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N-System Amino Acid Transport at the Blood–CSF Barrier

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Abstract: Despite L-glutamine being the most abundant amino acid in CSF, the mechanisms of its transport at the choroid plexus have not been fully elucidated. This study examines the role of L-, A-, ASC-, and N-system amino acid transporters in L-[^{14}C]glutamine uptake into isolated rat choroid plexus. In the absence of competing amino acids, approximately half the glutamine uptake was via a Na^+-dependent mechanism. The Na^+-independent uptake was inhibited by 2-amino-2-norbornane carboxylic acid, indicating that it is probably via an L-system transporter. Na^+-dependent uptake was inhibited neither by the A-system substrate α-(methylamino)isobutyric acid nor by the ASC-system substrate cysteine. It was inhibited by histidine, asparagine, and L-glutamate γ-hydroxamate, three N-system substrates. Replacement of Na^+ with Li^+ had little effect on uptake, another feature of N-system amino acid transport. These data therefore indicate that N-system amino acid transport is present at the choroid plexus. The V_{max} and K_m for glutamine transport by this system were 8.1 ± 0.3 nmol/mg/min and 3.3 ± 0.4 m*M*, respectively. This system may play an important role in the control of CSF glutamine, particularly when the CSF glutamine level is elevated as in hepatic encephalopathy. **Key Words:** Choroid plexus—Glutamine—Histidine—Rat—Influx.
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L-Glutamine is by far the most abundant amino acid present in CSF (see, e.g., Clarke et al., 1989), and increased cerebral glutamine concentrations appear to be important in the genesis of hepatic encephalopathy (Hawkins and Jessy, 1991; Takahashi et al., 1991; Hawkins et al., 1993). Despite its importance, our knowledge of glutamine transport at the blood–CSF and blood–brain barriers is incomplete. Glutamine is transported by the Na^+-independent, leucine-preferring (L-system) amino acid transporter from blood to CSF at the choroid plexus (Segal et al., 1990) and from blood to brain at the cerebral capillaries (Oldendorf and Szabo, 1976; Smith et al., 1987), tissues that are, respectively, sites of the blood–CSF and blood–brain barriers. However, whether glutamine transport at these tissues is also mediated by the Na^+-dependent A- and ASC-systems of Christensen (1975) is uncertain because these transporters participate in glutamine transport in some but not all cells (Thomas and Christensen, 1971; Kilberg et al., 1980; Handlogten et al., 1982; Hundal et al., 1987). In hepatocytes and skeletal muscle glutamine uptake is mediated by another Na^+-dependent transporter, the N-system (Kilberg et al., 1980; Hundal et al., 1987), so named because of the presence of a nitrogen in the side chain of its three naturally occurring substrates, glutamine, histidine, and asparagine (Kilberg et al., 1980). Whether N-system transport is present at the blood–brain barriers has not been determined.

In this study we have examined the role of the L-, A-, ASC-, and N-systems in glutamine uptake into isolated rat choroid plexus. For the N-system transport there are as yet no specific substrates, so we examined whether choroid plexus Na^+-dependent glutamine transport shares several properties found for the N-system in hepatocytes and skeletal muscle (Kilberg et al., 1980; Hundal et al., 1987).

MATERIALS AND METHODS

Experiments were performed on choroid plexuses isolated from pentobarbital (50 mg/kg)-anesthetized male Sprague–Dawley rats 30–50 days old. The methods used are based on those of Johanson and co-workers (Smith and Johanson, 1985; Parmelee and Johanson, 1989), which we have previously used to examine potassium transport (Keep et al., 1994; Keep and Xiang, 1995). The lateral ventricle plexuses were weighed and transferred to control buffer at 37°C. There was a 5-min recovery period before the beginning of any experiment.

Buffers

Most experiments were performed in bicarbonate buffers that were continuously bubbled with 5% CO_2/95% O_2 and contained 127 m*M* NaCl, 20 m*M* $NaHCO_3$, 2.4 m*M* KCl, 0.5 m*M* KH_2PO_4, 1.1 m*M* $CaCl_2$, 0.85 m*M* $MgCl_2$, 0.5 m*M* Na_2SO_4, and 5 m*M* glucose (pH 7.4). In experiments where a low-Na^+ buffer was used to investigate the role of Na^+ in

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Abbreviations used: BCH, 2-amino-2-norbornane carboxylic acid; MeAIB, α-(methylamino)isobutyric acid.

L-[^{14}C]glutamine uptake, buffer NaCl and $NaHCO_3$ were replaced by choline chloride and choline bicarbonate, respectively, producing a 1 m*M* Na^+ solution owing to the presence of Na_2SO_4. L-Glutamine, from the addition of isotope (700 n*M*), and selected competing amino acids (at 10 m*M*) were the only amino acids present in the buffer in most experiments. In one experiment, however, a physiological CSF was used with amino acids at the following concentrations: L-glutamine, 500 μM; L-glutamate, 8 μM; taurine, 6 μM; glycine, 7 μM; L-alanine, 26 μM; L-serine, 25 μM; L-threonine, 25 μM; L-lysine, 21 μM; L-arginine, 18 μM; L-histidine, 13 μM; L-valine, 16 μM; L-leucine, 11 μM; L-isoleucine, 4 μM; L-phenylalanine, 8 μM; L-tyrosine, 8 μM; L-methionine, 3 μM; L-asparagine, 8 μM; and L-aspartate, 0.2 μM [derived from the procedures of Berg et al. (1993) and Clarke et al. (1989)].

The effect of Na^+ replacement on [^{14}C]glutamine uptake was also examined in a Tris-based artificial CSF containing 140 m*M* NaCl, 3 m*M* KCl, 3 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 5 m*M* glucose, and 15 m*M* Tris (pH 7.4) that was bubbled with 100% O_2. This facilitated replacement of Na^+ with Li^+ as well as choline.

L-Glutamine uptake

After the recovery period, the plexuses were transferred to 0.95 ml of bicarbonate buffer with or without drug for 0.5 min. Uptake was started by addition of 0.05 ml of buffer with ~0.2 μCi of L-[^{14}C]glutamine and 0.1 μCi of [^{3}H]-mannitol (an extracellular marker). Uptake was terminated after 30 s by transferring the plexus to iced control buffer and filtering under reduced pressure. The filters (118 μm mesh) were washed three times with the same buffer. The filters and choroid plexuses were then soaked in 0.33 ml of 1 *M* hyamine hydroxide (a tissue solubilizer) for 30 min before addition of scintillation cocktail (Cytoscint) and counting with a dual-channel liquid scintillation counter (Model LS 3801; Beckman, Fullerton, CA, U.S.A.).

Preliminary experiments, where uptake time was varied from 0.25 to 10 min, showed that L-[^{14}C]glutamine uptake was only unidirectional for the first 30 s, and therefore experiments were limited to this time. The uptake of L-[^{14}C]-glutamine into choroid plexus (C_{cp}), in microliters per milligram wet weight, was calculated as

$$C_{cp} = \frac{C_t - C_f - [(H_t - H_f) \cdot \text{ratio}]}{C_{media}} \quad (1)$$

where C_t is the total L-[^{14}C]glutamine concentration in the plexus plus filter, C_f is a correction for the L-[^{14}C]glutamine binding to the filter, and C_{media} is the concentration of L-[^{14}C]glutamine in the external media. The term $(H_t - H_f) \cdot$ ratio is a correction for extracellular isotope, where H_t is the concentration of [^{3}H]mannitol in the plexus plus filter and H_f is the filter binding of mannitol. Multiplying the difference between these two parameters by the ratio of L-[^{14}C]glutamine to [^{3}H]mannitol in the external medium gives an estimate of the extracellular L-[^{14}C]glutamine content. The unidirectional influx rate for glutamine (v) was calculated by multiplying C_{cp} by the external unlabeled glutamine concentration and dividing by the duration of the experiment.

To determine the kinetic constants for Na^+-independent and -dependent uptake, a nonlinear curve-fitting program was used (Systat 5.2; Systat, Inc., Evanston, IL, U.S.A.). For Na^+-independent uptake a two-component model was used:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} + K_D \cdot [S] \quad (2)$$

where [*S*] is the glutamine concentration, V_{max} and K_m are the maximal velocity and affinity constant for saturable uptake, respectively, and K_D is a diffusion constant for nonsaturable transport. For the Na^+-dependent uptake, a simple Michaelis–Menten model was used, with

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad (3)$$

Materials

Amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). L-[^{14}C]Glutamine (260 mCi/mmol) was supplied by Amersham Life Science (Arlington Heights, IL, U.S.A.). [^{3}H]Mannitol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis). Hyamine hydroxide and Cytoscint were purchased from ICN (Costa Mesa, CA, U.S.A.).

Statistics

Comparisons were made using ANOVA and post hoc multiple comparisons tests (see text). Differences were considered significant at the $p < 0.05$ level (two tailed, unless otherwise stated). Data in the text are mean ± SE values.

RESULTS

Reducing the extracellular Na^+ concentration from 148 to 1 m*M* in bicarbonate buffer resulted in a 53% decline ($p < 0.001$) in choroid plexus L-[^{14}C]-glutamine uptake (5.08 ± 0.33 and 2.38 ± 0.13 μl/mg/min, respectively; media glutamine concentration, 700 n*M*), demonstrating both Na^+-dependent and -independent uptake. The mechanisms of Na^+-dependent and -independent L-[^{14}C]glutamine uptake were examined by addition of several potentially competing amino acids (at 10 m*M*) in either 148 (Fig. 1A) or 1 (Fig. 1B) m*M* Na^+. The effect on Na^+-dependent uptake was determined by difference (Fig. 1C).

Unlabeled L-glutamine inhibited total, Na^+-independent, and Na^+-dependent uptake. 2-Amino-2-norbornane carboxylic acid (BCH) inhibited total and Na^+-independent uptake but had no effect on Na^+-dependent uptake. BCH is an L-system substrate (Christensen et al., 1969), and these results indicate that the Na^+-independent L-[^{14}C]glutamine uptake is via this transporter. α-(Methylamino)isobutyric acid (MeAIB), an A-system substrate (Christensen et al., 1965), had no effect on total, Na^+-dependent, or Na^+-independent uptake, indicating that the A-system is not involved in [^{14}C]glutamine transport in this tissue. L-Cysteine reduced total and Na^+-independent uptake but had no effect on Na^+-dependent uptake. Cysteine is both a substrate for the Na^+-independent L- and the

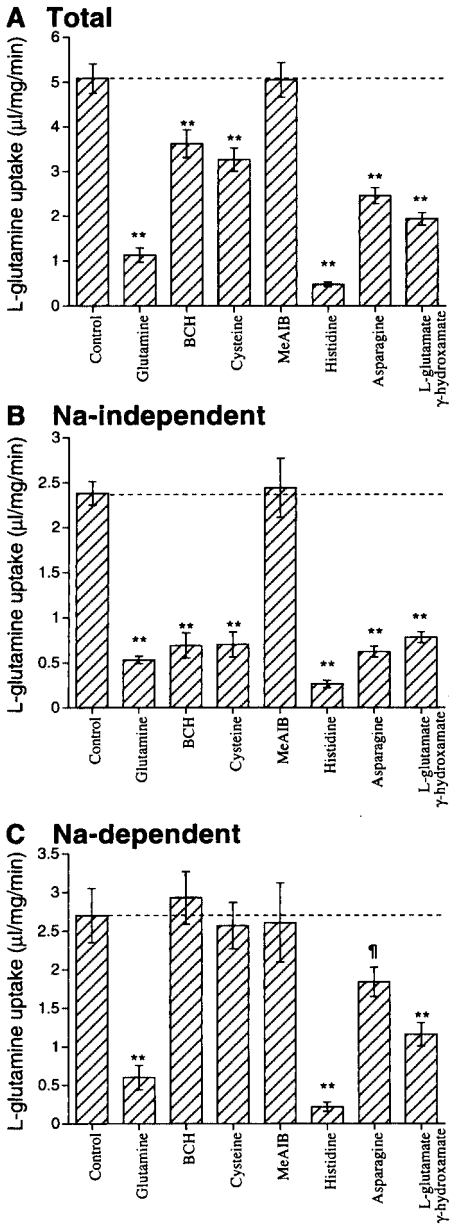

FIG. 1. L-[^{14}C]Glutamine uptake in the presence of (**A**) 148 or (**B**) 1 m*M* Na^+ in bicarbonate buffer. Measurements were made in the presence of different potentially competing amino acids (10 m*M*). Data are mean ± SE (bars) values (n = 5–22). **C:** The Na^+-dependent [^{14}C]glutamine uptake was determined by difference. Significant differences from the control (no competing amino acid) are indicated: $^{**}p < 0.01$ level. ¶$p < 0.05$, significant difference by one-tailed test. Comparisons with the control were made using Dunnett's (A and B) and Bonferroni (C) multiple comparison tests.

Na^+-dependent ASC-systems (Kilberg et al., 1979; Tayarani et al., 1987). The results on choroid plexus indicate that cysteine reduces [^{14}C]glutamine uptake via the former and that Na^+-dependent uptake is not via the ASC-system. L-Histidine reduced total, Na^+-independent, and Na^+-dependent uptake. Histidine is an L-, A-, and N-system substrate (Christensen, 1975; Kilberg et al., 1980). As the A-system does not appear to participate in glutamine uptake, these results suggest that histidine acts to inhibit [^{14}C]glutamine uptake by competing for both the Na^+-independent L- and the Na^+-dependent N-system.

As yet there are no specific substrates for the N-system transporter. However, work on other tissues has demonstrated certain properties of this system that we have also examined in choroid plexus. L-Asparagine is the third naturally occurring amino acid that is transported by the N-system in liver and skeletal muscle, although its affinity for the transporter appears to be less than that of either L-glutamine or L-histidine (Kilberg et al., 1980). Choroid plexus Na^+-dependent glutamine uptake was inhibited by L-asparagine ($p < 0.05$ by one-tailed t test), although to a lesser degree than by glutamine and histidine (Fig. 1). L-Glutamate γ-hydroxamate reduces N-system glutamine uptake in both hepatocytes and skeletal muscle (Kilberg et al., 1980; Low et al., 1991), and it has the same action on Na^+-dependent glutamine uptake in choroid plexus ($p < 0.01$; Fig. 1C). Finally, N-system uptake is less affected by Na^+ replacement with Li^+ than either the A- or ASC-system transporter (Kilberg et al., 1980). In choroid plexus Li^+ substitution in Tris buffer did not produce a significant reduction in choroid plexus [^{14}C]glutamine uptake, in contrast to the effect of choline substitution (Fig. 2).

The kinetics of L-glutamine uptake were examined in bicarbonate buffer by varying L-glutamine concentrations from 1 μM to 10 m*M* in the presence of 148 or 1 m*M* Na^+ (Fig. 3). Na^+-dependent uptake had a K_m of 3.3 ± 0.4 m*M* and a V_{max} of 8.1 ± 0.3 nmol/mg/min. Na^+-independent uptake was divided into two components: a saturable uptake system, with a K_m of 1.1 ± 0.6 m*M* and a V_{max} of 2.4 ± 0.7 nmol/mg/min, and an apparently nonsaturable mechanism, with a K_D of 0.32 ± 0.06 μl/mg/min. These measurements were made in the absence of possible competing amino acids that might normally be present in CSF. However, uptake in the presence of physiological CSF concentrations of amino acids in 148 and 1 m*M* Na^+ was not significantly different from uptake in the presence of glutamine alone (Fig. 4), which suggests that the con-

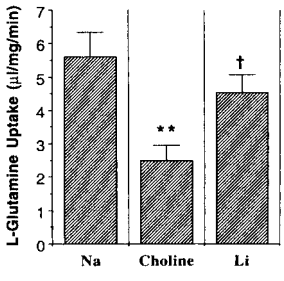

FIG. 2. L-[^{14}C]Glutamine uptake in Tris buffer in the presence of a normal Na^+ concentration or when Na^+ was replaced with choline or Li^+. Data are mean ± SE (bars) values (n = 5). $^{**}p < 0.01$ versus Na^+; †$p < 0.05$ versus choline. Comparisons between values are by ANOVA with Newman–Keuls multiple comparisons test.

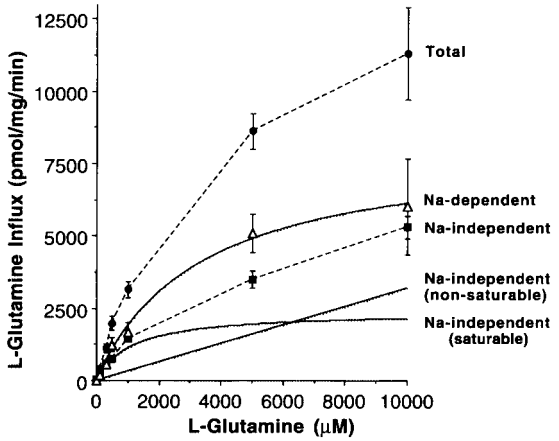

FIG. 3. Effect of external L-glutamine concentration on glutamine influx into choroid plexus in bicarbonate buffer. Measurements were made in the presence of 148 or 1 m*M* Na^+ (total and Na^+-independent uptake, respectively). Data are mean ± SE (bars) values (n = 5–22). The Na^+-dependent flux was determined by difference and fit to Michaelis–Menten kinetics. The Na^+-independent uptake was fit to a two-component model, with one system being saturable and the other nonsaturable.

centration of other amino acids in the CSF is normally not great enough to compete with glutamine for transport. It is possible, however, that some amino acids may be stimulating L-system transport, offsetting the effects of competition.

DISCUSSION

The results presented here indicate that L-glutamine uptake into the isolated rat choroid plexus is via both the Na^+-independent L-system and the Na^+-dependent N-system. L-system-mediated transport has previously been identified in mammalian choroid plexus (see, e.g., Davson et al., 1982; Segal et al., 1990). As with the blood–brain barrier (Betz and Goldstein, 1978), L-system transport appears present on both the blood- and brain-facing membranes of the choroid plexus and facilitates diffusion in either the blood-to-CSF (Segal et al., 1990) or CSF-to-blood (Davson et al., 1982) direction.

In contrast, N-system transport has not been previously identified at the choroid plexus. In terms of substrate specificity, Na^+ dependence, and Li^+ tolerance, the choroid plexus N-system transporter appears similar to that in hepatocytes and skeletal muscle (Kilberg et al., 1980; Hundal et al., 1987; Low et al., 1991). Also like in these tissues, L-glutamine transport at the choroid plexus is not mediated by either the A- or the ASC-system (Kilberg et al., 1980; Hundal et al., 1987). The capacity of the choroid plexus for glutamine transport is indicated by the similarity in the V_{max} for N-system transport (8 nmol/mg wet weight/min) to that found in hepatocytes [8 nmol/mg of intracellular water/min (Kilberg et al., 1980)].

In the sheep, glutamine uptake from blood to CSF is not Na^+ dependent (Segal et al., 1990). If this is also the case in the rat, it appears that the N-system transporter has an asymmetrical distribution, i.e., it is only present on the apical (brain-facing) membrane. It may therefore serve to move glutamine and the other naturally occurring substrates, histidine and asparagine, from the CSF to blood. Despite the presence of such a mechanism for glutamine transport at the blood–CSF barrier, the concentration of glutamine in CSF is only slightly less than that of plasma (see, e.g., Clarke et al., 1989). This suggests that glutamine clearance is being replaced by brain glutamine synthesis or that apical glutamine uptake is not matched under normal conditions by glutamine efflux across the basolateral (blood-facing) membrane into blood. In contrast, the level of histidine, an essential amino acid, is substantially less in CSF compared with plasma (see, e.g., Clarke et al., 1989).

The N-system transporter may be of particular importance in the clearance of CSF glutamine during hepatic encephalopathy, when hyperammonemia leads to cerebral glutamine synthesis and increased brain and CSF glutamine concentrations (for review, see Cooper and Plum, 1987). In this disease CSF glutamine content has been reported to increase from ~0.5 to 2.5 m*M* (Watanabe et al., 1984), which would be expected to increase clearance from the CSF through the N-system transporter because its K_m is ~3 m*M*. Indeed, Gjedde et al. (1978) have reported an increase in glutamine loss from the brain during hepatic encephalopathy induced by portacaval shunting, and Dejong et al. (1992) reported an increase during hyperammonemia induced by liver insufficiency. The increase in brain

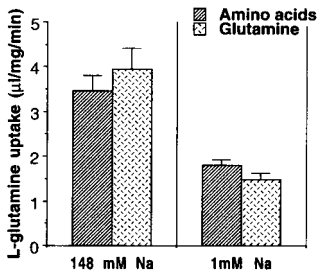

FIG. 4. L-[^{14}C]Glutamine uptake into isolated choroid plexus in the presence of either physiological concentrations of amino acids (including 500 μ*M* glutamine; see Materials and Methods) or 500 μ*M* glutamine alone. Measurements were made in the presence of 148 or 1 m*M* Na^+ (bicarbonate buffer). Data are mean ± SE (bars) values (n = 7–10). Na^+ removal produced a significant decrease in uptake with ($p < 0.01$) and without the presence ($p < 0.001$) of the other amino acids. The presence of the other amino acids made no significant difference in uptake in the presence of either 148 or 1 m*M* Na^+. Comparisons between values were by ANOVA with a Newman–Keuls multiple comparisons test.

glutamine concentration during hepatic encephalopathy appears to be necessary to the progression of this disease (Hawkins and Jessy, 1991; Takahashi et al., 1991; Hawkins et al., 1993) stressing the potential importance of this clearance mechanism. With a V_{max} of 8 nmol/mg wet weight/min, the N-system transporter in 6 mg of choroid plexus would be capable of clearing the whole glutamine content of a normal rat brain (~8,000 nmol) in ~3 h if the uptake were matched by glutamine efflux across the basolateral membrane into blood.

The mechanisms by which increases in brain glutamine levels may lead to hepatic encephalopathy are still uncertain. Brain glutamine, by acting as an osmolyte, may contribute directly to brain edema (Takahashi et al., 1991). However, the uptake of amino acid substrates of the L-system is also increased in hepatic encephalopathy (Zanchin et al., 1979; Mans et al., 1983). In isolated cerebral microvessels, intracellular glutamine can stimulate amino acid influx via the L-system (James and Fischer, 1981; Cangiano et al., 1983), suggesting that the increased transport in hepatic encephalopathy might result from the elevated brain glutamine concentrations. As several of the amino acids transported by the L-system carrier are neurotransmitter precursors, increased transport may lead to a derangement in brain function. If the N-system transporter is present at the blood–brain as well as the blood–CSF barrier, it may be a major determinant of intracellular glutamine level and hence the degree of L-system transport stimulation.

In summary, a major component of glutamine transport in the isolated choroid plexus is via the Na^+-dependent N-system. This may have important consequences for the regulation of the brain concentration of this amino acid under normal and pathophysiological conditions. Glutamine transport via this system will also affect the transport of other amino acids that are transported by either the N- or the L-system transporter.

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