Characterization of rapid and high-affinity uptake of L-serine in neurons and astrocytes in primary culture

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Abstract **The non-essential amino acid L-serine was shown to be required to support the survival of rat cerebellar Purkinje neurons because of lack of the expression of the L-serine biosynthesis enzyme 3-phosphoglycerate dehydrogenase in them. In the present study, we investigated L-[^{3}H]serine uptake in primary cultures of neurons and astrocytes from the rat telencephalon. In both neurons and astrocytes, L-[^{3}H]serine uptake was dependent on temperature and Na^+ ions, and exhibited a single component of high-affinity uptake sites (K_m = 15.0 and 17.2 μM for neurons and astrocytes, respectively). Kinetic analysis of L-[^{3}H]serine uptake also revealed that the uptake into neurons was faster than that into astrocytes. The selectivity of inhibition by amino acids of the L-[^{3}H]serine uptake resembled that of the system ASC transporters ASCT1 and ASCT2. Neutral amino acids L-alanine, L-serine, L-cysteine, and L-threonine strongly inhibited the uptake by both cell types. Furthermore, in astrocytes, but not in neurons, L-valine and L-proline also inhibited L-[^{3}H]serine uptake. Neither α-methyl aminoisobutyric acid (a system A-specific substrate) nor 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (a system L-specific substrate) inhibited the uptake of L-[^{3}H]serine in both neurons and astrocytes. Expression of ASCT transporters in both neurons and astrocytes was examined by use of reverse transcriptase polymerase chain reaction and immunoblot analysis. Whereas transcripts (mRNAs) of both ASCT1 and ASCT2 transporters were detected in astrocytes, only the mRNA of the former subtype was detected in neurons. Immunoblot analysis confirmed the presence of ASCT1 in both neurons and astrocytes. These findings indicate that neurons accumulate a high level of L-serine by using a Na^+-dependent, high-affinity transport system, operating predominantly through the ASCT1 transporter subtype.**

Key words: Serine transport; 3-Phosphoglycerate dehydrogenase; ASCT1; Reverse transcriptase polymerase chain reaction

1. Introduction

L-Serine is well-known to be a non-essential amino acid that can be synthesized by many cells. A recent study demonstrated that cerebellar Purkinje neurons had an essential requirement for extracellular L-serine to support their survival [1]. L-Serine is a key amino acid for the synthesis of the neurotransmitters glycine and D-serine in the brain [2,3], in addition to being a precursor for the biosynthesis of various protein and membrane lipid molecules. In the central nervous system, glycine and D-serine were shown to be especially important by acting as co-agonists in the regulation of *N*-methyl-D-aspartate receptor function [4–7]. L-Serine is synthesized from the glycolytic intermediate 3-phosphoglycerate by three enzymes, the initiating enzyme being 3-phosphoglycerate dehydrogenase (3PGDH) [8]. Recently, immunohistochemistry and in situ hybridization studies on 3PGDH demonstrated that 3PGDH expression was down-regulated in neurons of the developing mouse brain but highly expressed in astroglia [9]. In particular, cerebellar Purkinje neurons showed no detectable mRNA or immunoreactivity for 3PGDH [1,9]. Therefore, those 3PGDH-negative neurons require specific systems for the uptake of extracellular L-serine to support their survival and development.

The uptake of L-serine occurs through several transport systems, including Na^+-dependent transporters such as neutral amino acid transporter system ASC and system A, and Na^+-independent transporters, system L and system asc. System ASC transporters ASCT1 and ASCT2 have been identified and cloned from humans and mice [10–13]. Although the expression of the ASCT1, but not ASCT2, subtype of neutral amino acid transporter has been detected in brain, detailed aspects and its roles in L-serine transport in the brain remain to be clarified. In the present report, we provide a detailed characterization of L-serine transport into neurons and astrocytes, which is effected by Na^+-dependent and high-affinity transporters.

2. Materials and methods

2.1. Materials

L-[^{3}H]Serine (specific radioactivity 851.0 GBq/mmol) was purchased from NEN Life Science Products (Boston, MA, USA). 2-Methylaminoisobutyrate (MeAIB), 2-aminobicyclo(2,2,1)heptane-2-carbox-

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Abbreviations: 3PGDH, 3-phosphoglycerate dehydrogenase; MeAIB, α-methyl aminoisobutyric acid; BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid

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ylic acid (BCH), and all amino acids were obtained from Sigma-Aldrich. All other reagents used were analytical grade.

2.2. Cell cultures

Primary neuronal cell cultures were prepared from the fetal rat telencephalon (embryonic day 18 or 19) as described before [14]. Following dissociation for 15–20 min at 37°C with 0.25% trypsin (Difco) in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline containing 0.02% DNase I (Sigma), the cells were mechanically dispersed by repetitive pipetting and filtered through a nylon mesh. They were then rinsed with a defined culture medium, Dulbecco's modified Eagle's medium (DMEM), and plated on poly-L-lysine-coated wells of 96-well plates at a final density of $0.5–1.5\times10^6$ cells/ml. The cultures were maintained for 7 days in DMEM supplemented with 1 mg/ml bovine serum albumin (BSA), 10 μg/ml insulin, 1 nM 3,3′,5-triiodo-L-thyronine, 0.1 mg/ml human transferrin, 1 μg/ml aprotinin, 100 μM putrescine, 1 mM sodium pyruvate, 10 nM progesterone, 30 nM selenium, 0.1 mg/ml streptomycin sulfate, and 50 U/ml penicillin G potassium salt (Meiji Seika, Tokyo, Japan) in a humidified atmosphere of 5% CO_2 in air at 37°C. More than 95% of the cultured cells were negative for immunoreaction with anti-glial fibrillary acidic protein antibody (data not shown).

Astrocyte cultures were derived from the same preparation as the above described method preparing neuronal cultures. The dispersed cells were plated on poly-L-lysine-coated wells of 96-well plates and maintained for 14–21 days in DMEM containing 5% fetal calf serum and 5% horse serum supplemented with 0.1 mg/ml streptomycin sulfate and 50 U/ml penicillin G potassium salt.

2.3. L-[^{3}H]Serine uptake experiment

On the day of the experiment, the culture medium was aspirated from the neuronal or astrocyte cell cultures, and these cells were washed three times with 0.1 ml of uptake buffer containing 5 mM HEPES-NaOH (or KOH) (pH 7.2), 140 mM NaCl (or choline-Cl), 5 mM KCl, 1 mM KH_2PO_4, 1.8 mM $CaCl_2$, 0.4 mM $MgCl_2$, and 10 mM glucose. After the cells had been preincubated in the uptake buffer for 30 min at 37°C, they were incubated at 37°C for 5 (neuronal) or 10 (astrocytes) min in 0.1 ml of fresh uptake buffer supplemented with 20 nM (final concentration) L-[^{3}H]serine and different amounts of unlabeled amino acids. Uptake was terminated by rapid removal of the medium followed by quick washes with ice-cold phosphate-buffered saline. To measure the amount of L-[^{3}H]serine taken up into the cells, we added 50 μl of a 10% sodium dodecyl sulfate (SDS) solution to each well and solubilized the cells for 1 h at 37°C. The radioactivities were measured using a liquid scintillation counter. Each uptake was represented as the mean of three to four independent experiments, each done in quadruplicate. Na^+-independent uptake was carried out by incubation of cells in medium containing choline chloride instead of NaCl. Protein content was measured using a Bradford protein assay kit (Bio-Rad, Richmond, CA, USA) after the cells had been solubilized with 0.1 M NaOH.

2.4. RT-PCR analysis of ASCT transporter

Expression of ASCT1 and ASCT2 transcripts in neurons and astrocytes was examined by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA from cultured rat primary neurons and astrocytes was isolated using a Catrimox-14 RNA Isolation kit (TaKaRa, Osaka, Japan) according to the manufacturer's protocol. RT-PCR was performed with an RNA LA PCR kit (TaKaRa). RT was performed at 50°C for 30 min, followed by denaturation at 90°C for 5 min. PCR amplification of cDNAs of ASCT1, ASCT2, and GAPDH (as an internal standard) was then performed for 35 cycles of 94°C for 30 s for denaturation, 58°C for 30 s for primer annealing, and 72°C for 2 min for DNA extension. Sequences of the primers for ASCT1, ASCT2, and GAPDH cDNAs were as follow: for rat ASCT1: forward primer, 5′-GTTTGCGACGGCTTTTGCGACCTG-3′; reverse primer, 5′-GCATCCCCTTCCACGTTCACCACA-3′; product size, 399 bp (GenBank accession number AB103401), for rat ASCT2: forward primer, 5′-GCGCCTGGGCCCTGCTCTTTTT-3′; reverse primer, 5′-ACAATCTTGCCGGCCACCAGGAAC-3′; product size, 478 bp (GenBank accession number AJ132846); and for rat GAPDH: forward primer, 5′-GTCAGTGCCGGCCTCGTCTCATAG-3′ reverse primer 5′-GACCCTTTTGGCACCACCCTTCAG-3′; product size, 380 bp (GenBank accession number M17701). The PCR amplification products were separated on a 3% agarose gel, stained with ethidium bromide, and visualized under UV irradiation.

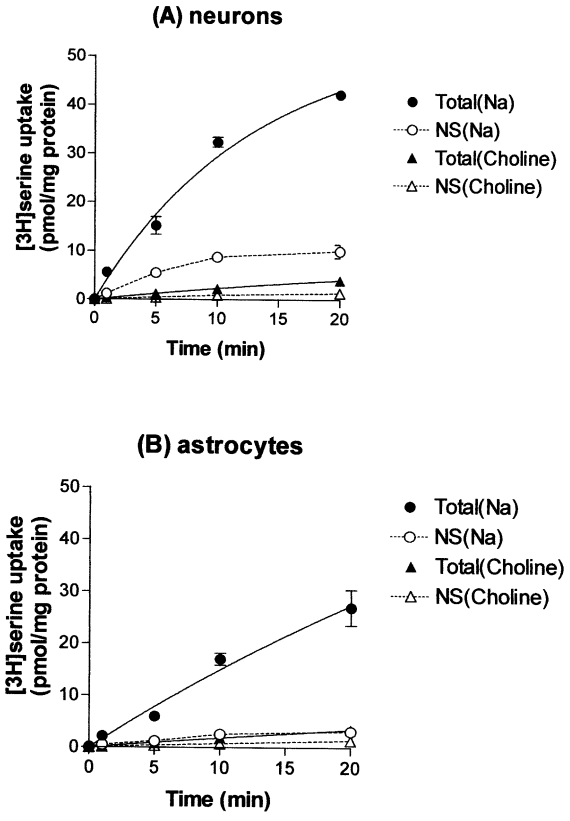

Fig. 1. Kinetics of L-[^{3}H]serine uptake into neurons (A) and astrocytes (B). L-[^{3}H]Serine uptake was assayed in primary neuron or astrocyte cultures in uptake buffer containing 10 μM L-[^{3}H]serine in the presence of either 140 mM NaCl or 140 mM choline-Cl. Nonspecific uptake (NS) was measured in the presence of 1 mM L-serine. Data are the mean ± S.E.M. of four independent experiments done in quadruplicate.

2.5. Immunoblotting

Both neuronal and astroglial cells were harvested and lysed in 50 mM Tris–HCl (pH 7.4) buffer containing 1 mM EDTA and complete protease inhibitor cocktail (Roche). After the homogenates had been solubilized in SDS-sample buffer and denatured at 100°C for 3 min, proteins were separated on 10% SDS–polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene difluoride membranes (ClearBlot, ATTO, Tokyo, Japan) by electroblotting. The blots were blocked with 3% BSA and 0.05% Tween 20 in Tris-buffered saline (TBS) at 4°C overnight and subsequently incubated at room temperature for 2.5 h with anti-ASCT1 antibody (1:500; Chemicon International, Temecula, CA, USA) in TBS (10 mM Tris–HCl [pH 7.5], 0.15 M NaCl) containing 0.1% BSA and 0.05% Tween 20 and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) (New England Biolabs) at room temperature for

Table 1
Kinetic characteristics of Na^+-dependent uptake of L-[^{3}H]serine in primary cultures of rat neurons and astrocytes

	K_m (μM)	V_{max} (nmol/min/mg protein)
Neurons	15.0 ± 1.6	2.456 ± 0.202
Astrocytes	17.2 ± 2.1	1.130 ± 0.272

Values of K_m and V_{max} are means ± S.E.M. calculated from a Lineweaver–Burk plot generated from the data obtained from saturation experiments using increasing concentrations of L-[^{3}H]serine (3–50 μM). Na^+-dependent uptake was defined as the difference between Na^+-containing buffer and choline-substituted buffer.

1 h. Immunoreactivities were visualized by the reaction of the blots with 0.1% diaminobenzidine, 0.012% H_2O_2, and 0.04% Ni-ammonium sulfate in 0.2 M Tris–HCl buffer, pH 7.5.

2.6. Calculations

Kinetic parameters were calculated by linear regression from Lineweaver–Burk plots using GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA, USA). IC_{50} values of amino acids were estimated by non-linear regression analysis with Prism 3.0.

3. Results

3.1. L-Serine uptake into primary neurons and astrocytes

At 37°C, L-[^{3}H]serine was taken up into primary neurons in a time-dependent manner, and its level reached a plateau after 20 min in the presence of Na^+ ions (Fig. 1A). In astrocytes, L-[^{3}H]serine was also taken up in a time-dependent manner and still increased linearly after 20 min (Fig. 1B). Furthermore, only a small amount of L-[^{3}H]serine uptake was detected in both primary neurons and astrocytes when choline-supplemented, Na^+-free uptake buffer was used (Fig. 1A,B), thus suggesting that most of the uptake occurred via a Na^+-dependent component. The initial rate of L-[^{3}H]serine uptake into neurons appeared to be faster than that into astrocytes ($t = 9.8$ min and 88 min, respectively).

L-[^{3}H]Serine uptake was concentration-dependent at 37°C (Fig. 2), and was dramatically suppressed at 4°C (data not shown). The uptake of L-[^{3}H]serine was saturable and followed Michaelis–Menten kinetics, which indicated a single uptake site. The K_m and V_{max} values (mean ± S.E.M.) were

Table 2
Effect of various amino acids on L-[^{3}H]serine uptake into rat neurons and astrocytes in primary culture

Amino acid	Astrocytes IC_{50} (μM)	Neurons IC_{50} (μM)
L-Ser	40.23 ± 10.87	67.03 ± 10.88
D-Ser	561.20 ± 149.8	> 1000
L-Ala	39.14 ± 14.14	90.19 ± 12.17
L-Cys	13.18 ± 3.47	35.71 ± 5.33
L-Thr	37.71 ± 8.42	64.64 ± 7.15
L-Asn	37.27 ± 7.33	141.5 ± 14.51
L-Val	76.38 ± 22.47	368.00 ± 15.20
L-Pro	176.25 ± 30.95	519.40 ± 61.60
L-Leu	241.00 ± 75.42	> 1000
L-Ile	267.9 ± 183.6	> 1000
L-Met	334.1 ± 38.74	> 1000
L-Gln	574.50 ± 77.73	> 1000
Gly	724.9 ± 113.8	> 1000
L-Asp	> 1000	> 1000
L-Glu	> 1000	> 1000
L-Phe	> 1000	> 1000
L-Tyr	> 1000	> 1000
L-Trp	> 1000	> 1000
L-His	> 1000	> 1000
L-Arg	> 1000	> 1000
L-Lys	> 1000	> 1000
MeAIB	> 1000	> 1000
BCH	> 1000	> 1000

IC_{50} values were calculated by non-linear regression analysis using the GraphPad program. Data are the mean ± S.E.M. of four to five separate experiments.

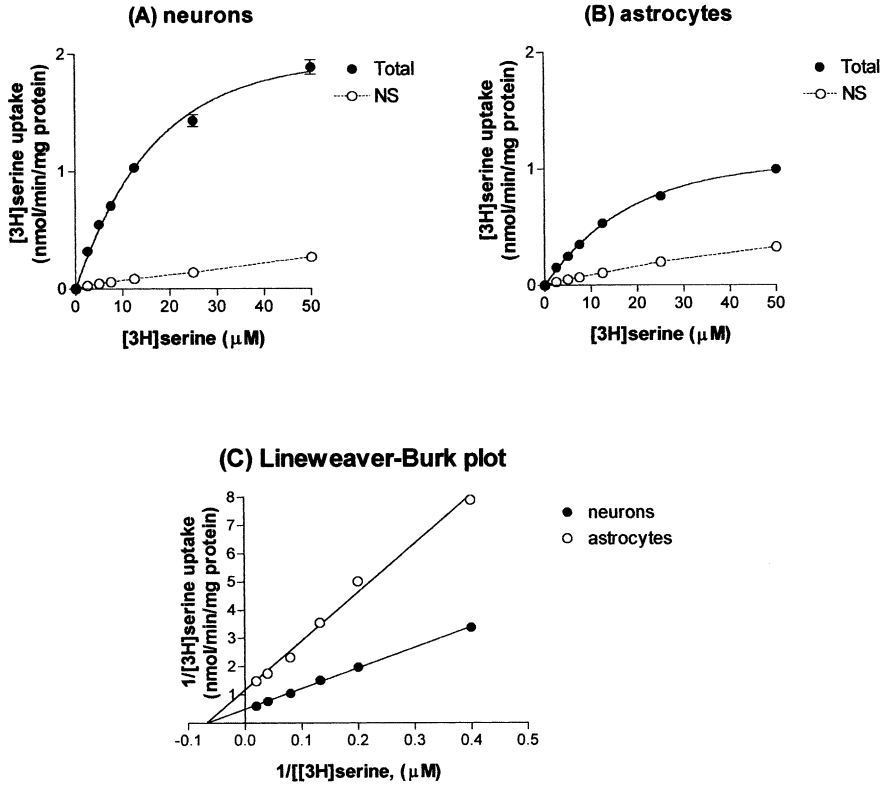

Fig. 2. Saturation analysis of L-[^{3}H]serine uptake into primary neurons (A) or astrocytes (B). Uptake was measured in the presence of increasing concentrations of L-[^{3}H]serine. C: Lineweaver–Burk plots were used to calculate kinetic parameters, which for the representative experiment shown were: $K_m = 14.7$ μM and $V_{max} = 2.013$ nmol/min/mg protein for neurons, and $K_m = 17.6$ μM and $V_{max} = 1.402$ nmol/min/mg protein for astrocytes.

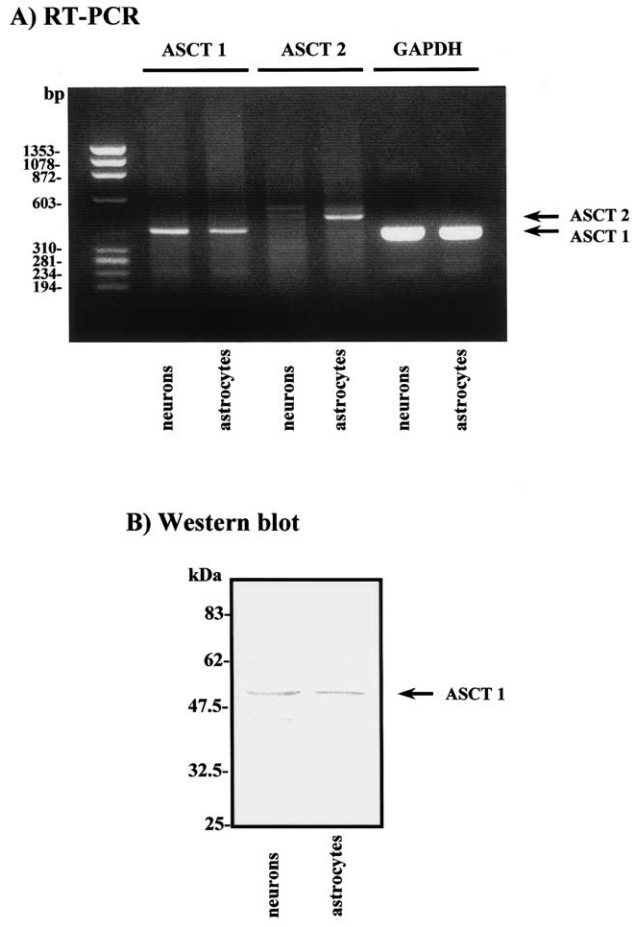

Fig. 3. RT-PCR and immunoblot analysis of ASCT expression in primary cultures of rat neurons and astrocytes. A: ASCT1 and ASCT2 subtype transcripts in neurons and astrocytes. Amplification products specific to ASCT1 (399 bp), ASCT2 (478 bp), and GAPDH (380 bp), the last as a control, were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The left lane is a molecular size marker. B: Immunoblot staining revealing ASCT1 (~55 kDa) expression in neurons and astrocytes.

15.0 ± 1.6 μM and 2.456 nmol/min/mg protein, respectively, for neurons and 17.2 ± 2.1 μM and 1.130 nmol/min/mg protein, respectively, for astrocytes (Fig. 2, Table 1).

3.2. Inhibition by various amino acids of the L-[^{3}H]serine uptake

To evaluate the specificity of the uptake system, the inhibitory potential of various amino acids on the uptake of L-[^{3}H]serine was measured for primary neurons and astrocytes. The neutral amino acids L-serine, L-alanine, L-cysteine, and L-threonine, but not glycine, strongly inhibited uptake in both primary neurons and astrocytes (Table 2). MeAIB and BCH, which are system A- and system L-specific substrates, respectively, did not inhibit the uptake. D-Serine was only weakly inhibitory in astrocytes. The strong inhibition of L-[^{3}H]serine uptake by L-alanine, L-serine, L-cysteine, L-threonine, and L-asparagine, and the lack of inhibition by MeAIB and BCH resemble the properties and specificity of ASCT-type transporters. In neurons, L-cysteine, L-serine, L-threonine, and L-alanine potently inhibited L-[^{3}H]serine uptake, with IC_{50} values in the range of 35–90 μM. Furthermore, L-asparagine exhibited an IC_{50} value of 141 μM, whereas L-valine and L-proline showed higher IC_{50} values (368–519 μM). On the other hand, in astrocytes, L-[^{3}H]serine uptake was also inhibited by L-asparagine and L-valine with high potency (IC_{50} values, 37–76 μM), in addition to being blocked by the above-mentioned neutral amino acids (L-cysteine, L-serine, L-threonine, and L-alanine; IC_{50} values, 13–40 μM). Also, L-proline, L-leucine, L-isoleucine, and L-methionine were weakly inhibitory (IC_{50} values, 176–334 μM). Other amino acids were inactive in preventing the uptake of L-[^{3}H]serine into both neurons and astrocytes. These inhibitory profiles of amino acids indicate that L-[^{3}H]serine may be taken up into astrocytes by both ASCT1 and ASCT2 transporters.

3.3. Expression of ASCT1 and ASCT2 in primary neurons and astrocytes

To confirm the possible involvement of ASCT1 and ASCT2 transporters in the uptake of L-[^{3}H]serine, we examined the expression of ASCT1 and ASCT2 at both mRNA and protein levels. Fig. 3A shows that ASCT1 transcripts were expressed by both rat neurons and astrocytes in primary culture, as examined by RT-PCR experiments employing primer pairs specific for each of the rat ASCT subtypes, but that ASCT2 mRNA could be amplified only from astrocytes. The weak-intensity bands of higher MW products in the ASCT2 lane were not ASCT2-specific fragments as determined by using sequence analysis. Also, we examined neurons and astrocytes for the presence of ASCT1 protein (~55 kDa) using anti-ASCT1 antibody and detected an immunoblot band representing this protein in samples from both cell types (Fig. 3B).

4. Discussion

This is the first detailed study of the mechanism of L-[^{3}H]serine uptake in neurons and astrocytes. In this study, we reported the kinetics and pharmacological characteristics of L-[^{3}H]serine uptake sites in rat primary neurons and astrocytes. L-Serine and other neutral amino acids, such as L-alanine and L-cysteine, are transported by several carrier systems, including the Na^+-dependent system ASC and system A, and also by the Na^+-independent system L and system asc. So far, it has been reported that ASCT-type transporters predominantly act in the Na^+-dependent uptake of small neutral amino acids [15]. Our present results clearly indicate that the major transport component of L-[^{3}H]serine uptake into both neurons and astrocytes acted mostly (>95%) in a Na^+-dependent manner and with high affinity (lower K_m values). Therefore, the contribution of the Na^+-independent system L and system asc to L-[^{3}H]serine uptake may be deemed negligible. This notion was further confirmed by the lack of inhibition by BCH, a system L-specific substrate. Moreover, L-[^{3}H]serine uptake into both neurons and astrocytes was not inhibited by MeAIB, a system A-specific substrate, indicating that members of the Na^+-dependent system ASC transporter family predominantly contribute to the uptake of L-[^{3}H]serine in both cell types.

In neurons, inhibition of Na^+-dependent L-[^{3}H]serine uptake was mostly restricted to that by amino acid substrates for the ASCT1 transporter subtype, such as L-cysteine, L-threonine, L-alanine, L-asparagine, and L-serine (in order of decreasing potency of inhibition). Moreover, the finding of the expression of ASCT1 mRNA and protein in neurons

strongly supports this notion. In addition, we further confirmed little or no expression of ASCT2 mRNA in primary neurons. Taken together, our data indicate that ASCT1 may specifically act as the major component of the transport system for L-serine uptake into neurons. On the other hand, the uptake of L-[^{3}H]serine in astrocytes could be also inhibited by L-valine, L-proline, L-leucine, and L-isoleucine, in addition to the above-mentioned ASCT1 substrate amino acids. These inhibitory profiles for amino acid selectivity indicate the additional contribution of the ASCT2 transporter in astrocytes, at least in part. Indeed, the RT-PCR experiment provided the evidence that ASCT2 mRNA was expressed in astrocytes, but not in neurons. The finding of the expression of ASCT2 mRNA in astrocytes is in agreement with a previous report [16]. Thus the present data clearly demonstrate that L-serine is transported into primary neurons and astrocytes differently, i.e. neurons predominantly use ASCT1, whereas astrocytes may use both ASCT1 and ASCT2 transporters.

L-Serine was taken up more rapidly into neurons than into astrocytes. Saturation kinetics revealed that the V_{max} value was two-fold greater in neurons than in astrocytes, which strongly indicates that neurons are capable of accumulating L-serine from extracellular spaces faster than astrocytes. This difference in the uptake rate of L-serine between neurons and astrocytes might correspond to the requirement of neurons for this amino acid. Indeed, most astrocytes highly expressed the enzyme of L-serine synthesis, 3PGDH, and thus can produce L-serine by themselves [1,9]. Thus, our finding of this rapid uptake of L-serine in neurons indicates that neurons have a strong need for extracellular L-serine released from neighboring astrocytes, which cells would contain higher levels of L-serine. L-Serine has been reported to have a marked effect on the survival and neurite outgrowth of neurons in culture [1,17]. Furthermore, it has been shown previously in culture studies that exogenous L-serine is required for the biosynthesis of phosphatidyl-L-serine and sphingolipids in hippocampal neurons [18]. Taken together, the facilitated uptake of L-serine is thought to be essential for maintaining neuronal function and survival.

In conclusion, the present study is the first to demonstrate that primary neurons and astrocytes differently take up L-serine by using Na^+-dependent, high-affinity transporter systems consistent with the ASCT1 and ASCT1/2 transporters, respectively. Furthermore, the data indicate that these differences in the L-serine transport system between neurons and astrocytes reflect the cellular requirement of this amino acid and that these transport mechanisms may play a key role in the regulation of protein and lipid biosynthesis in neurons, and in neuronal development and survival.

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