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Characterization of a Cell Line Model for D-Serine Uptake

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ABSTRACT

D-Serine is an important co-agonist of the *N*-methyl-D-aspartate (NMDA) receptors in the brain and its altered activity was identified in various pathological conditions. Modification of the extracellular D-serine level is suggested to be able to modulate the receptor function. Its transporters may thus serve as potential drug targets.

The aim of this work was to find an easily available human cell line model appropriate for screening molecules affecting D-serine transporters.

Characteristics of D-serine transport into SH-SY5Y human neuroblastoma cell line were studied and compared to those in cultured primary astrocytes. Uptake was followed by measuring intracellular D-serine concentration by capillary electrophoresis with laser induced fluorescence detection method.

We found that SH-SY5Y cells express functional ASCT-1 and ASCT-2 neutral amino acid transporters and show similar D-serine uptake kinetics to cultured astrocytes. Neutral amino acids inhibited D-serine uptake similarly in both cell types. Complete inhibition was achieved by L-alanine and L-threonine alike, while the two-step inhibition curve of trans-hydroxy-L-proline, a selective inhibitor of ASCT-1 supported the presence of functioning ASCT-1 and ASCT-2 transporters. Its higher affinity step corresponding to inhibition of ASCT-1 was responsible for about 30% of the total D-serine uptake.

Based on our results human SH-SY5Y cell line shows similar uptake characteristics to primary astrocytes and thus can serve as a suitable model system for testing of compounds for influencing D-serine uptake into astrocytes.

1. Introduction

Proper function of *N*-methyl-D-aspartate (NMDA) receptors of glutamate is crucial in neuronal development and plasticity, the molecular basis of several higher neuronal functions such as memory formation, learning, etc. Dysregulation of NMDA receptor activity leads to numerous pathological conditions. Its hypofunction is well established e.g. in schizophrenia while its over-activation results in excitotoxicity and consequential neurodegeneration [1].

NMDA receptor activation requires the binding of a co-agonist in addition to glutamate that can be either glycine or D-serine depending on receptor subtype and its cellular localization in various brain areas. D-Serine is regarded to be the primary co-agonist on the synaptic NMDA receptors in the hippocampus and prefrontal cortex [2]. Reduced D-serine concentration in blood of schizophrenia patients was first reported by Hashimoto et al. [3] and later confirmed by several research groups (for review see [4]). Furthermore, high dose D-serine supplementation either alone [5] or in adjunct to antipsychotic drug therapy [6] was shown to improve the symptoms of the disease.

Promising preclinical and clinical data initiated an intensive research to better understand the synthesis, release and elimination of D-serine in the central nervous system. Serine racemase (SR) was identified as the unique source of D-serine *de novo* synthesis [7]. SR expression was first shown in astrocytes where D-serine was also found highly abundant, so originally it was regarded as a gliotransmitter [8]. However, recently SR was shown being

Abbreviations: Asc-1, alanine-serine-cysteine-1; ASCT-1, alanine-serine-cysteine-threonine-1; ASCT-2, alanine-serine-cysteine-threonine-2; CE-LIF, capillary electrophoresis laser induced fluorescence; DAAO, D-amino acid oxidase; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPA-β-CD, 6-monodeoxy-6-mono(3-hydroxy) propylamino-β-CD; NBD-F, 4-fluoro-7-nitrobenzofurazan; NMDA, *N*-methyl-D-aspartate; RIPA, radioimmunoprecipitation assay buffer; SR, serine racemase; TBSS, Tris buffered salt solution; TBST, Tris-buffered saline containing 0.1% Tween 20; t-Pro, trans-hydroxy-L-proline.

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predominantly expressed in neurons [9] and its neuron specific knockdown resulted in a more considerable reduction of D-serine level compared to its glia specific knockdown [10]. Based on these results neurons are regarded now as the primary site of D-serine synthesis. Its neuronal release was demonstrated to be induced by depolarization: however, it was found calcium independent suggesting a non-vesicular mechanism [11]. Alanine-serine-cysteine 1 (Asc-1) transporter was identified to mediate D-serine release from neurons [12]. Asc-1 is a sodium independent small neutral amino acid exchange transporter that shows high affinity to D-serine [13]. Its expression is restricted to neurons in the central nervous system and is only abundant in the presynaptic membrane [14].

D-Serine was also identified to be substrate of alanine-serine-cysteine-threonine-1 and -2 (ASCT-1, ASCT-2), sodium dependent hetero-exchange transporters for small neutral amino acids. Their expression was demonstrated in both astrocytes and neurons [15,16]. Ribeiro and co-workers showed first that D-serine uptake into astrocytes is mediated by ASCT type transporters and suggested they play an important role in the regulation of extracellular level of the co-agonist [17].

Degradation of D-serine is catalyzed by the peroxysomal flavoprotein, D-amino acid oxidase (DAAO). The distribution of the enzyme in the body is uneven, its expression in higher brain areas is insignificant resulting in considerable D-serine level [18].

The possibilities for restoration of the altered NMDA receptor activation in various pathological conditions are extensively studied. Since fine tuning of receptor activity is required modulation of the co-agonist level is a promising approach. In case of D-serine its synthesizing and metabolizing enzymes as well as its transporters may serve as potential drug targets. Inhibitors of DAAO were used concomitantly with D-serine supplementation to reduce its rapid elimination. However, species differences were observed suggesting different role of DAAO in D-serine metabolism in various animals [19]. Regulation of D-serine synthesis by SR is not well characterized so far, thus the way of its pharmacological modulation is not clear. As the transporters play a major role in the regulation of extracellular D-serine concentration they seem to be more promising drug targets. However, for studying the effect of drug candidates acting on the transporters, a simple model system would be required. Previously mainly primary astrocyte and neuronal cultures were applied for characterization of the transporters [17,20]. Their extensive use for testing high number of molecules is difficult and raises ethical issues. Cell lines could be more appropriate for screening. Previously rat C6 glioma cell line was commonly used for studying transporters of various amino acids, e.g. glutamine [21]. D-Serine transport into these cells was demonstrated by Sikka et al [22]. They showed that the cells express SR and ASCT-2 and in the absence of D-amino acid oxidase ASCT-2 is an important player in the regulation of D-serine homeostasis. Its mRNA expression was shown to be dependent on D-serine availability. The role of ASCT-1 in the transport was not addressed. The other widely used model for studying D-serine transport are rat Müller cell line (rMC-1) and mouse primary Müller cells. This model was chosen because D-serine and SR were detected in Müller cells of retina and astrocytes in vertebrates and D-serine increases NMDA induced excitotoxicity in these cells [23]. Its transport was characterized by Dun et al [24] and was found sodium-dependent and inhibited by the substrates of ASCT transporters. They showed that the cells contained mRNA of both ASCT-1 and ASCT-2 but because glutamine, regarded specific for ASCT-2 transporter, inhibited D-serine transport they focused only on ASCT-2 and claimed it being important in the regulation of NMDA receptor activity of the neighboring neurons. The role of ASCT-1 was not explored. Foster at al [20] were the first demonstrating that both ASCT-1 and ASCT-2 contributes to D-serine transport into rat hippocampal astrocytes and HEK293 cells transfected with human ASCT-1 or ASCT-2. They demonstrated that L-glutamine is a preferential but not a specific substrate of ASCT-2 in the transfected cells raising the possibility of species difference in the affinity of the substrates to the ASCT transporters. They also found differing affinity of substrates to transporters in astrocytes and cells transfected with human ASCT transporters. This was explained by the possible difference in transporter density on the cell surface during transfection which can be a limitation of using transfected cells as a model.

Regarding the suggested species difference, the goal of our present study was to find a human cell line expressing ASCT-1 and ASCT-2 to establish a model system for rapid screening of compounds for influencing D-serine uptake into astrocytes. We also aimed at clarifying the contribution of ASCT-1 transporter to D-serine uptake. SH-SY5Y human neuroblastoma cell line was thus studied and its D-serine transport characteristics was compared to those of cultured primary astrocytes.

2. Materials and methods

2.1. Chemicals

D-Serine, L-alanine, L-threonine, L-glutamine, t-Pro, buffer components, trypsin, trypan blue, acetonitrile, L-cysteic-acid, acrylamide were purchased from Sigma-Aldrich (St. Louis, MO, USA) and choline chloride was from Alfa Aesar (Haverhill, MS, USA). Fluorescent reagent, 4-fluoro-7-nitrobenzofurazan (NBD-F) was provided by TCI (Tokyo, Japan). 6-Monodeoxy-6-mono(3-hydroxy) propylamino-β-CD (HPA-β-CD) was purchased from Cyclolab Ltd. (Budapest, Hungary). Ultrapure water from MilliQ Direct 8 water purification system (Merck Millipore, Billerica, MA, USA) was applied for all experiments.

For preparation of cell culture medium, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) and fetal bovine serum (FBS) were provided by Corning (Tewksbury, MA, USA) and Biosera (Nuaille, France), respectively and stable glutamine was obtained from Pan Biotech (Aidenbach, Germany).

For western blot analysis Novex 4-12% polyacrylamide gels (Thermo Fisher Scientific, Waltham, MA, USA) were used. ASCT-1 and ASCT-2 primary antibodies were supplied by Sigma-Aldrich and Cell Signaling (Danvers, MA, USA), respectively. Horseradish peroxidase conjugated secondary anti-rabbit antibody and Pierce enhanced chemiluminescence (ECL) detection reagent from Thermo Fisher Scientific were used.

2.2. Cell cultures

Wistar rat pups of 1-3 days old were purchased from Toxi-Coop Ltd. (Budapest, Hungary) and used for establishment of primary astrocyte culture according to the protocol of Mecha et al. [25]. Animal experiments were approved by the Institutional Animal Care and Use Committee of Semmelweis University (approval number: PE/EA/850-2/2016). Procedures were in harmony with the EU Council Directive on laboratory animals (86/609/EEC). Astrocytes were used after reaching confluency of P1 subculture.

SH-SY5Y cells were acquired from The European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained according to the recommendation of the provider.

2.3. Analysis of cellular D-serine uptake

2.3.1. Time- and concentration-dependence of the uptake into SH-SY5Y cells

On the day of the experiment cells were trypsinized and suspended in Tris buffered salt solution (TBSS) at concentration of 1 million cells in 0.5 mL. Cell suspensions were incubated with 0, 25, 50 and 200 μM of D-serine for 0, 15, 30, 60 and 120 minutes at

37 °C. After completion of incubation uptake was terminated by cooling the samples on ice followed by centrifugation (630 g, 4 °C, 5 min) and washed twice with ice cold TBSS. Pelleted cells were resuspended in 35 μL mixture of acetonitrile:water (2:1, v/v) and the precipitated protein was removed by centrifugation (3000 g, 4 °C, 20 min). Supernatant was collected and stored at −80 °C until analysis.

2.3.2. Time and sodium dependence of the uptake

For the analysis of sodium independent transport sodium chloride was substituted by choline chloride. Samples containing 1 million cells were incubated with 50 μM D-serine for 0, 15, 30, 60 and 120 minutes at 37 °C. After completion of incubation time uptake was terminated and the samples were processed as described in section 2.3.1.

2.3.3. Characterization of D-serine transport kinetics

Cell suspensions were incubated in the presence of various concentrations of D-serine (0-10 000 μM) for 15 min at 37 °C to study the transport kinetics. After completion of incubation time uptake was terminated and the samples were processed as described in section 2.3.1.

2.3.4. Inhibition of D-serine uptake by neutral amino acids

Cells were incubated with 25 μM D-serine in the presence of various concentrations of L-alanine, L-threonine, L-glutamine and t-Pro, respectively for 15 min at 37 °C. After completion of incubation time uptake was terminated and the samples were processed as described in section 2.3.1.

2.4. Determination of intracellular D-serine concentration

Capillary electrophoresis coupled to laser induced fluorescence detection (CE-LIF) analysis of D-serine content of cell extracts was performed according to our previously published validated method [26]. Briefly, 5 μL samples were derivatized by mixing with 5 μL ethanolic NBD-F solution (3 mg/mL) and 5 μL borate buffer (pH 8.5; 20 mM) containing 5 μM L-cysteic acid as internal standard and heating for 20 min at 65 °C. Prepared samples were stored at −20 °C until CE analysis.

All measurements were carried out on a P/ACE MDQ capillary electrophoresis system operated by 32 Karat software version 5.0 (Beckman Coulter, Brea, CA, USA). LIF detector with Argon-ion laser source was applied. Excitation and emission wavelengths were 488 and 520 nm, respectively. Separation took place in fused silica capillaries of 75 μm id and 365 μm od (Agilent Technologies, Santa Clara, CA, USA). Before use capillaries were coated with linear polyacrylamide. Representative electropherograms of D-serine separation is shown in Fig. 1.

2.5. Western blot analysis

Expression of ASCT-1 and ASCT-2 in SH-SY5Y cell line was studied by western blot analysis. About 5 million cells were extracted by 200 μL radioimmunoprecipitation assay buffer (RIPA, Thermo Fisher Scientific) and the samples were separated in 4-12% gradient gel using Tris-glycine-EDTA buffer and transferred to PVDF membrane. Membranes were blocked by 5% non-fat dry milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour and probed with 1 μg/mL anti-ASCT-1 or 4 μg/mL anti-ASCT-2 antibodies in 5% non-fat dry milk dissolved in TBST overnight at 4 °C. Membranes were washed three times with TBST and then incubated with horseradish peroxidase conjugated secondary anti-rabbit antibody for 1 hour at room temperature. ASCT-1 and ASCT-2 were detected on autoradiography films using Pierce ECL reagent.

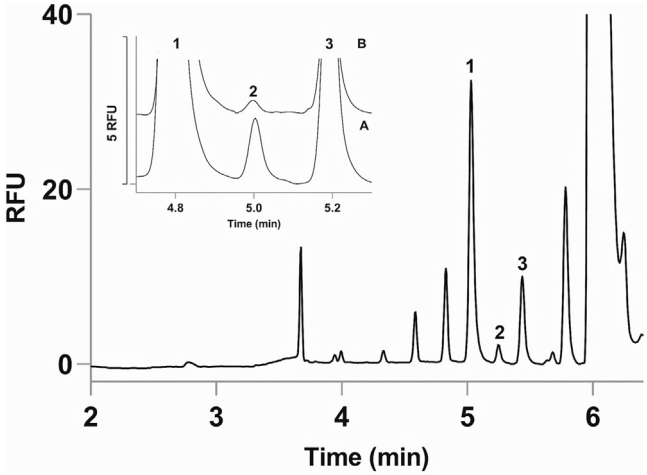

Fig. 1. Electropherograms demonstrating determination of intracellular D-serine content of human SH-SY5Y neuroblastoma cells. Numbered peaks indicate NBD-F labelled glycine (1), D-serine (2) and L-serine (3). In the insert the electropherograms of samples (**A**) after incubation the cells with 25 μM D-serine for 15 min and (**B**) after incubation the cells with 25 μM D-serine for 15 min in the presence of 3 mM of L-alanine are shown. Separation conditions: 50 mM pH 7 HEPES buffer containing 6 mM HPA-β-CD; 30/40 cm x75 μm id coated fused-silica capillary, −24 kV. Sample injection: 3474 Pa pressure for 5 sec.

2.6. Data evaluation

GraphPad Prism 8 for Windows (GraphPad Software, La Jolla, CA, USA) was used for data analysis and curve fitting. Exponential plateau fitting scheme, fit logIC50 fitting method and Michaelis-Menten curves were applied for analysis of time dependence, inhibition and kinetics of D-serine uptake, respectively.

For estimation of intracellular D-serine concentration 6 and 7 μm radius of SH-SY5Y cells and astrocytes were used, respectively [27].

3. Results and Discussion

D-Serine uptake characteristics of SH-SY5Y cells were evaluated and compared to those of primary astrocytes. First the cell line was incubated in the presence of 25, 50 and 200 μM D-serine, respectively for up to 120 minutes and its uptake was assessed by measuring its intracellular level. The transport was found concentration and time dependent. It was saturated after 30 minutes incubation when 200 μM D-serine was used as substrate, while in case of its lower concentrations only a tendency of saturation was seen after 2 hours (Fig. 2).

In sodium free buffer a very limited D-serine uptake was detected indicating overwhelming dominance of sodium dependent transport mechanism into SH-SY5Y cells and astrocytes alike (Fig. 3A and B). Sodium independent portion of the uptake was less than 10% into each cell type probably due to a non-specific process. ASCT transporters are known responsible for sodium dependent uptake of D-serine into cells and were reported to be expressed on astrocytes [16]. These findings suggest that the undifferentiated SH-SY5Y cell line may show similar D-serine transport characteristics to those previously reported for cultured astrocytes [20].

To establish the similarity, the kinetics of D-serine transport into SH-SY5Y cells and astrocytes were compared. Incubation of each cell type with increasing concentrations of D-serine showed a proportional elevation in the uptake rate according to Michaelis-Menten kinetics and reached plateau at similar concentration range (Fig. 4). Estimated K_M values and maximal uptake velocity (v_{max}) were not significantly different indicating comparable affinity and capacity of D-serine transport in these cell types (Table 1). We can

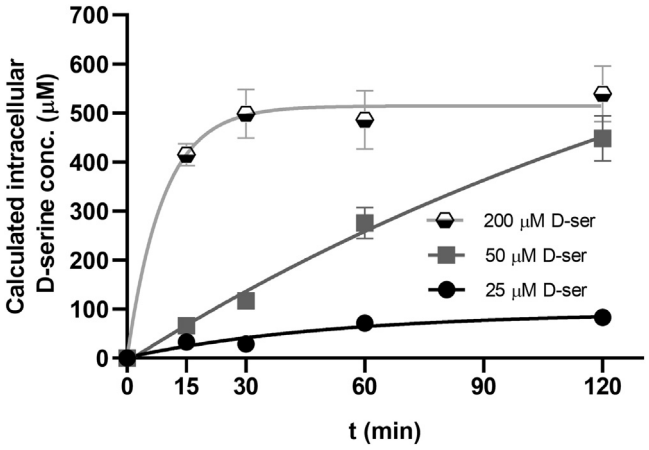

Fig. 2. Concentration dependent D-serine uptake into SH-SY5Y cells. Intracellular D-serine content in SH-SY5Y cells was determined after their incubation with various concentrations (25, 50, 200 μM) of D-serine for different periods of time (0, 15, 30, 60, 120 min). Data are shown as mean ± SEM of three experiments (n = 3).

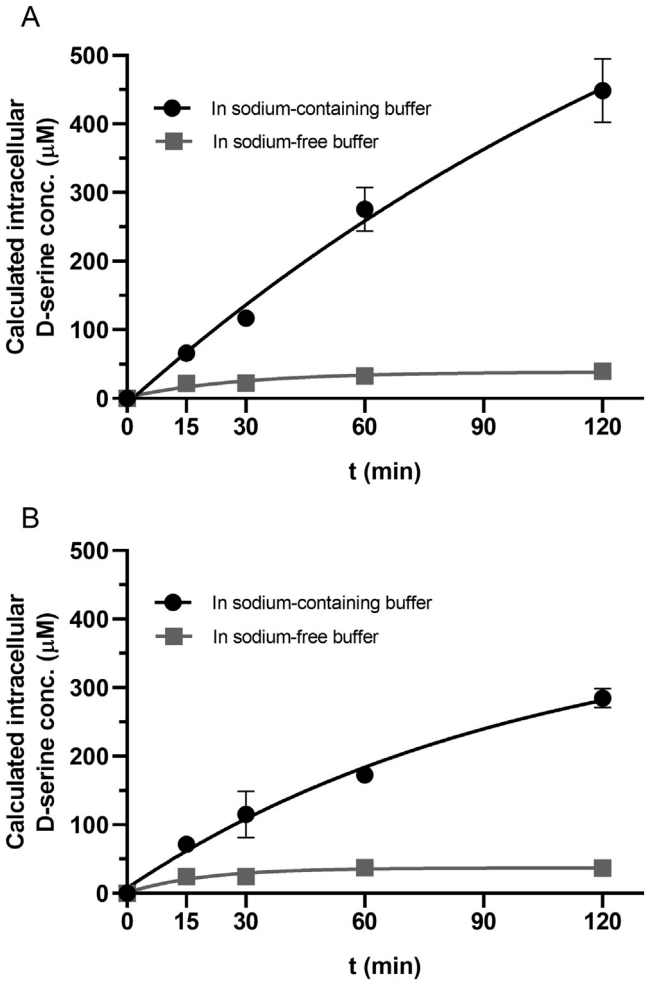

Fig. 3. Sodium dependence of D-serine uptake into SH-SY5Y and astrocyte cells. Uptake of D-serine into SH-SY5Y cells (A) and cortical astrocytes (B) was measured over a period of 120 minutes in sodium containing or sodium free buffers. D-Serine concentration was 50 μM. Data are shown as mean ± SEM of three experiments (n = 3).

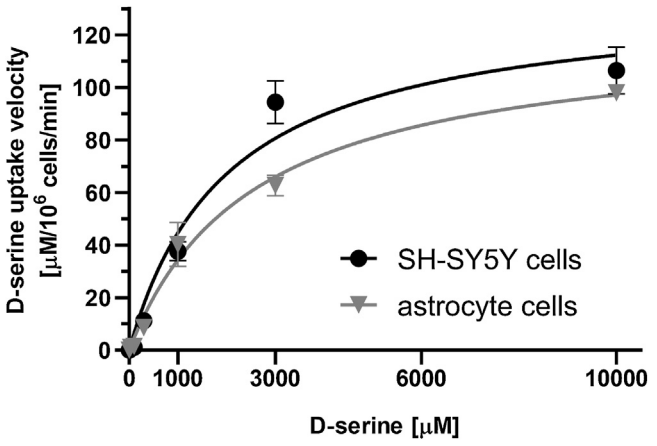

Fig. 4. Kinetic of D-serine uptake into SH-SY5Y and astrocyte cells. Intracellular D-serine concentration measured in SH-SY5Y cells and rat cortical astrocytes in the presence of increasing concentrations of D-serine. Data are shown as mean ± SEM of three experiments (n = 3). Michaelis-Menten curves were fitted.

Table 1
Kinetic parameters of D-serine uptake into SH-SY5Y and astrocyte cells.

	SH-SY5Y		astrocyte		
	mean	95% CI	mean	95% CI	p
Km (μM)	2025	1081-3884	2545	1714-3847	0.5131
Vmax (μM/10^6 cells/min)	135.0	108.4-172.0	121.9	105.8-142.8	0.4339

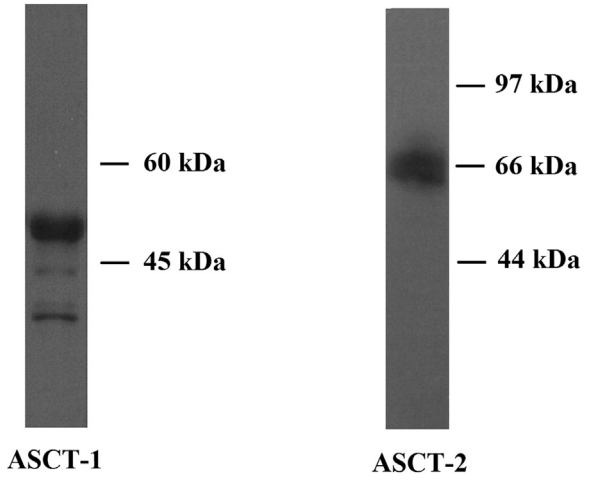

Fig. 5. Western blot analysis of ASCT-1 and ASCT-2 in SH-SY5Y cells. SH-SY5Y cell lysate (40 μg protein) was analyzed by western blot using 4-12% gradient gel and probed by rabbit anti-human ASCT-1 and rabbit anti-human ASCT-2 antibody. ASCT-1 and ASCT-2 were detected at about 52 and about 66 kDa, respectively.

assume that D-serine uptake into SH-SY5Y cells is highly similar to that of astrocytes thus it can serve as model of the astrocytic transport of this D-amino acid.

To further confirm the role of ASCT transporters in the D-serine uptake into SH-SY5Y cells their expression was studied by western blot analysis. Expression of both transporters was detected in SH-SY5Y cells. The most intensive band appeared at about 52 kDa using anti-human ASCT-1 antibody (Fig. 5), which is comparable to the predicted 55 kDa molecular weight reported earlier in anterior cingulate cortex [28]. Anti-human ASCT-2 antibody detected an immunoreactive band at 66 kDa (Fig. 5) which is in line with the previously reported molecular weight of the glycosylated protein [15].

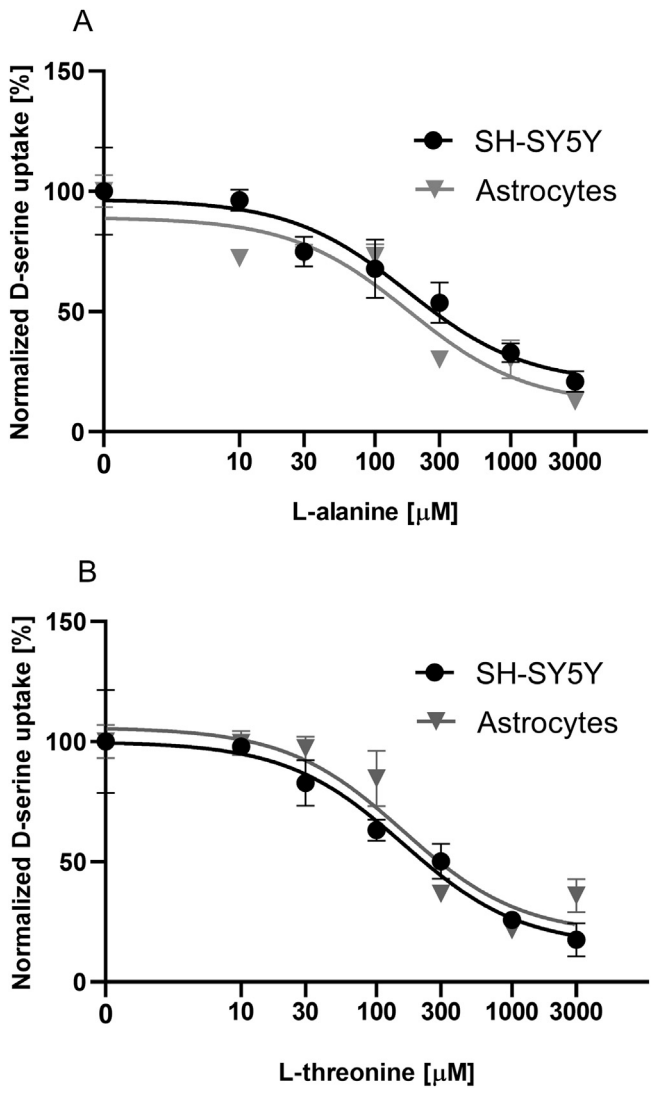

Fig. 6. Inhibition of D-serine transport into SH-SY5Y cells and rat cortical astrocytes by L-alanine and L-threonine. L-Alanine (A) and L-threonine (B) concentration dependently inhibited the D-serine uptake. One-step inhibition curve was observed in case of both cell types. In all experiments uptake of 25 μM D-serine was studied for 15 min. Intracellular D-serine concentration in the absence of inhibitor was regarded as 100% uptake. Data are shown as mean ± SEM of three experiments (n = 3).

Table 2
Estimated IC50 values of various neutral amino acid inhibitors on D-serine uptake into SH-SY5Y and astrocyte cells.

	SH-SY5Y		astrocyte		
	mean	95% CI	mean	95% CI	p
L-Ala IC50 (μM)	174.4	40.21-772.4	177.7	66.21-470.4	0.982
L-Thr IC50 (μM)	157.9	50.52-521.8	158.1	74.47-335.5	0.998
L-Gln IC50 (μM)	97.47	53.12-182.5	67.95	26.12-206.3	0.515
t-Pro IC50 (μM)*	8.218	1.98-26.5	3.975	0.16-19.7	0.522

* for ASCT1

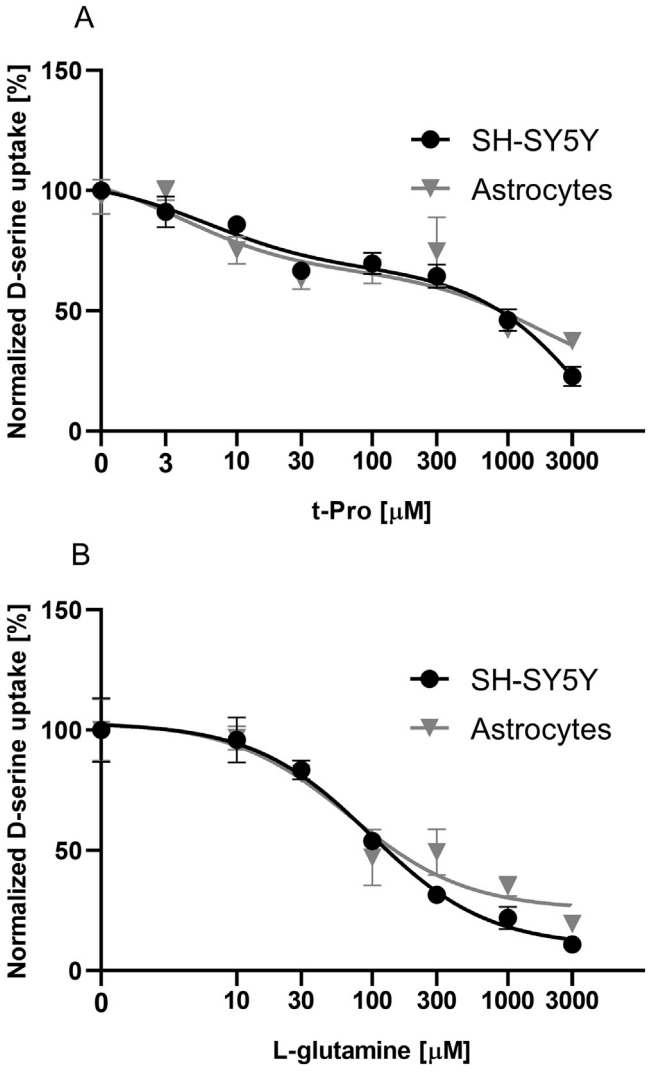

Fig. 7. Inhibition of D-serine transport into SH-SY5Y cells and rat cortical astrocytes by t-Pro and L-glutamine. T-Pro resulted in two-step inhibition curve in both cell types (A). One-step inhibition curve was observed in the presence of L-glutamine in both SH-SY5Y cells and cultured astrocytes (B). Intracellular D-serine concentration in the absence of inhibitor was regarded as 100% uptake. Data are shown as mean ± SEM of three experiments (n = 3).

As our ultimate intention is studying drug candidates acting by inhibition of D-serine uptake into astrocytes we compared the inhibitor sensitivity of the transporters on the two cell types.

Natural substrates of ASCT transporters, L-alanine and L-threonine concentration dependently inhibited D-serine uptake into both SH-SY5Y cells and astrocytes and almost complete inhibition was achieved. Comparable IC50 values indicate their similar inhibitor affinity to the transporters on the two cell types (Fig. 6A and B, Table 2). Based on literature data these neutral amino acids have similar affinity to ASCT-1 and ASCT-2 [20]. In line of this finding we observed a one-step sigmoidal inhibition curve, not allowing the distinction of the two transporters and having insight if both ASCT transporters are involved in D-serine uptake or only one of them is functioning.

The relative contribution of the individual transporters to the D-serine uptake was thus assessed by using their selective substrates. t-Pro was previously shown having considerably higher affinity to ASCT-1 [29], while L-glutamine was reported being a preferential substrate of ASCT-2 [30]. Inhibition of D-serine uptake by t-Pro showed a two-step curve indicating contribution of both ASCT transporters (Fig. 7A). The IC50 values of the higher affinity component corresponding to the inhibition of ASCT-1 were comparable in the two cell types (Table 2). Because of the weak inhibition of ASCT-2 by t-Pro complete inhibition of the uptake was not reached in the studied concentration-range and the estimation of IC50 values of the second step suffers from high error (data not shown). Based on the height of the first step the contribution of ASCT-1 to D-serine transport was about 30% in both cell types. For a long time,

the involvement of ASCT-1 in D-serine uptake was neglected. However, using transfected cells or cultured astrocytes it was shown to be substrate of both transporters [12,20]. Our present results further confirm that ASCT-1 is partially responsible for D-serine uptake into cultured astrocytes and similar result was found in SH-SY5Y cells. Using L-glutamine however, a single-step inhibition curve was detected contrary to the findings of Foster et al [20], who observed slight selectivity towards ASCT-2. The contradiction is probably explained by the rather small difference in the affinity of L-glutamine to the ASCT transporters (Fig. 7B).

4. Conclusion

Our present results demonstrate that human SH-SY5Y neuroblastoma cells express functional ASCT-1 and ASCT-2 transporters and show comparable uptake kinetics of D-serine to cultured astrocytes. As similar inhibition characteristics of the established competitive uptake inhibitor small neutral amino acids were observed in both cell types, the undifferentiated SH-SY5Y cells seem to be an appropriate model system for screening potential inhibitor substances of D-serine uptake into astrocytes by ASCT transporters. The advantages of using this cell line are the easy availability of functioning human transporters, its inexpensive maintenance and the avoidance of sacrificing laboratory animals.

Author statement

Eva Szoko: Supervision of the project, data evaluation, preparation of the publication; **Istvan Vincze**: Planning and performing the experiments, CE measurements, data analysis, preparation of the draft of the manuscript and the figures; **Peter P. Lakatos:** Performing some experiments, data analysis, preparation of the figures; **Fruzsina Bagamery**: Maintenance of the cells, preparation of the primary culture, performing some of the experiments; **Tamas Tabi**: Evaluation of experimental data, preparation of the publication

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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