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Research report

# Glial transport of the neuromodulator D-serine

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**Abstract**

D-Serine is an endogenous agonist of NMDA receptors that occurs in astrocytes in gray matter areas of the brain. D-Serine is synthesized from L-serine by the activity of a glial enriched serine racemase, but little is known on the properties of D-serine transport and factors regulating its synaptic concentration. In the present report we characterize the transport of D-serine in astrocytes. In primary astrocyte cultures, D-serine uptake is dependent on sodium ions and exhibits both low affinity and low specificity for D-serine. The kinetics of D-serine transport resembles that of ASCT type transporters as several small neutral amino acids strongly inhibit the uptake of D-serine. D-Serine fluxes are coupled to counter-movement of L-serine and to a less extent to other small neutral amino acids. Thus, addition of D-serine to cell cultures elicits robust efflux of intracellular L-serine. Conversely, physiological concentrations of L-serine induce efflux of preloaded D-serine from astrocytes. L-Serine was more effective than kainate, which have been previously shown to induce D-serine release from astrocytes upon stimulation of non-NMDA type of glutamate receptors. The features of D-serine transport we describe reveal possible new mechanisms controlling the synaptic concentration of D-serine. 

*Theme:* Neurotransmitters, modulators, transporters and receptors

*Topic:* Uptake and transporters

*Keywords:* D-Serine; Serine racemase; Astrocyte; L-Serine; D-Amino acid oxidase; *N*-Methyl D-aspartate; Glutamate receptor

## 1. Introduction

High levels of D-serine are found in mammalian brain where it is an endogenous agonist of the glycine site of *N*-methyl D-aspartate (NMDA) type of glutamate receptors [6,16,19]. NMDA receptor requires co-activation at a 'glycine site', at which D-serine is at least as potent as glycine [3]. Immunohistochemical studies localized D-serine to protoplasmic astrocytes in areas enriched in NMDA receptors, while glycine densities are highest in the brainstem and spinal cord [20]. Extracellular concentrations of D-serine are comparable to glycine in several brain areas, while in the striatum, D-serine levels are more than twice the values for glycine [8]. Selective destruction of the endogenous D-serine by applying D-amino acid oxidase decreases the activity of NMDA receptors measured both biochemically and electrophysiologically, suggesting that endogenous levels of D-serine are required for the normal activity of NMDA receptors [16].

As an endogenous co-agonist of NMDA receptors, D-serine may play a role in several pathological conditions related to NMDA receptor dysfunction. NMDA receptor hypofunction has been shown to play a role in the pathophysiology of schizophrenia [15]. In schizophrenic subjects treated with conventional neuroleptics, administration of D-serine greatly improves the negative, positive and cognitive symptoms of the disease [23]. By contrast, D-serine might be deleterious in conditions in which overstimulation of NMDA receptors leads to excitotoxicity and cell death [2]. D-Serine is released into cerebroventricular fluid in animal models of stroke [13] and drugs that block the 'glycine site' of NMDA receptors prevent stroke damage [3].

D-Serine is synthesized from L-serine by serine racem-

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ase, a pyridoxal 5′-phosphate-dependent enzyme enriched in brain glial cells [4,27,28]. The distribution of mouse and human serine racemase closely resembles that of D-serine and pharmacological inhibition of the enzyme diminishes D-serine levels in astrocyte cultures, suggesting that serine racemase physiologically synthesizes D-serine to regulate NMDA receptors [17,28].

Though the biosynthetic pathway for D-serine has been established, several aspects of D-serine disposition in the brain remain to be clarified. The mechanisms of D-serine transport and release are poorly understood and the fate of D-serine released in the synapse is currently unknown. It has been proposed that upon stimulation of non-NMDA receptors, D-serine is released from astrocytes to regulate NMDA receptors in nearby synapses [19]. Removal and recycle of released D-serine would require a specific transport system. However, studies of the properties and specificity of D-serine uptake in astrocytes have not been done yet. The few studies showing D-serine transport in cells other than astrocytes exhibit differences regarding the requirement for sodium ions. A sodium-dependent transport of D-serine has been detected in C6 glioma cells [10], while Fukasawa et al. identified a putative sodium-independent amino acid transporter that also transports D-serine when expressed in *Xenopus* oocytes [5].

In the present report, we provide a detailed characterization of D-serine transport system in cultured astrocytes. We found D-serine transport to be dependent on sodium ions and show that D-serine elicits robust release of L-serine from cultured astrocytes. Both the influx and the efflux of D-serine are coupled to L-serine counter-movement and to a less extent to other neutral amino acids, suggesting that D-serine transporter works as an antiporter. The properties of D-serine transport we describe will help to understand the factors controlling the synaptic concentration of D-serine.

## 2. Materials and methods

### 2.1. Astroglia-rich primary cultures

Post-natal day 0–1 Wistar rats were deeply anesthetized in ether and decapitated. The brains were rapidly removed and placed in phosphate-buffered saline supplemented with 10% glucose. Then, each cerebral cortex was dissected and freed of meninges. Cells were mechanically dissociated and plated in 24-well plates (Costar) at a density of $1\times10^6$ cells/well in Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum, 5 mM glutamine and penicillin-streptomycin. Every 2 days, cultures were vigorously washed several times with media to dislodge microglia and neurons. Cultures were used after 14 days and contained negligible amounts of neurons. Identical results were obtained utilizing a purified type 1 astrocyte culture, in which the contaminating microglia and oligodendrocyte precursors were removed by overnight shaking of culture flasks as described [12]. All experiments were carried out in accordance with the European Communities Council directive 86/609/EEC.

### 2.2. D-Serine uptake

For D-serine uptake in culture cells, media were aspirated and the cells were washed twice with 1.0 ml uptake buffer containing 10 mM HEPES–Tris (pH 7.4), 100 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl and 1 mM $CaCl_2$. Then, the cells were incubated with 0.3 ml of uptake medium supplemented with 0.1 μCi D-[$^3$H]serine and different amounts of unlabeled D-serine for 10–20 min at 37 °C. Uptake was stopped by aspiration of the medium followed by two quick washes with 2 ml uptake medium. To quantify the amount of D-serine taken up by the cells, 0.2 ml of a 1% Triton X-100 solution was added to each well and further incubated for 10 min. The detergent solution containing the radioactive amino acid was counted on a scintillator counter. Each point was done in triplicate or quadruplicate and the amount of D-serine taken up was calculated by normalizing the values to the protein content. Blanks were carried out by incubation of sample on ice in medium containing choline chloride instead of NaCl. Blanks never exceeded 10% of the sample counts. The effects of amino acids on D-serine uptake were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test.

### 2.3. Amino acid determination

Cells or uptake media were treated with 5% trichloroacetic acid (TCA) to precipitate proteins and extract the free amino acids. Samples were spun at 20 000 *g* for 5 min, the supernatants were extracted three times with water-saturated diethyl ether to remove TCA and the samples were analyzed by high-performance liquid chromatography (HPLC) [7].

### 2.4. Amino acid release

To examine the amino acid efflux induced by D-serine, astrocyte cultures were incubated in uptake medium supplemented with different concentrations of non-radioactive D-serine. After 10–20 min, an aliquot of the medium was collected and several amino acids in the medium were analyzed by HPLC. The values of amino acid release elicited by D-serine were calculated by subtracting the amounts of amino acids that non-specifically leaked from the cells (basal efflux) in media not containing D-serine. In some experiments, cellular amino acids were analyzed by HPLC before and after D-serine addition to monitor the decrease in cellular amino acids levels caused by the efflux induced by D-serine. For D-serine release experiments, cells were first loaded with 200 μM D-[$^3$H]serine in uptake

medium for 30 min at 37 °C. Then, cells were washed twice to remove the D-serine that was not taken up, and the medium was changed to a fresh one containing test drugs. After different times, an aliquot was removed and counted on a scintillation counter.

### 2.5. Staining for D-serine

For immunocytochemistry, primary astrocytes were plated on two-well glass-slides and incubated with D-serine in uptake medium, The cells were subsequently fixed with 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and 0.1% sodium metabisulfite for 30 min at 37 °C. After reduction of free aldehyde groups by 20 min incubation in 0.5% sodium borohydride, cells were incubated with a purified anti-D-serine antibody (1:2000 dilution) raised against a reduced glutaraldehyde conjugate of D-serine [19]. To avoid any cross reactivity with L-serine present in the cells, incubations were carried out in the presence of 0.5 mM L-serine–glutaraldehyde conjugate. Pre-absorption of the antibody with 0.5 mM D-serine–glutaraldehyde conjugate abolished immunoreactivity. Staining was developed with immunoperoxidase ABC Elite kit (Vector, CA, USA) using 3,3′-diaminobenzidine as substrate.

### 2.6. Materials

L- and D-amino acids, α-(methylamino)isobutyric acid and kainate were obtained from Sigma (St. Louis, MO, USA). DNQX was purchased from Tocris (Balwin, MO, USA). Cell culture reagents were purchased from Life Technologies. D-[3-$^3$H]Serine was purchased from Moravek (CA, USA). The batch was analyzed by HPLC and was found to be of 98% purity. L-[$^3$H]Serine was obtained from New England Nuclear.

## 3. Results

We found that primary astrocytes cultures take up D-serine in the presence of sodium ions (Fig. 1A). Kinetic analysis indicate a single $K_m$ of 650±100 μM and a maximal velocity of 17±3 nmol/mg min (Fig. 1B). HPLC analysis of intracellular amino acids reveals that D-serine is not significantly metabolized in the course of the experiment (data not shown). D-Serine taken up by astrocytes can also be revealed by immunocytochemistry using an antibody that selectively recognizes D-serine (Fig. 2B). Astrocytes incubated in the absence of D-serine exhibit much lower immunoreactivity, presumably by the presence of low levels of endogenous D-serine in the cells (Fig. 2A). All cells take up D-serine at similar levels, suggesting that the transport system is widely and evenly expressed in the culture. Pre-absorption of the antibody with D-serine glutaraldehyde conjugate abolishes immunoreactivity (Fig. 2C).

To examine the specificity of the transporter, we tested the effects of several L- and D-amino acids on D-serine uptake (Fig. 3). D-Serine uptake seems to be mediated by a neutral amino acid transport system. Transport of D-serine is inhibited by L-amino acids, following the order: L-Cys> L-Thr=L-Ser=L-Ala>L-Met>L-Gln>L-Leu>L-Val>Gly= L-Pro. Charged and aromatic amino acids have practically no effect (Fig. 3). D-Serine transport is also unaffected by excess (2 mM) 2-(methylamino)-isobutyric acid, a specific substrate for the system A amino acid transporter (data not shown). D-Amino acids are less effective than L-amino acids, as only D-Cys, D-Thr and D-Ser significantly inhibit

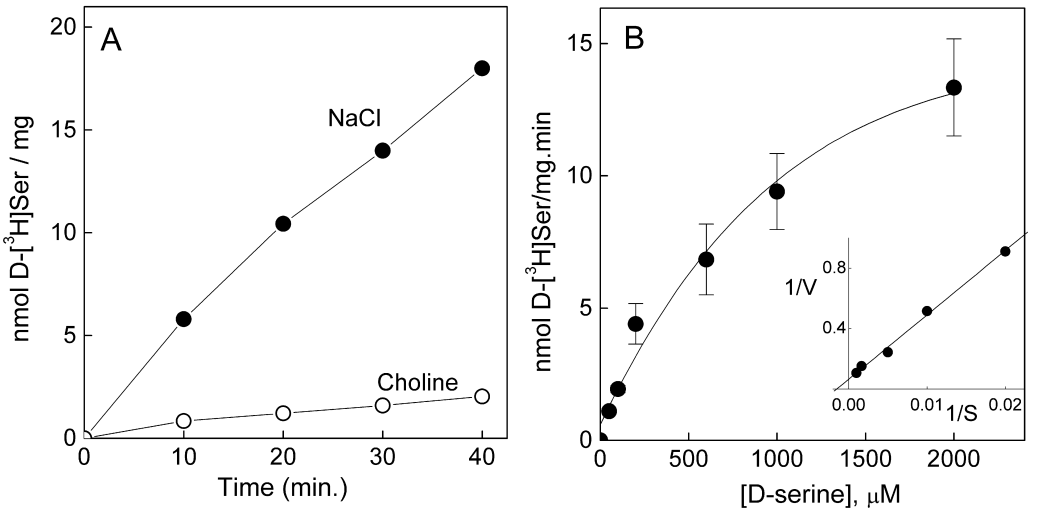


Fig. 1. Kinetics of D-serine uptake by astrocytes. (A) D-Serine uptake was assayed in primary astrocyte cultures in media containing 10 mM HEPES–Tris (pH 7.4), 1 mM $MgCl_2$, 2 mM KCl, 1 mM $CaCl_2$, 20 μM D-[$^3$H]serine in the presence of 100 mM of either NaCl (●) or CholineCl (○). (B) Kinetic analysis of D-serine uptake in the presence of NaCl. Uptake was measured after 20 min. Inset shows the double-reciprocal plot of B.

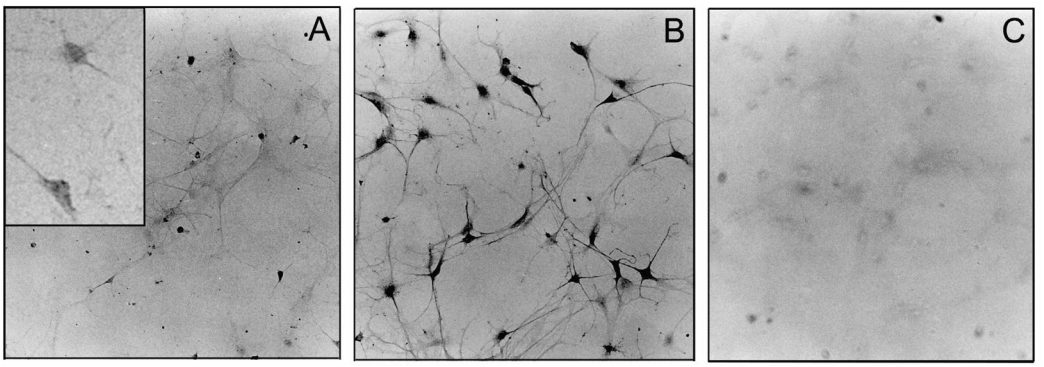


Fig. 2. Immunocytochemical analysis of astrocyte cultures. Incubation of the cells with D-serine increases D-serine immunoreactivity. Primary astrocyte cultures were incubated for 20 min in media containing 10 mM HEPES–Tris (pH 7.4), 1 mM $MgCl_2$, 2 mM KCl, 1 mM $CaCl_2$, 100 mM NaCl and either no (A) or 20 μM D-serine (B, C). Media were washed and the cells were fixed and stained for D-serine. In C, immunoreactivity was abolished by preabsorption of the antibody with 0.5 mM D-serine glutaraldehyde conjugate.

D-serine uptake. Among the D-amino acids tested, D-serine is the most effective, second only to D-cysteine, suggesting that the carrier exhibit some selectivity toward D-serine among D-amino acids. Nonetheless, D-serine is less effective than L-Ser, L-Ala and L-Thr in inhibiting the uptake of D-[$^3$H]serine (Fig. 4). This implies that D-serine transport is mediated by a carrier that prefers L-amino acids, exhibiting a higher affinity for neutral L-amino acids than for D-serine itself.

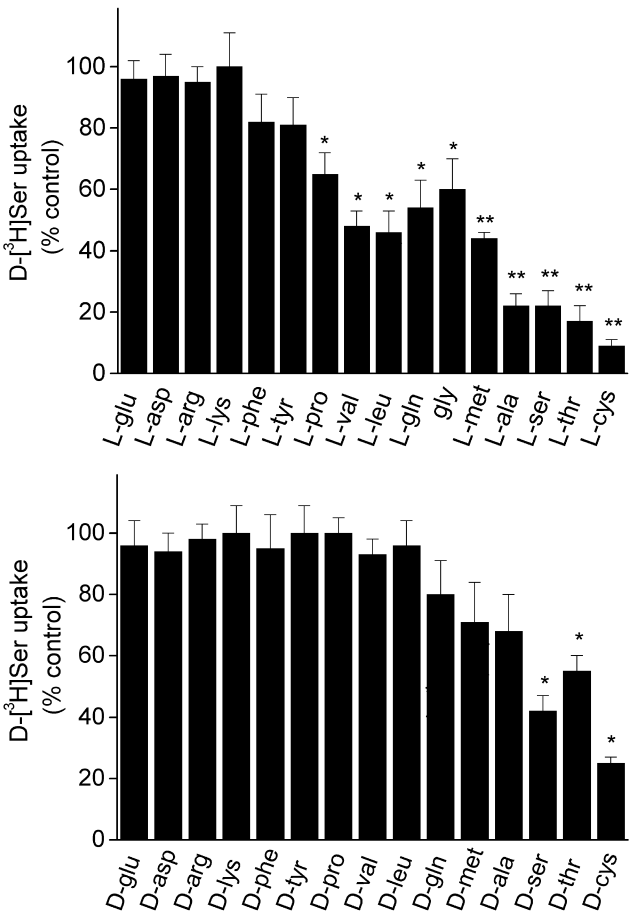


Fig. 3. Specificity of D-serine transport in astrocytes. Sodium-dependent uptake of D-serine was assayed as in Fig. 1, except that media contained 1 mM of different amino acids. The values represent the mean±S.E.M. of three independent measurements done in triplicates. *, Different from control at $P<0.050$. **, Different from control at $P<0.010$.

The strong inhibition of D-serine uptake by L-Ala, L-Ser, L-Cys and L-Thr, the lack of inhibition by 2-(methylamino)-isobutyric acid and the sodium requirement resemble the properties and specificity ASCT-type transporter, which normally catalyses a sodium-dependent amino acid exchange, working as an antiporter [24]. To examine whether D-serine uptake is coupled to counter-movement of other amino acids, we monitored the amounts of amino acids that are released to the medium during the uptake of D-serine. Accordingly, we found that D-serine uptake is associated with an efflux of neutral amino acids from the cells. The release of L-serine elicited by D-serine is at least threefold higher than of other amino acids (Table 1). Though the intracellular levels of L-serine are higher than other amino acids, the fractional release is also the highest, implying that L-serine is the main counter-transported amino acid. High release rates are also observed for L-alanine and L-glutamine (Table 1). D-Serine does not seem to affect the osmotic balance of astrocytes, as it does not elicits taurine efflux (data not shown), which is known to be sensitive to changes of intracellular osmolarity [14].

Though L-Asp and L-Glu are poorly recognized by the transporter under our experimental conditions (Fig. 3), they are also released at significant levels by the addition of D-serine (Table 1). Thus, D-serine is likely to promote alterations on excitatory amino acid homeostasis as well. At present, the mechanisms underlying the release of L-Asp and L-Glu elicited by D-serine are not clear.

In case D-serine transporter mediates an heteroexchange of amino acids by a counter-transport mechanism, neutral amino acids should stimulate the efflux of D-serine as well. Accordingly, we found that L-Ser, L-Thr, L-Ala and L-Gln greatly increase the rate of D-serine efflux (Fig. 5).

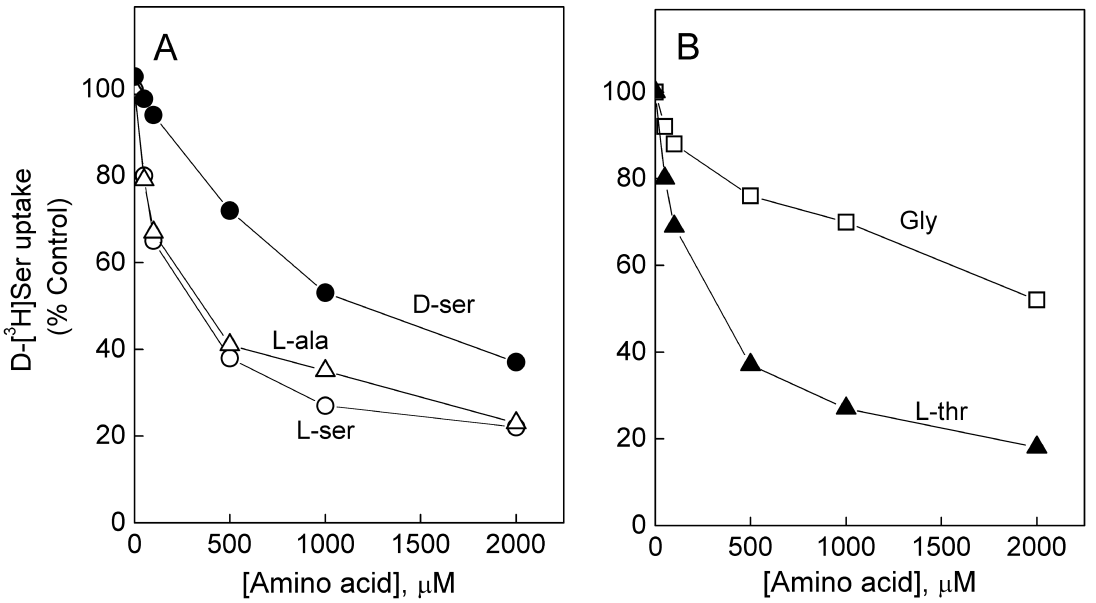


Fig. 4. Inhibition of D-serine uptake in astrocytes by small neutral amino acids. Sodium-dependent D-serine uptake was assayed as in Fig. 1, except that media contained different amounts of L-serine (○), D-serine (●), L-alanine (△), glycine (□) or L-threonine (▲). The values are representative of three measurements using three different culture preparations.

Substitution of NaCl by KCl has no effect (Fig. 5). We confirm previous finding that D-serine efflux is stimulated by kainate and is blocked by DNQX, an antagonist of kainate/AMPA type of glutamate receptors (Fig. 6A and Ref. [19]). We now demonstrate that L-serine at physiological extracellular concentration (20 μM) is also effective in promoting D-serine efflux from astrocytes (Fig. 6B). A similar effect is observed with L-alanine and L-threonine, while glycine is much less effective (Fig. 6B).

To evaluate whether D-serine and L-serine fluxes in glia are linked, we tested the effect of D-serine on the transport of L-[$^3$H]serine into astrocytes. Accordingly, D-serine inhibited the uptake of L-[$^3$H]serine (Fig. 7). Uptake of L-[$^3$H]serine is inhibited following the order: L-Ser=L-Ala=L-Thr>D-Ser=Gly. Similar to that observed for D-[$^3$H]serine uptake, the transport of L-serine seems to be mediated by a carrier system that prefers neutral L-amino acids.

## 4. Discussion

This is the first detailed characterization of D-serine uptake mechanism by astrocytes. Recently, it has been shown that a new transporter type, designated Asc-1, catalyzes a sodium-independent uptake of neutral amino acids, including D-serine, when expressed in *Xenopus* oocytes [5]. However, there is no data on cellular expression of such transporter in the brain. We observe only a very low uptake of D-serine when sodium is replaced by choline, indicating that the major D-serine transporter

Table 1
D-Serine elicits release of intracellular amino acids

| Amino acid | Release induced by D-serine (nmol/mg) | Total intracellular content (nmol/mg protein) | Fractional release (%) |
|---|---|---|---|
| L-Serine | 142±15 | 548 | 26 |
| L-Alanine | 44±8 | 245 | 18 |
| L-Glutamine | 27±4 | 443 | 6 |
| L-Aspartate | 24±2 | 262 | 9 |
| L-Glutamate | 16±2 | 223 | 7 |
| L-Threonine | 10±2 | 147 | 7 |

Astrocyte cultures were incubated with NaCl-containing uptake media as in Fig. 1, supplemented with 50 μM D-serine. To monitor the amino acids released during uptake of D-serine, an aliquot of the medium was collected after 20 min incubation and analyzed by HPLC. The values for amino acid release were obtained by subtracting the basal efflux measured in the absence of D-serine from the values in the presence of 50 μM D-serine. The total intracellular content of amino acids was determined as in Materials and methods. Fractional release was calculated using the formula: intracellular content/amino acid released×100. The values are the mean±S.E.M. of three different measurements.

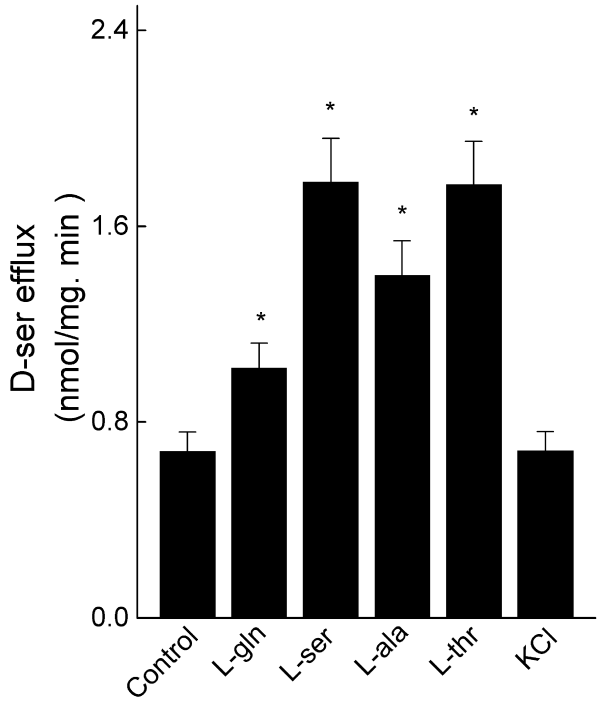


Fig. 5. Release of D-serine elicited by small neutral amino acids. Astrocyte cultures were pre-loaded with D-[$^3$H]serine as in Fig. 1. After uptake, the cells were washed and exposed to fresh media containing 0.3 mM of test agents or 100 mM KCl instead of NaCl. L-gln, L-Glutamine; L-ser, L-serine; L-ala, L-alanine; L-thr, L-threonine. The values represent the average±S.E.M. of four different measurements. *, Different from control at $P<0.05$.

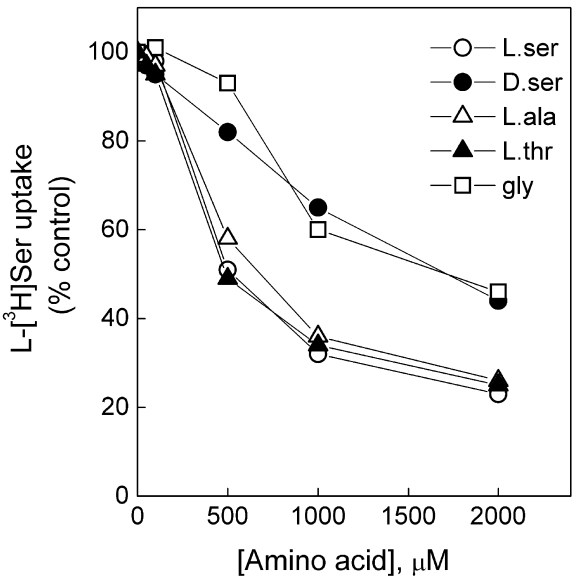


Fig. 7. Inhibition of L-serine uptake in astrocytes. Uptake media and conditions were the same as in Fig. 4, except that L-[$^3$H]serine was used instead of D-[$^3$H]serine. Inhibition of L-[$^3$H]serine by L-serine (○), D-serine (●), L-alanine (△), glycine (□) or L-threonine (▲). The values are representative of three measurements done in triplicates using three different culture preparations.

present in our cultures requires sodium and is different from Asc-1.

In contrast to the neuronal localizations and transport of D-aspartate, which is the second most abundant D-amino

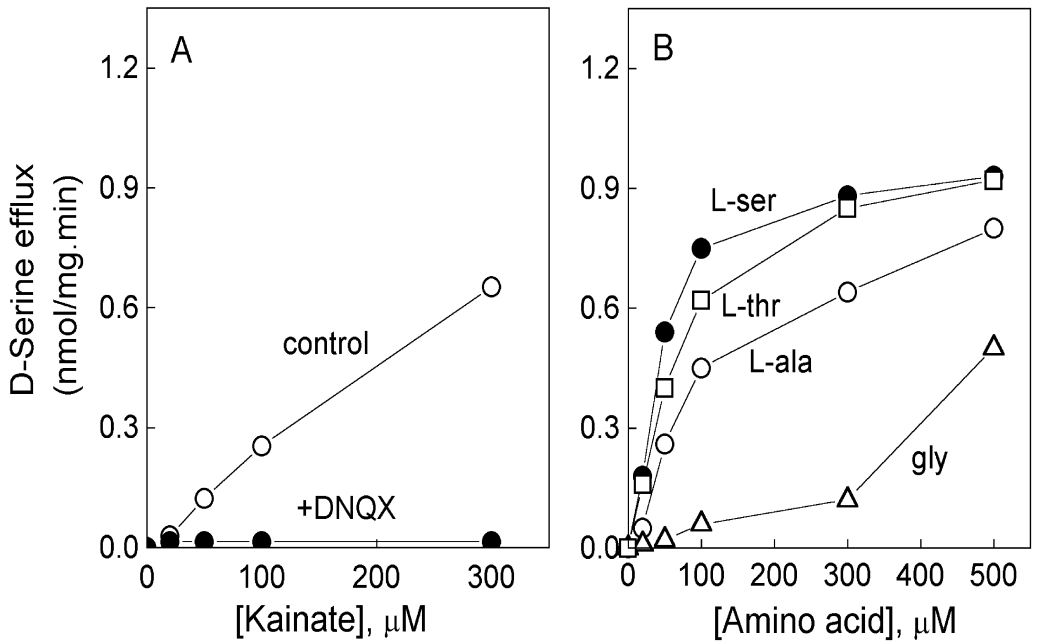


Fig. 6. Concentration-dependence for the effects of kainate and neutral amino acids on the release of D-serine. Astrocyte cultures were pre-loaded with D-[$^3$H]serine in NaCl-containing media as in Fig. 1. Media were washed and substituted by a fresh one containing different amounts of test agents. (A) Kainate induces D-serine efflux (○), which is blocked by 30 μM DNQX (●). (B) Concentration-dependence for L-serine (●), L-threonine (□), L-alanine (○), glycine (△). The values of basal D-serine efflux measured in the absence of test agents were subtracted from each point and corresponded to an average of 0.7 nmol/mg min. The values are representative of three measurements done in triplicates using three different culture preparations.

acid [29], D-serine occurs in astrocytes [19,26]. The specificity of D-serine transport we observed resembles that of ASCT transporters [24]. ASCT1 has similar amino acid specificity to ASCT2 but practically does not recognize D-serine [21]. ASCT2 transporter might account for transport of D-serine we observed in astrocyte cultures as well as the uptake of D-serine detected in C6 glioma cells [10]. Detailed localization of ASCT2 in the brain is not known, but reverse transcription polymerase chain reaction (RT-PCR) studies revealed its abundant message in astroglia [1].

We demonstrate that at the transport level, D-serine fluxes are coupled to L-serine counter-movement, suggesting that D-serine transport is mediated by an antiporter mechanism. We believe that such mechanism of transport is of physiological relevance, since it is observed at concentrations of L-serine normally found in the extracellular medium [8]. Like D-serine, highest levels of L-serine occur in astrocytes [25,30] and serves as a substrate for the mammalian serine racemase to synthesize D-serine [4,27,28]. Thus, the coupling of D-serine efflux with L-serine influx would provide L-serine required for de novo synthesis of D-serine by serine racemase.

Though L-serine seems to be the major amino acid counter-transported in exchange with D-serine, other small neutral amino acids are released during D-serine uptake, including glutamine. The efflux of L-glutamine during D-serine uptake (Table 1) raises the possibility that an increase on glutamine efflux from astrocytes may improve the removal of extracellular D-serine. Such increase on glutamine efflux from astrocytes is observed during intense neuronal activity, in which glutamate released from neurons is converted back to glutamine in astrocytes [18]. Thus, it is possible that neuronal activity influences D-serine levels. For instance, Hashimoto et al. recently demonstrated that extracellular levels of D-serine measured by microdialysis significantly decrease upon prolonged neuronal depolarization with NMDA, kanate or veratridine [9]. Our results suggest that amino acid fluxes between astrocytes and neurons, such as glutamine cycling, might regulate extracellular D-serine concentration, providing a plausible explanation for Hashimoto's group findings.

Free D-serine concentrations in the extracellular medium of pre-frontal cortex and striatum are 4 and 7 μM, respectively. In striatum, extracellular D-serine values are more than twice the values for glycine and glutamate [8]. Our present report shows that the D-serine transport system has low affinity and low specificity for D-serine, indicating that removal of synaptic D-serine might be less effective than of other amino acids. As a result, high concentrations of D-serine might accumulate in the synaptic cleft due to the low-efficiency kinetics of the transporter. This provides an explanation on why extracellular D-serine levels measured by microdialysis in some brain regions are higher than those of more abundant intracellular amino acids, such as glutamate, aspartate and glycine.

Previous studies failed to detect significant levels of D-amino acid oxidase, an enzyme that degrades D-serine, in forebrain areas [11,19]. The low affinity and low-specificity transport of D-serine we observed and the absence of D-amino acid oxidase in the forebrain suggest that the activity of the biosynthetic enzyme, serine racemase, might directly influence the synaptic concentration of D-serine. An inhibitor of serine racemase was effective in decreasing D-serine levels in cell culture system, providing a new strategy to decrease NMDA receptor co-activation by D-serine, with therapeutic implications for stroke [17]. This resembles other non-conventional neural messengers systems, such as nitric oxide and carbon monoxide, that lack specific degrading and storage systems and their extracellular levels are regulated mostly by the activities of the biosynthetic enzymes [22].

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