Study on mechanisms regulating D-serine levels – possible drug targets

Doctoral short thesis

Dr. István Vincze

Semmelweis University
Doctoral School of Pharmaceutical Sciences

Supervisor: Dr. Éva Szökő, D.Sc., Professor

Opponents: Dr. Róbert Gáspár, PhD, Associate Professor
Dr. Tibor Zelles, PhD, Associate Professor

Chair of Final Committee: Dr. Imre Klebovich, D.Sc., Professor Emeritus
Members of Final Committee: Dr. Eszter Duca, PhD., Associate Professor
Dr. Gergely Völgyi, PhD., Associate Professor

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1. Introduction

D-serine possesses co-agonist properties at the N-methyl-D-aspartate (NMDA), ionotropic glutamate receptors which play an important role in neurotransmission. The disturbed function of the NMDA receptors has been identified in a number of central nervous system diseases, accordingly altered levels of D-serine have also been identified in various central nervous system disorders (summarized in Table 1.)

Table 1.: Alteration of D-serine concentrations found in various central nervous system diseases

Diseases	Hypothesized changes in the level of D-serine in the central nervous system
ALS	↑
Alzheimer's disease	↑
Epilepsy	↕
Schizophrenia	↓
Parkinson's disease	↓

In the mentioned diseases D-serine concentration was determined from different biological matrices. ALS: spinal cord, Alzheimer's disease: cerebrospinal fluid, schizophrenia: plasma, Parkinson's disease: cerebrospinal fluid, epilepsy: cerebrospinal fluid, various brain areas (animal experiment)

Serine racemase enzyme (SR), D-amino acid oxidase enzyme (DAAO) and various transporters play an important role in the regulation of D-serine concentration. SR enzyme occurs primarily in neurons and its most important function is expected to form D-serine from L-serine. Its altered function has been shown in various diseases, however, its applicability as a drug target has been investigated only in few studies.

DAAO, which is functioning with the cofactor flavin adenine dinucleotide (FAD), is responsible for the metabolism of D-serine. The enzyme is most abundant in the central nervous system in the lower brainstem and cerebellum. Numerous DAAO enzyme inhibitor compounds have recently been developed and tested in *in vitro* experiments, thereafter in preclinical studies, primarily in animal models of schizophrenia. Beside encouraging results, ineffectiveness is also reported in the literature. Only sodium

benzoate, showing a weak DAAO enzyme inhibitory property has been tested in clinical trials. The development and investigation of novel molecules still seems mandatory.

In the central nervous system, various transporters are responsible for transfer of D-serine across membranes. The alanine-serine-cysteine-1 (asc-1) transporter was found primarily in neurons, acts in Na^+-independent manner and possesses high affinity for D-serine. The most important function of the transporter is to release D-serine from the cell into the extracellular space.

The presence of alanine-serine-cysteine-threonine-1 and -2 (ASCT-1 and -2) transporters are mainly characteristic of astrocyte cells. They function in Na^+-dependent manner and have low affinity for D-serine. The suggested most important roles of these transporters are D-serine uptake and a paralel L-serine release (antiporter function). Selective or preferred substrates can be used to distinguish ASCT-1 and ASCT-2 transporters. Trans-4-hydroxy-L-proline (t-Pro) is considered to be the preferred substrate for ASCT-1 transporter, but it also inhibits ASCT-2 transporter at high concentrations. L-glutamine and L-asparagine are supposed to be the slightly preferred substrates for ASCT-2.

Recently, ketamine has been used to treat people with depression due to its rapid effect. Ketamine is a racemic compound consisting of R- and S-ketamine enantiomers. S-ketamine is suggested to be responsible for the dissociative side effect experienced during ketamine treatment. Its selective, mixed competitive, non-competitive inhibitory effect on ASCT-2 transporter was proposed as a possible mechanism. Our aim was to characterize the properties of the transporters and to study the effect of various compounds on D-serine transport *in vitro,* using cell cultures as models ensuring quick and simple workflow.

2. Aims

The aim was to study the possibilities of influencing intra- and extracellular D-serine concentration, , since decreased, as well as increased amounts of D-serine may play a role in various central nervous system diseases. Our goal was to investigate potential drug targets and to develop a cell model which is suitable for testing potential drug candidates.

- Investigation of the effect of a novel D-amino acid oxidase (DAAO) enzyme inhibitor, 6-fluoro-1H-indazol-3-ol on the change of D-serine level *in vivo*
- Characterization of the SH-SY5Y human neuroblastoma cell line as a possible D-serine transport model
- Comparison of D-serine transport of SH-SY5Y human neuroblastoma cell line and rat astrocyte cell culture
- Investigation of the effect of S-ketamine on D-serine transport in the SH-SY5Y cell line

3. Methods

3.1 Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12, (DMEM / F12), fetal bovine serum (FBS), stabilized glutamine, and non-essential amino acid solutions were used to maintain the cell culture.

Novex 4-12% polyacrylamide gel was applied for Western blot analysis. Primary antibodies to ASCT-1 and ASCT-2 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Cell Signaling (Danvers, MA, USA), respectively. Employed secondary antibody was against rabbit antigen for Western blot analysis.

3.2 *In vivo* testing of the 6-fluoro-1H-indazol-3-ol DAAO enzyme inhibitor was performed in male NMRI mice. Five animals per group were treated intraperitoneally with 30 mg/kg D-serine and 30 mg/kg 6-fluoro-1H-indazol-3-ol or compound-free isotonic saline. D-serine concentrations of plasma were determined at 10, 60, and 120 minutes.

The animals were treated with the approval of Ethics Committee for Animal Experiments at Semmelweis University and the acceptance decision of National Food Chain Safety Office (NÉBIH, Budapest, Hungary) (22.1/606/001/2010, 5 February 2010). All experiments were in accordance with the European Council Regulation on the protection of animals used for experimental and other scientific purposes (86/609/EEC).

3.3 Quantitative determination of D-serine from biological samples was performed by the capillary electrophoresis laser-induced fluorescence (CE-LIF) method previously developed and validated in our institute. 4-fluoro-7-nitrobenzofurazan (NBD-F) fluorescent reagent was applied for derivatization, and L-cysteic acid (2 μM) as an internal standard was added to the borate buffer used for derivatization (pH 8.5; 20 mM). The samples were heated at 65 ° C for 20 minutes for the reaction and then cooled. Samples were stored at -20 ° C until analysis.

Analytical measurements were performed on a P/ACE MDQ CE instrument coupled with LIF detector equipped with an Ar-ion laser source. The separation was performed in a 75 μm inner diameter silica capillary coated with linear polyacrylamide. The effective and total lengths of the capillaries were 30 and 40 cm, respectively.

3.4 Wistar rat pups (1-3 days old) were used to prepare the primary astrocyte cell culture. Animals were treated with the approval of the Ethics Committee for Animal Experiments at Semmelweis University and the acceptance decision of National Food Chain Safety Office (NÉBIH, Budapest, Hungary) (22.1/606/001/2010, 5 February 2010). The experiments complied in all respects with the Council of Europe Regulation on the protection of animals used for experimental and other scientific purposes (86/609/EEC). After reaching confluence, the astrocyte cells were used for experiments

SH-SY5Y human neuroblastoma cell line was also employed during the investigations. It was obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK).

3.5 Western blot analysis was performed to test the expression of ASCT-1 and ASCT-2 transporters in SH-SY5Y cells. Samples were separated on a 4-12% gradient gel. Following electrophoresis, proteins were blotted onto a PVDF membrane. Membranes were incubated in 5% skimmed milk powder solution containing 1 μg/mL anti-ASCT-1 and 4 μg/mL anti-ASCT-2 primary antibody, respectively, at 4 °C overnight. Thereafter the membranes were incubated with horseradish peroxidase conjugated to a secondary antibody produced against rabbit antigen. After washing three times for 10 min with TRIS-buffered saline containing Tween 20 (TBST) transporter proteins were visualized on X-ray film by chemiluminescence using Pierce ECL Western blot reagent.

3.6 On the day of the experiment, cells were treated with trypsin and resuspended in Tris-adjusted HEPES buffer (THB) in a concentration of 1 million cells/500 μL solution. Cell suspension was incubated with 0, 25, 50, and 200 μM D-serine for 0, 15, 30, 60, and 120 min at 37 °C. After incubation, the suspension was cooled in ice-water

bath and centrifuged (630 g, 4 °C, 5 minutes) then washed twice with cold THB solution. The cell pellets were finally resuspended in 35 µL of acetonitrile:water (2:1 v/v) and the precipitated protein was removed by centrifugation (3000 g, 4 °C, 20 min). The supernatant was collected and stored at -80 °C until analysis.

3.7 To study Na^+-independent transport, the NaCl content of the buffer solution was replaced with choline chloride. The cell suspension at a concentration of 1 million cells/500 µL solution was incubated with 50 µM D-serine for 0, 15, 30, 60 and 120 minutes at 37 °C. At the end of the incubation period, the samples were processed as described in Section 3.6.

3.8 To investigate the kinetics of D-serine transport, a cell suspension with a concentration of 1 million cells/500 µL solution was incubated with various concentrations of D-serine (0-10000 µM) for 15 minutes at 37 °C. At the end of the incubation period, the samples were processed as described in Section 3.6.

3.9 Cells were incubated with 25 µM D-serine in combination with various concentrations of L-alanine, L-threonine, L-glutamine, t-Pro and L-serine, respectively at 37 °C to study their inhibitory effect on D-serine uptake. At the end of the incubation period samples were processed as described in Section 3.6.

3.10 Adherent SH-SY5Y cells were maintained in medium containing 100 µM D-serine for 24 hours. Trypsin was used to collect the cells which were resuspended in THB to get the final concentration of 1 million cells/500 µL solution. Cells were incubated with 300 µM L-serine containing medium for 0, 5, 15, and 30 minutes at 37 °C. At the end of the incubation period, the samples were processed as described in Section 3.6.

3.11 Cells were incubated with 25 μM D-serine along with various concentrations of S-ketamine (0, 2, 4, 10, 20, 50, 75, and 100 μM) for 15 minutes at 37 °C. At the end of the incubation period, the samples were processed as described in Section 3.6.

3.12 Adherent SH-SY5Y cells were incubated with 0, 25, and 50 μM S-ketamine for 72 h. After incubation S-ketamine was removed and the cells were resuspended in THB producing the final composition of 1 million cells/500 μL solution. Cells were then incubated for 15 minutes at 37 °C employing different concentrations of D-serine (300 and 1000 μM). At the end of the incubation period, the samples were processed as described in Section 3.6.

3.13 Results are presented as mean ± standard error of the mean (SEM) based on the values of at least three parallel measurements. Statistical differences were analyzed using a two-sample t-test or one-way analysis of variance (ANOVA) followed by a Dunnett post-hoc test. F-test was employed to compare the different curve fits, or the parameters calculated from the fits. The differences were considered statistically significant when $p<0.05$.

4. Results

4.1 We have participated in a project of DAAO inhibitor development of Medicinal Chemistry Research Group (Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest Hungary). Based on preliminary experiments, the molecule 6-fluoro-1H-indazol-3-ol was selected for *in vivo* testing. Mice were treated with D-serine or D-serine and 6-fluoro-1H-indazol-3-ol intraperitoneally, and plasma D-serine concentrations were determined by CE-LIF. At 10 min post-administration, 13.81 ± 1.20 μM D-serine was measured in the group treated with D-serine alone. Co-administration of D-serine with a DAAO inhibitor resulted in a significant, 33% increase in plasma D-serine. One hour after treatment, the plasma concentration of D-serine was 11.11 ± 1.69 μM in D-serine-treated mice, which was significantly increased by 27% with the addition of a DAAO inhibitor. After two hours, D-serine levels returned to the control (Figure 1).

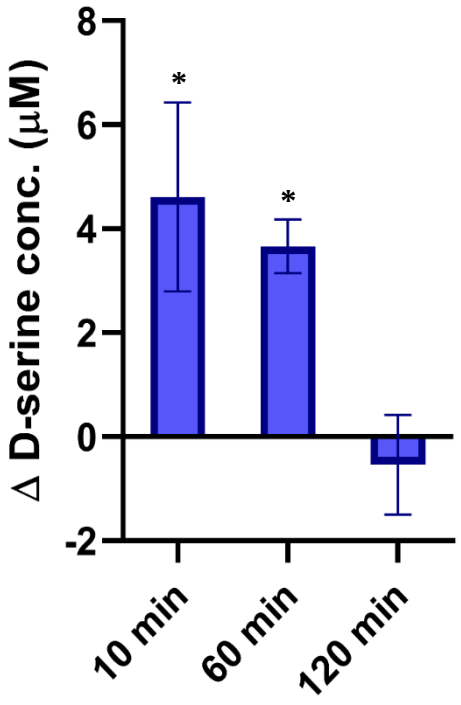

Figure 1: Difference in D-serine concentrations after treatment with 6-fluoro-1H-indazol-3-ol molecule (novel DAAO inhibitor) in the plasma of mice. Mice received intraperitoneally 30 mg/kg D-serine in isotonic saline or 30-30 mg/kg D-serine and 6-fluoro-1H-indazol-3-ol, respectively. Plasma D-serine concentrations were determined at 10, 60, 120 minutes after administration. The difference in D-serine concentrations were obtained by subtracting the values measured for D-serine and saline treatment from the D-serine levels measured in the case of 6-fluoro-1H-indazol-3-ol and D-serine treatment. Two-sample t-test was employed, in which the results of D-serine and saline solution treatment were compared with the values obtained from D-serine and 6-fluoro-1H-indazol-3-ol coadministration. The analysis was considered statistically significant at $p<0.05$. Results are expressed as mean ± SEM (10 min, n=5; 60 min, n=4, 120 min, n=5).

4.2 D-serine uptake of the SH-SY5Y neuroblastoma cell line was tested applying different concentrations of D-serine (25, 50, and 200 μM) and incubation times (0, 15, 30, 60, and 120 min). Increasing the employed D-serine concentration resulted in higher intracellular D-serine levels. In parallel, increasing incubation time was also associated with rising intracellular D-serine content. Based on these results concentration- and time-dependent transport was present in SH-SY5Y cell line. The 15 min incubation time was in the dynamic phase for all three concentrations applied therefore this incubation time was used for inhibition and kinetic studies.

4.3 Na^+-dependence of D-serine uptake by SH-SY5Y and astrocyte cells was also tested. The neuronal asc-1 transporter functions in Na^+-independent manner, while ASCT-1 and ASCT-2 transporters (mainly located to astrocyte cells) operates in Na^+-dependent manner. Substitution of sodium ions with choline ions in the incubation medium resulted in significantly 66–91% lower D-serine uptake at all time points in both cell types. Based on our observation predominantly Na^+-dependent D-serine uptake takes place both in SH-SY5Y cell line and astrocyte culture. Proteins of ASCT-1 and ASCT-2 transporters were detected in SH-SY5Y cell line by Western blot analysis. Functioning ASCT-1 and ASCT-2 transporters are suggested in this cell type.

4.4 Comparison of D-serine uptake kinetics in SH-SY5Y and astrocyte cells was performed. Both cell types were incubated with different concentrations of D-serine (0, 10, 30, 100, 300, 1000, 3000, and 10.000 μM) for 15 min. Increasing D-serine concentration resulted in rising intracellular D-serine content in both SH-SY5Y and astrocyte cells. The uptake curves followed the Michaelis-Menten kinetics (Fig. 2). The calculated kinetic parameters, Km and V_{max} values did not show significant difference in the two cell types, thus similar affinity and capacity of D-serine transport is assumed for SH-SY5Y and astrocyte cells.

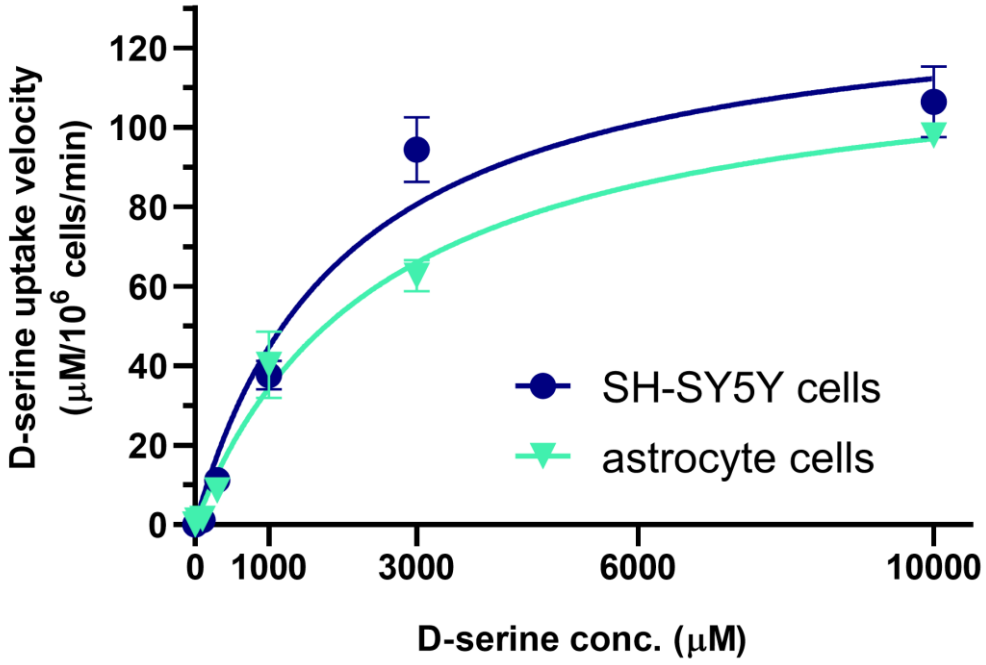

Figure 2: Kinetic characterization of D-serine uptake in SH-SY5Y and astrocyte cells. The Figure shows the rate of D-serine uptake in SH-SY5Y cells and rat cortical astrocyte cells when different concentrations of D-serine were used (0-10.000 μM) in Na^+-containing incubation buffer. Data are presented as mean ± SEM ($n \geq 3$). A Michaelis-Menten curve was applied for fitting.

4.5 Uptake of D-serine was tested in SH-SY5Y and astrocyte cells, in the presence of L-alanine or L-threonine, substrates of the ASCT-1 and ASCT-2 transporters. For both amino acids, concentration-dependent inhibition and at the highest employed concentration close to maximal supression of D-serine uptake were observed in both SH-SY5Y and astrocyte cells. Since two transporters may play role in D-serine uptake, the possibility of one- or two-step inhibition curve was also examined. In the statistical comparison, one-step fitting appeared to be the preferred model in case of both amino acids in SH-SY5Y and astrocyte cells as well. Calculated IC_{50} values of inhibition also showed no statistical difference for L-alanine and L-threonine in SH-SY5Y and astrocyte cells, as well.

T-Pro possesses charachter of ASCT-1 selectivity. This amino acid inhibited the uptake of D-serine in a concentration-dependent manner in both cell types (Fig. 3. A). Fitted

inhibition curves were compared and the two-step model proved to be a better fit for both SH-SY5Y and astrocyte cells. Around 30% decrease was registered in the uptake of D-serine in the higher affinity inhibition phase, which may correspond to the ASCT-1 transporter. IC_{50} values of abovementioned higher affinity inhibition phase were not statistically different in SH-SY5Y and astrocyte cells. At high concentrations t-Pro also inhibited the ASCT-2 transporter, as shown in the second phase of the inhibition curve. The highest employed concentration of t-Pro did not cause complete inhibition of D-serine uptake. IC_{50} values were calculated based on the results of the second phase, however, large error appeared, therefore statistical comparison could not be performed.

L-glutamine resulted in a concentration-dependent, almost complete inhibition of transport of D-serine in both cell types (Fig. 3. B). L-glutamine is suggested to be a preferred substrate for the ASCT-2 transporter, however in our experiment the one-step model characterized better the inhibition curves in tcase of SH-SY5Y and astrocyte cells as well. Consequently, L-glutamine probably possesses only a moderate affinity difference for the ASCT-1 and ASCT-2 transporters. The calculated IC_{50} values did not differ significantly comparing SH-SY5Y and astrocyte cells.

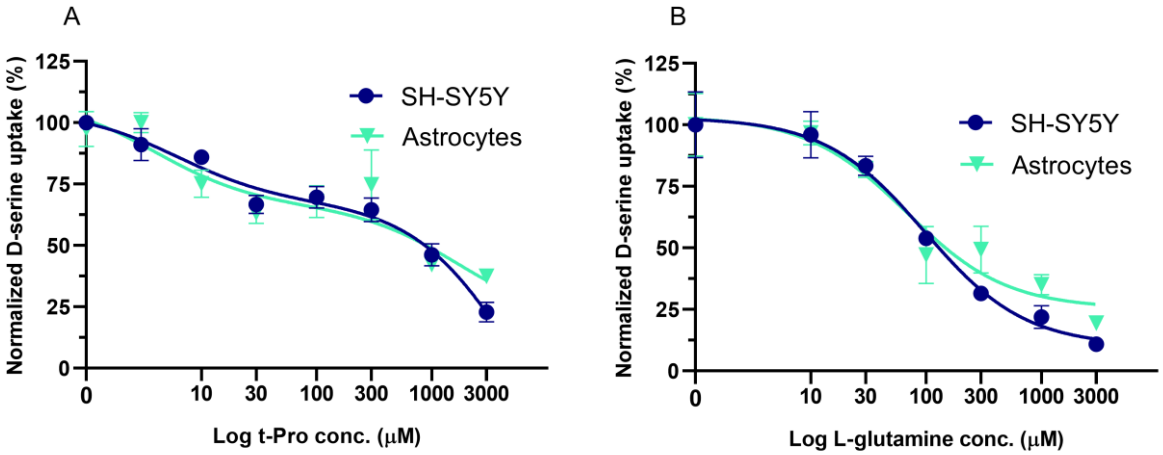

Figure 3: Inhibition of D-serine uptake in SH-SY5Y and rat cortical astrocyte cells by t-Pro and L-glutamine. T-Pro resulted in a two-step inhibition curve in both cell types (A). A one-step inhibition curve was observed for L-glutamine in SH-SY5Y and astrocyte cells (B). Intracellular D-serine concentration measured in the absence of inhibitor was considered as 100% of the D-serine uptake. Data are presented as mean ± SEM (n ≥ 3).

4.6 Following comparative measurements of SH-SY5Y and astrocyte cells, further characterization of D-serine transport was performed in SH-SY5Y cell line. Based on the literature data ASCT-1 and ASCT-2 transporters operate as antiporters, the uptake of an amino acid is accompanied by the release of another one. Treatment with L-serine caused time-dependent release of D-serine in SH-SY5Y cells pre-loaded with D-serine. This result was supported by measuring the decrease of intracellular D-serine concentration and also the increase of extracellular D-serine level (Fig. 4).

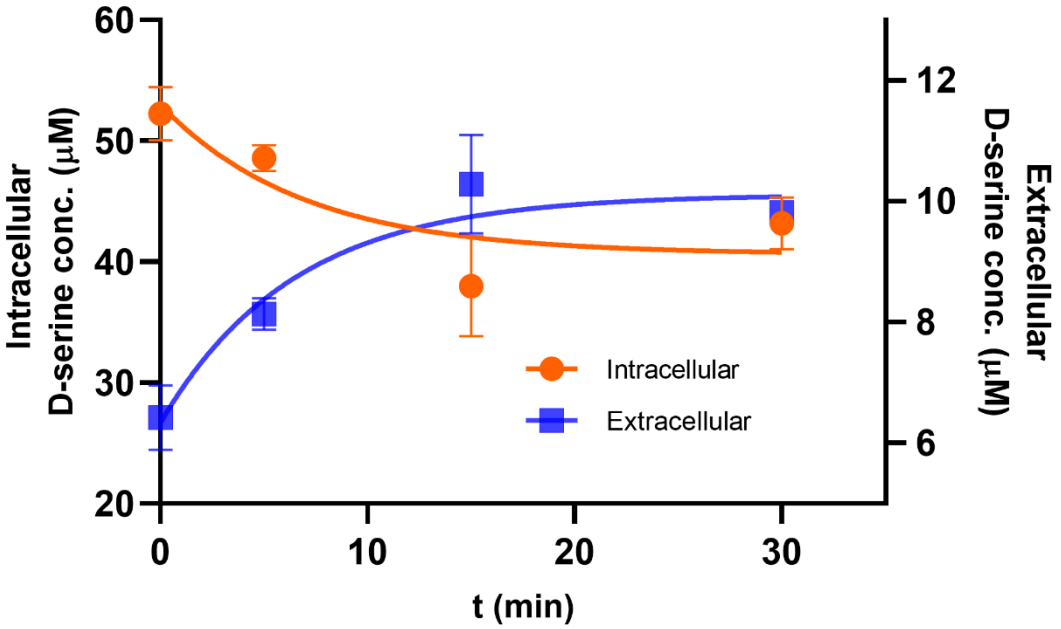

Figure 4: Realese of D-serine in SH-SY5Y astrocyte model cells induced by incubation with L-serine. Both intracellular and extracellular D-serine concentrations were determined. Adherent SH SY5Y cells were treated with medium containing 100 μM D-serine for 24 hours. After washing and suspending the cells, 300 μM L-serine was employed for 30 minutes. Data are presented as mean ± SEM (n ≥ 3).

4.7 Molecules supposed to affect D-serine transport of astrocyte cells were tested. In a recent *in vitro* experiment, the inhibitory effect of acute treatment with S-ketamine on D-serine transport was reported and the inhibition was linked to the ASCT-2 transporter. However, in our investigation incubation buffer containing 25 μM D-serine supplemented with increasing concentrations of S-ketamine did not significantly change the uptake of D-serine into SH-SY5Y cells.

The effect of long-term S-ketamine pretreatment of the cells on D-serine uptake was also examined. SH-SY5Y cells were incubated for 72 h with or without 25 or 50 μM S-ketamine, and after removal of the incubation medium, 300 or 1000 μM D-serine containing medium were employed for 15 min and levels of the intracellular D-serine were determined. Compared to the control group which were not pre-incubated with S-ketamine, 25 and 50 μM S-ketamine pre-treatment caused no significant change in D-serine uptake (Fig. 5).

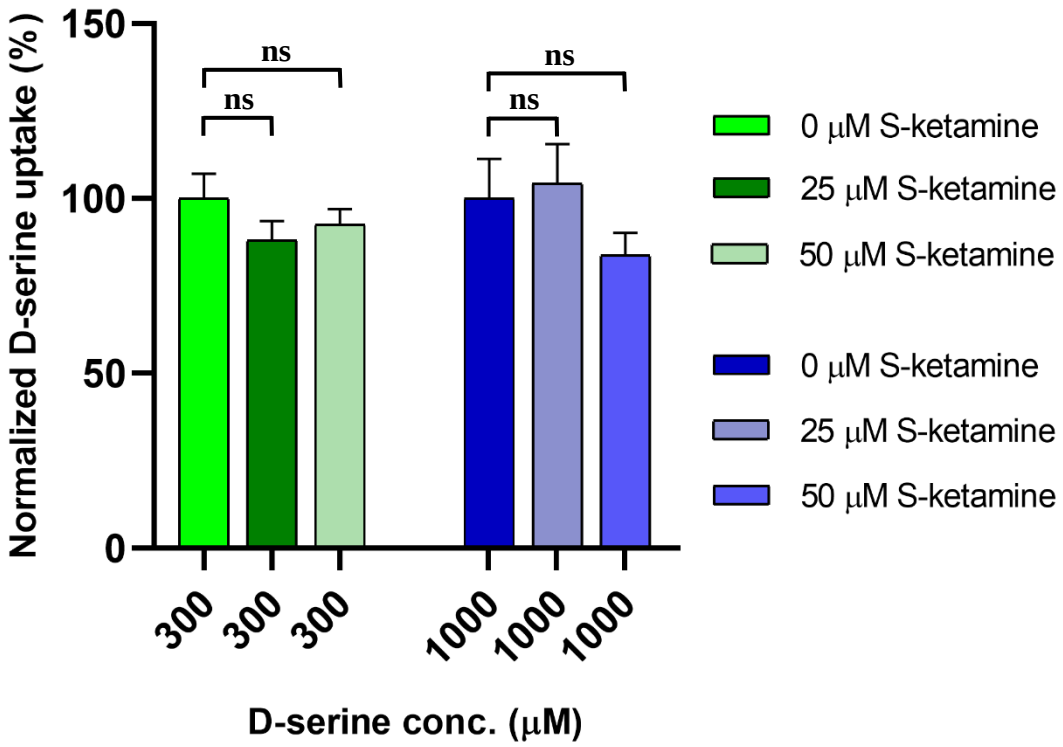

Figure 5: Effect of long-term S-ketamine pre-treatment on D-serine transport in SH-SY5Y cells. Adherent SH-SY5Y cells were maintained with 0, 25 or 50 μM S-ketamine for 72 hours. The cells were washed and resuspended and incubated with 300 or 1000 μM of D-serine for 15 minutes. Data are presented as mean ± SEM (n ≥ 3).

5. Conclusions

The aim of the present work was to study the possibilities of influencing intra- or extracellular D-serine concentrations in *in vivo* and *in vitro* experiments. First a novel DAAO inhibitor compound was tested *in vivo*. Thereafter D-serine transport of the SH-SY5Y cells was characterized and compared to that of astrocyte cells to determine if the cell line is suitable for modeling astrocyte-like D-serine transport. Finally, the pharmacological effect of S-ketamine on D-serine transport was investigated.

Based on preliminary studies, 6-fluoro-1H-indazol-3-ol was selected for *in vivo* testing. Our aim was to investigate the change in plasma D-serine concentration in mice treated with the new DAAO inhibitor. The tested molecule, 6-fluoro-1H-indazol-3-ol significantly increased the plasma concentration of D-serine in the animals. The effect developed as early as 10 minutes after intraperitoneal treatment and was maintained at 60 minutes. Our result confirmed the *in vivo* DAAO inhibitory effect of 6-fluoro-1H-indazol-3-ol.

We demonstrated that the SH-SY5Y human neuroblastoma cell line is suitable for the study of D-serine transport. Transport was compared with D-serine transport of astrocyte cells with the following observations:

- Predominantly Na^+-dependent D-serine transport was found in SH-SY5Y cell line similarly to astrocyte cells, and ASCT-1 and/or ASCT-2 transporters were supposed to be involved. Both transporter proteins were identified in SH-SY5Y cells.
- Two-step inhibition of D-serine uptake in both cell types caused by t-Pro, preferred substrate of ASCT-1, indicate that both ASCT-1 and ASCT-2 transporters are involved in D-serine transport.

- L-glutamine did not prove to be the preferred substrate for ASCT-2 transporter on either SH-SY5Y or astrocyte cells.
- ASCT substrate amino acids (L-alanine, L threonine, t-Pro, L-glutamine) similarly inhibited D-serine uptake in both SH-SY5Y and astrocyte cells. Calculated IC_{50} values did not show significant difference comparing the two cell types. This was confirmed by the similar kinetics of D-serine uptake in both SH-SY5Y cell line and astrocytes. These results support the applicability of the SH-SY5Y cell line as an astrocyte model to study D-Serine transport.

Further characterization of the SH-SY5Y astocyte-like cell model was performed by experiments with L-serine.

- L-serine treatment caused D-serine release from cells pre-loaded with D-serine, confirming the antiporter function of ASCT-1 and ASCT-2 transporters in the SH-SY5Y cell line.

The effect of S-ketamine on D-serine uptake was tested in the SH-SY5Y cell model.

- Astrocyte-like D-serine transport was not influenced by either acute treatment or prolonged pre-incubation with S-ketamine in the SH-SY5Y cell line.

Possibilities to influence the extracellular level of D-serine may be promising in numerous central nervous system diseases. The most studied are the inhibitors of DAAO enzyme, however, the clinical breakthrough is yet to come.

Another possible way to modulate the levels of D-serine is through its transporters, in which astrocyte cells play an important role in the central nervous system. Based on our results, the SH-SY5Y neuroblastoma cell line is a suitable astrocyte model providing an easy-to-use, fast, and robust workflow for studying the effect of various compounds on astrocyte-like D-serine transport avoiding the need to sacrafice laboratory animals.

6. Publications

Publications in the topic of dissertation:

1. **Vincze I,** Lakatos PP, Bagaméry F, Tábi T, Szökő É
   Characterization of a Cell Line Model for d-Serine Uptake.
   JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
   Volume: 187, pages: 113360, 2020, doi:10.1016/j.jpba.2020.113360
   Scientific article
   IF: 3.209 (2019)

2. Lakatos PP**, Vincze I,** Nyariki N, Bagaméry F, Tábi T, Szökő É
   The effect of L-theanine and S-ketamine on D-serine cellular uptake
   BIOCHIMICA ET BIOPHYSICA ACTA-PROTEINS AND PROTEOMICS
   Volume: 1868, pages: 140473, 2020, doi:10.1016/j.bbapap.2020.140473
   Scientific article
   IF: 2.371 (2019)

3. Szilagyi B, Kovacs P, Ferenczy GG, Racz A, Nemeth K, Visy J, Szabo P, Ilas J, Balogh GT, Monostory K, **Vincze I**, Tábi T, Szökő É, Keserű MGY
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   BIOORGANIC & MEDICINAL CHEMISTRY
   Volume: 26, page: 1579-1587., 2018, doi:10.1016/j.bmc.2018.02.004
   Scientific article
   IF: 2.802

Other original publications - not related to the topic of the dissertation:

1. Bagaméry F, Varga K, Kecsmár K, **Vincze I**, Szökő É, Tábi T
   The Impact of Differentiation on Cytotoxicity and Insulin Sensitivity in Streptozotocin Treated SH-SY5Y Cells
   NEUROCHEMICAL RESEARCH
   In press p. In press (2021), doi:10.1007/s11064-021-03269-2
   Scientific article
   IF: 3.038 (2019)

2. **Vincze I**, Rudge J, Vasarhelyi B, Karvaly GB
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Volume: 12, page:1243-1261., 2020, doi:10.4155/bio-2020-0179
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IF: 2.371 (2019)

3. Karvaly, GB, Neely M, Kovács K, **Vincze I**, Vásárhelyi B, Jelliffe RW
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4. Bagaméry F, Varga K, Kecsmár K, **Vincze I**, Szökő É, Tábi T
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