrit of 0.35 was used for these studies. Supplemental pentobarbital (22 mg/kg) was given just before perfusion to prevent the animals from awakening during the perfusion. In addition, pentobarbital at a concentration of 10 μg/ml was included in the perfusate to maintain anesthesia. Femoral and carotid blood pressures were recorded continuously through transducers connected to the arterial catheters. Blood pressure and EEG were recorded by using a Biopac MP100 data-acquisition system. The body temperature was maintained between 37.5 and 38.5°C by using a rectal thermistor connected to a temperature monitor/controller. Animal use for these studies was approved by the University of Michigan University Committee on Use and Care of Animals and meets NIH guidelines.

Measurement of blood-to-brain transport during in situ perfusion

Blood-to-brain transfer constants (K_i) for L-[^{14}C]glutamine (0.025 μCi/ml) were determined by using a 5-min perfusion. After the perfusion, the brain was removed, the hemispheres bisected, and dissected into frontal, parietal, and occipital cortices and diencephalon, cerebellum, and brainstem. Brain and perfusion fluid samples were dissolved in methylbenzthonium hydroxide and prepared for liquid scintillation counting.

Composition of perfusion fluids

Blood was stored at 4°C until the day of use. Whole blood was centrifuged at 1,660 *g* for 10 min at 8°C, and the plasma and white blood cells were discarded. The lightly packed RBCs were washed twice by centrifugation in 1.5 volumes of 0.9% NaCl and twice by centrifugation in 1.5 volumes of perfusate saline. After the final spin, the RBCs were resuspended at a hematocrit of 0.35 in saline. The composition of the control saline used to make the RBC-containing perfusate was (m*M*) 117 NaCl, 4 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 25.1 $NaHCO_3$, 2.5 $CaCl_2$, and 5 glucose. The perfusate also contains the following 19 amino acids at the average physiological levels (m*M*) (Jeppsson et al., 1985; Milakofsky et al., 1985; Smith et al., 1987; Hageman and Eriksson, 1990) found in plasma: 0.3 alanine, 0.22 arginine, 0.07 asparagine, 0.02 cysteine, 0.06 glutamate, 0.42 glutamine, 0.23 glycine, 0.07 histidine, 0.09 isoleucine, 0.16 leucine, 0.44 lysine, 0.06 methionine, 0.08 phenylalanine, 0.22 serine, 0.1 taurine, 0.37 threonine, 0.07 tryptophan, 0.07 tyrosine, and 0.18 valine. The perfusate containing radioactive isotopes was gently bubbled with a mixture of oxygen and carbon dioxide to maintain the pH in the range 7.35–7.45.

Two different Na replacement experiments were performed. In one set, mixtures of Tris-HCl and Tris-base and 24 m*M* Tris-carbonate were used as replacement for NaCl and $NaHCO_3$ (1 m*M* Na experiments). In the second set, Na substitution experiments used LiCl and Li_2CO_3 in place of NaCl and $NaHCO_3$ (143 m*M* LiCl experiments). Na and K concentrations were confirmed by using a flame photometer (IL 943).

Normocapnic perfusates with either a low or high pH were created by changing the amount of $NaHCO_3$, along with appropriate changes in the amount of NaCl included in the perfusate, to maintain a constant osmolality. The low pH perfusate contained (in m*M*) 132 NaCl, 4 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 9.98 $NaHCO_3$, 2.5 $CaCl_2$, and 5 glucose, with a pH of 7.05 ± 0.03. The high pH perfusate contained (m*M*) 79 NaCl, 4 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 62.9 $NaHCO_3$, 2.5 $CaCl_2$, and 5 glucose, with a pH of 7.75 ± 0.01.

Calculation of rate constants for BBB permeability to glutamine

The K_i for movement of solute across the BBB was calculated from the following equation:

$$K_i = A_b/(C_p \times T) \quad (1)$$

A_b is the amount of solute in the brain per unit mass of tissue (dpm/g), C_p is the perfusate solute concentration (dpm/ml), and T is time. In the present experiments C_p is constant.

The K_i values measured in this study were <4% of the rate of measured blood flow. Consequently, K_i closely approximates the permeability–surface area (*PS*) product as described by Fenstermacher et al. (1981). The *PS* for a substance at the BBB is the product of the permeability (*P*) (cm/min) and the surface area (*S*) (cm^2/g).

Uptake data were corrected for the amount of tracer that remains within the vascular space of the brain by the following equation:

$$A_b = A_T - (pCPV \times C_p) \quad (2)$$

A_T is the amount of solute in the brain per unit mass of tissue (dpm/g). The *pCPV* is the perfusate cerebral plasma volume. The *pCPV* was determined for each animal from the amount of [^{3}H]inulin (2-min circulation) in the brain samples (assuming no tissue uptake).

The time of isotope perfusion was 5 min. The transfer constant for glutamine was measured with in situ perfusion of sheep RBCs in the following four groups of rats: group 1, animals perfused with a saline containing either 15 m*M*

mannitol (n = 6), or 5 m*M* mannitol and 10 m*M* BCH, a model amino acid substrate for the L-system transporter (n = 5), or 5 m*M* cysteine, a substrate for the A-, ASC-, and L-system transporters, and 10 m*M* BCH (n = 6); group 2, animals perfused with a saline containing either 142 m*M* Na (n = 9), or 1 m*M* Na (n = 9), or 143 m*M* LiCl (n = 6); group 3, animals perfused with a saline containing 2.5 m*M* histidine with either 142 m*M* Na (n = 4), or 1 m*M* Na (n = 4), and 10 m*M* glutamine with either 142 m*M* Na (n = 6), or 1 m*M* Na (n = 6); group 4, animals perfused with saline with a pH of 7.4, containing 25.1 m*M* $NaHCO_3$ (n = 9), or saline with a pH of 7.0 containing 10 m*M* $NaHCO_3$ (n = 5), or saline with a pH of 7.8 containing 63 m*M* $NaHCO_3$ (n = 5).

The transfer constant for glutamine was also determined from a multiple time/graphical analysis. The following equation describes the relationship between the amount of tracer in the brain (A_b) and its concentration in plasma:

$$A_b = K_i \times \int C_p dt + V_b \times C_p(T) \qquad (3)$$

V_b is the sum of the plasma and rapidly filling spaces ($V_b = pCPV + V_r$). V_r is termed the rapidly filling space because it rapidly and reversibly exchanges with plasma (Patlak et al., 1983). Under initial velocity conditions, a plot of $A_b/C_p(T)$ versus perfusion time is a straight line with the slope = K_i and the y intercept = V_b. The time of isotope circulation ranged between 0.5 and 10 min.

The uptake of glutamine by the sheep RBCs was also measured in each experiment. During the time course of the experiments shown in Fig. 1, there was no significant uptake of glutamine by sheep RBCs. The glutamine space in the RBC, relative to the plasma, averaged −0.04 ± 0.044 $\mu l/\mu g$ (n = 6) at 5 min of circulation.

In addition, the transfer constant for L-[^{14}C]glutamine was measured in vivo, using Eqs. 1 and 2 as described previously (Betz et al., 1989). The effect of changing plasma pH was measured in the following four groups of animals (each n = 5) 1 h after an intraperitoneal injection, at a dose of 20 ml/kg, of: 235 m*M* NaCl, 235 m*M* HCl, 235 m*M* $NaHCO_3$, or 235 m*M* NH_4Cl.

Perfusate cerebral blood flow (*pCBF*)

The *pCBF* was calculated by using [^{14}C]isopropyliodoamphetamine ([^{14}C]IMP) as the blood flow indicator. IMP has a high effective partition coefficient between brain and blood that approaches 25:1 (Lear et al., 1982). Other investigators have found that IMP uptake in brain represents cerebral blood flow (CBF) for at least the first 15 min after tracer administration (Rapin et al., 1984). Under these conditions, the *pCBF* was calculated as follows:

$$pCBF = A_b/(C_p \times T) \qquad (4)$$

Animals (n = 4) were preperfused for 2.5 min with the appropriate solution before determining the *pCBF*. Radiolabeled IMP was perfused into the external carotid arteries for 30 s in order to measure *pCBF*. The CBF was also measured in vivo by using Eq. 4 and [^{14}C]IMP with a 30-s circulation time. The in vivo CBF was measured in a group of four animals. In addition to the cortical samples, basal ganglia, and brainstem, the choroid plexuses from the lateral and the fourth ventricle were sampled.

Statistical analysis

Data from single time point experiments (Eq. 1) were analyzed using a model 1 ANOVA with either an unpaired two-tailed t test, or with a two-tailed Bonferroni post hoc test for multiple comparisons. The data for the effect of pH on the in vivo or in situ uptake of glutamine into the cortex were analyzed by least-squares linear regression. The results were subjected to an analysis of covariance with a two-tailed t test. The Systat statistical software was used for these comparisons. A p value of <0.05 was considered significant. All data are mean ± SD values, except where noted as SEM.

Chemicals and radiotracers

[^{3}H]Inulin, L-[^{14}C]glutamine, and [^{14}C]IMP were purchased from Du Pont–NEN (Boston, MA, U.S.A.). All other chemicals were obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

RESULTS

Table 1 presents the average perfusate values for blood gases, physiological parameters, osmolality, and Na, K, and glucose concentrations. These data were measured just before the beginning of a perfusion. The data are presented in groups that correspond to the major change made in the perfusate. For example, the Na concentration for the 1 m*M* Na group was 2.2 ± 0.9 m*M* in comparison with the control value of 137 ± 5 m*M* ($p \leq 0.001$). The only unanticipated differences were in the pH and P_{CO_2} between the control and the 1 m*M* Na groups and the P_{CO_2} between control and the pH 7.0 groups. These were small changes that would not affect glutamine transport.

Table 2 presents a comparison of cerebral plasma volume, measured as the inulin space, and blood flow measured both in vivo and in situ (with control perfusate). Plasma volume in the cortex during bilateral in situ perfusion at a rate of 2.5 ml/min with washed sheep RBCs was reduced 17% ($p \leq 0.01$) compared with in vivo values. In a similar manner, CBF in the cortex was reduced 18% ($p \leq 0.01$) compared with in vivo values. These small differences in perfusion during in situ perfusion should not adversely effect determination of the mechanism of transport (see below for a comparison of effect of pH on glutamine transport). The inulin space and blood flow were also measured in the lateral and fourth ventricle choroid plexuses. The inulin space for the choroid plexus actually represents a combination of the residual vascular volume and the extracellular space of this tissue. Table 3 presents the combined value for these tissues. The in vivo and in situ values for the choroid plexuses are very similar to published values (Deane and Segal, 1985; Szmydynger-Chodobska et al., 1994).

The plasma volume (Table 3) during in situ perfusion was measured in groups that correspond to the major change made in the perfusate, simultaneously with glutamine K_i. The animals in which the plasma bicarbonate level was reduced to 10 m*M* showed a statistically significant increase in plasma volume when compared with control animals ($p \leq 0.001$).

Graphical analysis of glutamine transport

Graphical analysis of multiple time data was used to confirm that glutamine transport in the presence of

TABLE 1. *Physiological parameters*

	Control (n = 45)	1 m*M* Na (n = 19)	LiCl (n = 6)	pH 7.0 (n = 5)	pH 7.8 (n = 5)
Weight (g)	304 ± 23	287 ± 27	287 ± 27	300 ± 34	282 ± 20
Perfusion pressure (mm Hg)	108 ± 29	122 ± 25	93 ± 25	110 ± 18	126 ± 22
Perfusate pH	7.38 ± 0.04	7.48 ± 0.03[a]	7.37 ± 0.04	7.05 ± 0.03[a]	7.74 ± 0.01[a]
Perfusate P_{CO_2} (mm Hg)	37 ± 4	51 ± 5[a]	38 ± 4	48 ± 9[a]	37 ± 2
Perfusate P_{O_2} (mm Hg)	215 ± 37	189 ± 55	241 ± 13	232 ± 16	206 ± 10
Perfusate hematocrit (%)	34 ± 1	32 ± 1	35 ± 1	34 ± 0.5	33 ± 2
Perfusate glucose (mg/dl)	89 ± 13	87 ± 8	80 ± 5	81 ± 9	89 ± 4
Perfusate Na (mEq/L)	137 ± 5	2.2 ± 0.9[a]	1 ± 0.5[a]	133 ± 1	137 ± 3
Perfusate K (mEq/L)	4.1 ± 0.6	4.1 ± 0.1	4.1 ± 0.1	3.7 ± 0.1	3.8 ± 0.1
Perfusate osmolality (mOsm/L)	286 ± 14	287 ± 13	274 ± 9	286 ± 6	281 ± 11

Data are mean ± SD values. Perfusion pressure is the carotid pressure minus the pressure due to the carotid cannula. n = number of animals.
[a] $p \leq 0.001$.

physiological concentrations of 19 of the most common amino acids (including 0.42 m*M* glutamine) remained unidirectional during the typical uptake experiment. Figure 1 presents a graphical analysis for glutamine uptake in the frontal cortex during in situ perfusion, using time points of 0.5, 2.0, 5, and 10 min of circulation time. Inspection of the regression line for linearity indicates that during the 10 min of the perfusion, glutamine transport remained unidirectional. The transfer constant for glutamine during perfusion with a perfusate containing physiological concentrations of amino acids was 10.4 ± 0.4 μl/g/min with a rapidly filling space of 13.1 ± 2.3 μl/g. Data are mean ± SEM values. The K_i during in situ perfusion, using a single time point analysis, with a 5-min time point, was 10.8 ± 0.6 μl/g/min. The results validate the use of 5-min, single time point studies for the remainder of this study.

Glutamine transport at the luminal membrane of the BBB

Glutamine uptake was measured in the presence of either 15 m*M* mannitol (control), 10 m*M* BCH with 5 m*M* mannitol, or 10 m*M* BCH and 5 m*M* cysteine (Fig. 2). The K_i of L-[^{14}C]glutamine uptake into the cortex was 11.6 ± 1.04 ml/g/min (mean ± SD). To determine if this rather large influx was due to transport by the L-system transporter, an excess (10 m*M*) of BCH, the model amino acid for system-L, was added to the perfusate. BCH failed to significantly reduce the K_i of L-[^{14}C]glutamine transport into the cortex, basal ganglia, cerebellum, or brainstem. In a similar manner, 10 m*M* BCH and 5 m*M* cysteine, a substrate for the ASC- and A-system transporters, did not inhibit transport L-[^{14}C]glutamine into the cortex or basal ganglia. However, the combination of BCH and cysteine did slightly reduce uptake of L-[^{14}C]glutamine into the cerebellum and brainstem. These results indicate that glutamine transport, in the presence of this complement of amino acids, does not enter the brain through either the L- or the ASC-system at the luminal membrane of the BBB.

Effect of Na and Li concentration on glutamine transport

None of the known L-amino acid transport systems at the luminal membrane of the BBB show Na-dependent transport. Of the described carrier-mediated systems at the luminal membrane of the BBB, only β-taurine transport is Na-dependent (Benrabh et al., 1995). Na-dependent glutamine transport was tested by substituting Tris-HCl for NaCl and Tris-carbonate

TABLE 2. *Comparison of inulin space and CBF*

	Inulin space (μl/g)		CBF (ml/g/min)	
Region	In vivo	In situ	In vivo	In situ
Cortex	5.9 ± 0.5[a]	4.8 ± 0.4[b]	0.56 ± 0.06	0.46 ± 0.02[a]
Basal ganglia	7.0 ± 0.7	5.8 ± 0.6[a]	0.73 ± 0.11	0.48 ± 0.02[b]
Cerebellum	8.2 ± 0.9	6.7 ± 0.4[a]	0.73 ± 0.13	0.51 ± 0.03[a]
Brainstem	9.1 ± 3.3	6.3 ± 0.7	0.81 ± 0.11	0.50 ± 0.02[b]
Choroid plexus	217 ± 27	309 ± 45[a]	4.8 ± 1.1	6.8 ± 1.3[a]

The inulin space for the choroid plexus is a combination of residual vascular volume and extracellular space of the tissue, whereas for the other tissues it just represents the vascular volume. Data are mean ± SD values, n = 4, for both in vivo and in situ.
In vivo vs. in situ: [a] $p \leq 0.05$; [b] $p \leq 0.01$.

TABLE 3. *Inulin space in the cortex during in situ perfusion*

Group	Inulin space (μl/g)	n
Control	6.1 ± 1.2	36
1 m*M* Na	5.0 ± 0.5	10
LiCl	5.3 ± 0.4	6
10 m*M* $NaHCO_3$ (pH 7.0)	8.2 ± 0.8[a]	5
63 m*M* $NaHCO_3$ (pH 7.8)	5.1 ± 0.7	5

Data are mean ± SD values.
[a] $p \leq 0.01$ vs. control.

for $NaHCO_3$ (Ennis et al., 1996). The final Na concentration in these experiments was 2.4 ± 1.0 m*M* (n = 9). Na replacement reduced glutamine transport by 62, 58, 58, and 54% (all $p \leq 0.001$) in the cortex (Fig. 3), basal ganglia, cerebellum, and brainstem, respectively.

The Na-dependent glutamine transporter (system-N) shows varying tolerance for Li substitution for Na, depending on the tissue studied. Using in situ perfusion, we found that Li substitution resulted in a 44% decrease ($p \leq 0.001$) in the Na-dependent portion of glutamine uptake into the cortex (Fig. 3). In a similar manner, Li substitution reduced glutamine transport by 44, 48, and 53% in the basal ganglia, cerebellum, and brainstem, respectively.

Na-dependent L-[^{14}C]glutamine transport: effect of histidine and glutamine

N-system transport in other tissues has a restricted range of substrates that includes glutamine, histidine, and asparagine. Of these three, histidine and glutamine are the most effectively transported by the N-system (Kilberg et al., 1993). The effect of adding either 2.5 m*M* histidine or 10 m*M* glutamine to the perfusate on the total and Na-independent uptake of L-[^{14}C]glutamine is presented in Fig. 4. The Na-dependent uptake was calculated by subtracting the total and Na-independent values. Both histidine and glutamine significantly reduced the total uptake by 50 and 64%, respectively (both $p \leq 0.001$). The Na-independent transport of L-[^{14}C]glutamine in the presence of histidine was significantly less ($p \leq 0.001$) than the total (in the presence of histidine). This was most likely because an additional 2.5 m*M* histidine did not completely inhibit Na-dependent glutamine transport. Na-dependent glutamine transport was statistically higher in the presence of 2.5 m*M* histidine than in the presence of 10 m*M* glutamine ($p \leq 0.01$). As reported above, Na replacement reduced L-[^{14}C]glutamine uptake by 62% into the cortex. Neither histidine nor glutamine had any further statistically significant effect on Na-independent transport of L-[^{14}C]glutamine, indicating that its transport in the present experiments is through the Na-dependent pathway. The Na-dependent portion of glutamine transport was reduced 63% by 2.5 m*M* histidine and 90% by 10 m*M* glutamine (both $p \leq 0.001$, com-

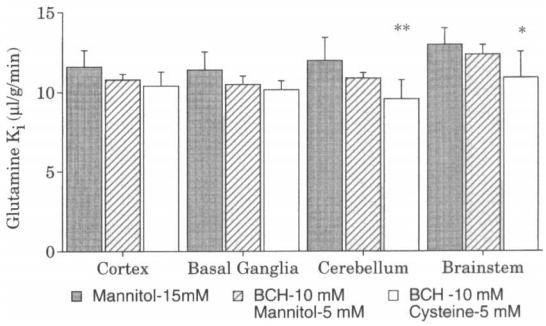


FIG. 2. Effect of substrates of the L- (BCH) or A- and ASC- (cysteine) transport systems on the K_i for L-[^{14}C]glutamine at the BBB. In the presence of physiological concentrations of amino acids (including 0.42 m*M* glutamine) in the perfusate, the K_i for L-[^{14}C]glutamine uptake into the brain was 11.6 ± 1.1 μl/g/min. Neither BCH, the model substrate for the L-system transporter, nor a combination of BCH and cysteine substantially reduced the glutamine K_i. Data are mean ± SD values, n = 6 for 15 m*M* mannitol, n = 5 for 10 m*M* BCH and 5 m*M* mannitol, and n = 6 for 10 m*M* BCH and 5 m*M* cysteine. *p* values are shown for comparison with the mannitol control group. $^*p \leq 0.05$; $^{**}p \leq 0.01$.

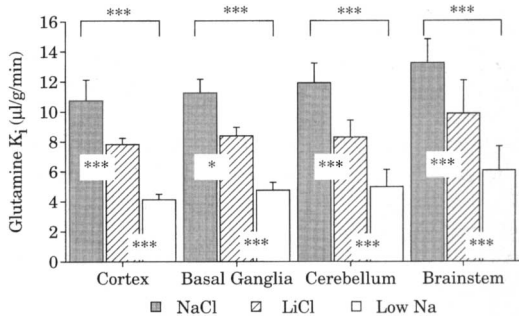
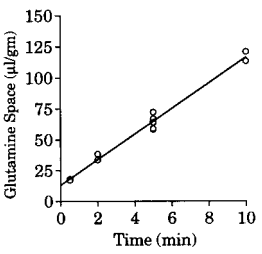


FIG. 3. Effect of either reducing perfusate Na from an average of 138 ± 2 to 2.4 ± 1 mEq/L or replacing perfusate Na (0.7 ± 0.6 mEq/L) with LiCl. Physiological concentrations of amino acids (including 0.42 m*M* glutamine) were included in the perfusate. Decreasing perfusate Na from 140 to 2.4 m*M* reduced the cortical K_i for L-[^{14}C]glutamine by 62% ($p < 0.001$). In contrast, replacing Na with Li resulted in a 27% decrease in the cortical K_i for L-[^{14}C]glutamine. Data are mean ± SD values, n = 9 for NaCl, n = 6 for LiCl, and n = 9 for low Na. *p* values are shown for between pairs (inserts) or for the comparison of low Na to NaCl (brackets). $^*p \leq 0.05$; $^{***}p \leq 0.001$.

FIG. 1. Graphical analysis of in situ uptake of L-[^{14}C]glutamine into the cortex in the presence of physiological concentrations of amino acids. Data were best fit by linear-regression analysis to Eq. 3. The slope of the line is the transfer constant (K_i) and the intercept is V_r, K_i = 10.4 ± 0.4 μl/g/min, and V_r = 13.1 ± 2.3 μl/g, r = 0.992. Data are mean ± SEM values.



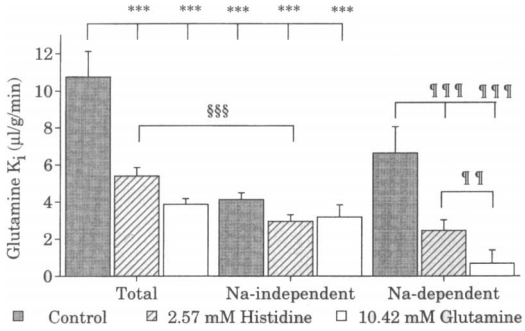


FIG. 4. Effect of adding 2.5 m*M* histidine or 10 m*M* glutamine on the total, Na-independent or the Na-dependent transport of L-[^{14}C]glutamine into the cortex. The Na-dependent transport was calculated by subtraction of the total and Na-independent transport. These experiments were all performed in the presence of physiological concentrations of amino acid, including 0.42 m*M* glutamine and 0.07 m*M* histidine. Data are mean ± SD values, n = 9 for experiments with normal amino acid composition, n = 4 for 2.57 m*M* histidine experiments, and n = 6 for 10 m*M* glutamine experiments. *p* values are shown for comparison of Total to the indicated group (***$p \leq 0.001$), or histidine with and without Na (§§§$p \leq 0.001$), or Total Na-dependent glutamine transport with either histidine or glutamine (¶¶¶$p \leq 0.001$), or Na-dependent glutamine transport with histidine compared with Na-dependent glutamine transport with glutamine (¶¶$p \leq 0.01$).

pared with control). These results indicate that in the presence of physiological concentrations of the amino acid glutamine, the transport at the luminal membrane of the BBB is through a Na-dependent carrier that is probably the N-system transporter.

Effect of pH on L-[^{14}C]glutamine uptake: comparison of in vivo and in situ transport

Glutamine transport through the hepatic N-system shows a marked sensitivity to extracellular pH, with a 60% reduction in transport at pH 7.0 and a 60% increase in transport at pH 8.0, both compared with the rate at pH 7.5 (Kilberg et al., 1980). Figure 5 presents the regression lines describing the relationship between extracellular pH and glutamine transport into the cerebral cortex both in situ (A) and in vivo (B). During in situ perfusion, we varied the perfusate pH by changing the perfusate content of $NaHCO_3$, while holding the CO_2 approximately constant. The BBB was exposed to a changed pH for only 6 min. At a pH of ~7.0 (pH = 7.05 ± 0.03, mean ± SD; n = 5), the glutamine K_i was reduced to 43% of the value at pH 7.4 (pH = 7.39 ± 0.03, n = 9), while a pH of 7.8 (pH = 7.75 ± 0.01, n = 5) increased the glutamine pH to 139% of the value at pH 7.4. To verify that uptake of glutamine at the BBB is pH dependent we also measured glutamine uptake in vivo by imposing a metabolic acidosis or alkalosis 1 h before isotope injection. Over the narrower pH range that these manipulations produced, glutamine K_i again varied as a function of pH, but the slope of the line describing this relationship was larger in vivo than in situ ($p \leq 0.001$). We believe that this difference is due to the longer exposure time to an altered blood pH in vivo and a larger pH gradient between blood and the endothelial cells of the BBB and to small changes in plasma glutamine concentration (not measured) during manipulation of blood pH (Welbourne, 1987).

DISCUSSION

Comparison of in vivo and in situ plasma volume and CBF

In situ perfusion has proven valuable in clarifying mechanisms of transport at the luminal membrane of the BBB. It allows for precise control of the composition of the perfusate, avoids recirculation of radioactive metabolites from the systemic circulation, and allows for constant plasma levels of radioactive tracers to be delivered to the brain. Mechanisms for amino acid (Smith, 1991), sodium (Ennis et al., 1996), and peptide transport (Zloković et al., 1989; Zloković, 1995), have been successfully determined using this method. However, in situ perfusion relies on artificial perfusates and sets CBF and perfusion pressure with an external pump. Consequently, it is necessary to validate the parameters for perfusion by using in vivo methods. Table 2 presents a comparison of cerebral plasma volume and CBF measured both in vivo and in situ. Both the plasma volume and the CBF showed minimal (<20%) reductions compared with in vivo values. These results validate the flow rate and the use of sheep RBCs during in situ perfusion in order to approximate in vivo cerebral perfusion. The use of a bilateral perfusion also allowed us to measure the plasma volume/extracellular space and blood flow in the lateral and fourth ventricle choroid plexuses. The in vivo and in

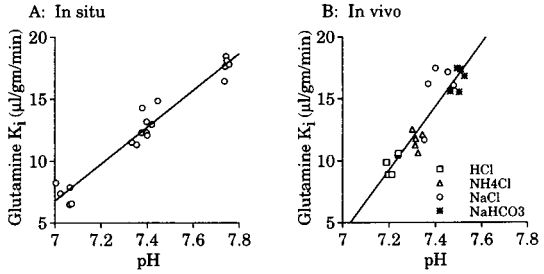


FIG. 5. Comparison of the effect of perfusate pH on the cortical K_i L-[^{14}C]glutamine measured either in situ (**A**) or in vivo (**B**). During in situ perfusion physiological concentrations of amino acids were included in the perfusate. The in situ glutamine K_i for the cortex was measured after changing the perfusate pH through manipulation of the $NaHCO_3$ concentration. The time of exposure to the perfusate in situ was 6 min. The in vivo glutamine K_i for the cortex was measured 1 h after intraperitoneal injections (20 ml/kg) of 235 m*M* NaCl, $NaHCO_3$, HCl, or NH_4Cl. The line describing K_i as a function of pH in situ is: $K_i = 14.8 \times pH - 96.9$, $r^2 = 0.95$, and in vivo K_i varies as a function of pH: $K_i = 25.7 \times pH - 175.5$, $r^2 = 0.84$. The difference in the slope of these two lines ($p \leq 0.001$) probably reflects the different times of exposure of the BBB to an altered pH.

situ values are very similar to published values (Deane and Segal, 1985; Szmydynger-Chodobska et al., 1994) and indicate that this vital portion of the barrier system of the brain is functioning during the present experiments.

Glutamine transport at the luminal membrane of the BBB

This study introduces evidence that a transporter for glutamine is present at the luminal membrane of the rat BBB. We have tentatively identified this transporter as the N-system based on the following evidence: (1) Glutamine transport is Na-dependent and shows moderate Li tolerance; (2) glutamine transport at the BBB shows marked pH sensitivity both in vivo and in situ; (3) Na-dependent glutamine uptake shows self-inhibition; (4) BCH, a model substrate for the L-system, and cysteine, a substrate for A- and ASC-systems (both Na-dependent), does not inhibit glutamine transport; and (5) Na-dependent glutamine transport is inhibited significantly by histidine.

The known Na-independent amino acid carriers at the luminal membrane include the L-system for phenylalanine, tryptophan, and leucine (Smith et al., 1987), the basic (cationic or γ^+) for lysine and arginine (Stoll et al., 1993), and the acidic (x^-) amino acid transporter for glutamine and aspartate (Davson et al., 1990; Al-Sarraf et al., 1995). There is evidence that transport of amino acids at the luminal membrane of the brain capillary endothelial cell is not through either the A- (alanine-preferring; Na-dependent) or ASC- (alanine-serine-cysteine preferring; Na-dependent) systems (Ennis et al., 1994; Benrabh and Lefauconnier, 1995). The Na dependence of glutamine transport, combined with the observation that excess (10 m*M*) BCH did not further reduce the glutamine K_i, indicates that glutamine transport into the brain is most probably through a novel transporter. Adding 10 m*M* glutamine to the perfusate inhibited Na-dependent glutamine transport into the cortex by ~90%. Glutamine uptake at the luminal membrane of the BBB was measured in the presence of physiological concentrations of 19 of the most common amino acids (including 0.42 m*M* glutamine). Smith et al. (1987) calculated that a amino acid composition similar to the one we used would saturate the L-system transporter at the BBB. The absence of any major inhibition of glutamine uptake by BCH does not imply that glutamine cannot be transported by the L-system transporter in the absence of competing amino acids. It does indicate that under physiological conditions, luminal L-system transport is not a major contributor to brain glutamine uptake.

Glutamine transport at the luminal membrane of the BBB showed moderate Li tolerance and marked sensitivity to intravascular pH, and inhibition by histidine, all characteristics of N-system transport in liver (Kilberg et al., 1980), astrocytes (Nagaraja and Brookes, 1996), and choroid plexus (Keep and Xiang, 1995; Xiang et al., 1998). The Na-dependent glutamine transporter (system-N) shows varying tolerance for Li substitution of Na, depending on the tissue studied (Kilberg et al., 1980; Hundal et al., 1987; Nagaraja and Brookes, 1996; Tamarappoo et al., 1997). The system-N transporter in rat brain neurons and perfused rat hindlimb have been described as only weakly tolerant of Li substitution whereas those in rat liver hepatocytes, rat sarcolemmal vesicles from skeletal muscle, and mouse astrocytes are characterized as strongly Li tolerant. In the choroid plexus, the system-N-like glutamine transport was also tolerant of Li but showed a 33% decrease in Na-dependent transport that was not statistically significant (Keep and Xiang, 1995). Of the known Na-dependent transporters only the A-system also shows a marked pH sensitivity (Kilberg et al., 1980), and this is not present at the luminal membrane of the BBB (Ennis et al., 1994; Benrabh and Lefauconnier, 1995).

Smith et al. (1987) have also used in situ brain perfusion to measure BBB glutamine transport. Their influx in the presence of normal concentrations of plasma amino acids (4.1 nmol/g/min) is very similar to that found in this study (4.9 nmol/g/min). These authors also determined kinetic constants for glutamine transport (V_{max} of 43.2 nmol/g/min, a K_m of 0.88 m*M*, and a K_D of 2.4 μl/g/min) (Smith et al., 1987). In analyzing the effect of competition of other neutral amino acids on glutamine uptake, these investigators assumed that glutamine transport was only through the L-system amino acid carrier. However, the results of the present study indicate that a second carrier (N-system) also transports glutamine at the luminal membrane of the BBB. Consequently, the V_{max} and K_m for glutamine transport calculated by Smith et al. (1987) should represent some combination of the maximum velocity and the affinity of glutamine for the L- and N-system amino acid transporters at the BBB. The K_D calculated by Smith et al. (1987) should represent an accurate value for glutamine diffusion at the BBB.

Only two other groups have investigated glutamine transport at the luminal membrane of the BBB (Oldendorf, 1971; Pardridge and Mietus, 1982). Pardridge and Mietus (1982) found a faster rate of glutamine transport in newborn rabbit brain compared with adult rat (Oldendorf, 1971). In contrast to the present report, they found that glutamine transport was Na-independent using the carotid injection technique and a Na concentration of 50 m*M* (Pardridge and Mietus, 1982). In addition to the different species used, another possible reason for the different results may be that we used a Na concentration of 1 m*M* to test the Na dependence of glutamine transport. Alternatively, the results of Pardridge and Mietus (1982) raise the interesting possibility that Na-dependent glutamine transport by the N-system at the BBB may not develop until later in neonatal life.

A very recent report has provided evidence, using fractionated vesicles from bovine brain endothelial

cells, that the N-system transporter is probably present on the abluminal as well as the luminal membrane (Lee et al., 1998). The role an abluminal transporter plays in the overall nitrogen balance of the brain remains to be clarified.

Role of N-system transport at the BBB

The combined unidirectional flux of L-amino acids into the brain has been calculated as ~53 nmol/g/min (Smith et al., 1987). Our calculated unidirectional glutamine flux out of an artificial plasma of 4.9 nmol/g/min is ~10% of this total L-amino acid flux and highlights the potential importance of glutamine transport into the brain for its overall nitrogen balance.

Much of the interest in glutamine in the brain centers on hepatic encephalopathy where detoxification of ammonia in the brain results in a buildup of glutamine (Cooper and Plum, 1987). This accumulation of glutamine appears to be involved in the pathophysiology of this condition as methionine sulfoximine, an inhibitor of glutamine synthetase can prevent the metabolic symptoms, brain edema (Hawkins and Jessy, 1991; Hawkins et al., 1992), and brain amino acid changes associated with hyperammonemia (Rigotti et al., 1985).

The rate of glutamine synthesis in the brain of normal, pentobarbital-anesthetized rats is markedly higher (40 nmol/g/min; Sibson et al., 1998) than the unidirectional influx of glutamine from blood (5 nmol/g/min; this study). However, the luminal N-system transporter may have an important role in hepatic encephalopathy, for the rate of glutamine accumulation after portacaval shunting in the rat, a model of hepatic encephalopathy, is ~5–6 nmol/g/min for the first 24 h (Mans et al., 1990). This suggests that alterations in blood-to-brain glutamine transport could significantly alter brain glutamine accumulation in this condition. In addition, during hepatic encephalopathy, there is an increased uptake of L-system substrates that are precursors for neurotransmitters and this has been hypothesized to lead to a derangement of brain (Zanchin et al., 1979; Mans et al., 1983). Changes in brain and brain endothelial cell glutamine concentration, via trans-stimulation, have been implicated as a cause of this change in L-system-mediated transport (James and Fischer, 1981; Jeppsson et al., 1983; Jeppsson et al., 1985). The Na-dependent N-system transporter may be a major determinant of brain endothelial cell glutamine concentration and, thus, the degree of trans-stimulation.

In summary, a major component of glutamine transport at the luminal membrane of the BBB is through the N-system amino acid transporter. This transporter shows the characteristics of Na dependence, Li tolerance, pH sensitivity, and inhibition by histidine, the other major N-system substrate.

Acknowledgment: This study was supported by grants from the National Institutes of Health (NS37409 and NS23870) and a grant from the American Diabetes Association. We also thank Dr. A. L. Betz for many useful discussions and suggestions about this study.

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