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D-Serine Uptake and Release in PC-12 Cells Measured by Chiral Microchip Electrophoresis-Mass Spectrometry

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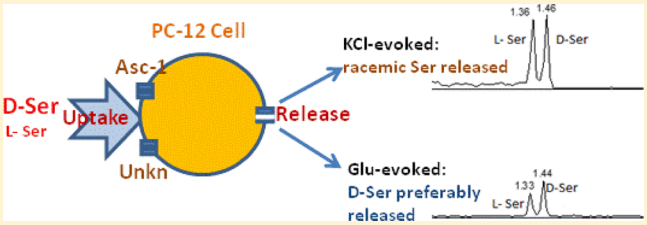
ABSTRACT: Previous work has established that D-serine (D-Ser) plays important roles in certain neurological processes. Study on its uptake/storage and release by neuronal cells is highly significant for elucidating relevant mechanisms. In this work, PC-12 cells were incubated with racemic Ser (100 μM each enantiomer). After incubation, both intra- and extracellular levels of D-Ser and L-Ser were quantified by chiral microchip electrophoresis with mass spectrometric detection. It was found the cells preferably took up D-Ser over L-Ser. After 120 min incubation, D-Ser percentage ([D-Ser]/([D-Ser] + [L-Ser]) in the culture media changed from 50% to 9% while inside the cells it increased from 13% to 67%. Small neutral amino acids such as threonine impaired D-Ser uptake. Ser release was studied by using PC-12 cells preloaded with D-Ser. KCl, Glu, and Gly evoked Ser release. Interestingly, while depolarization by KCl evoked release of Ser as a D-Ser/L-Ser mixture of 1:1 ratio, the stereoisomeric composition of Ser released due to Glu exposure varied with the exposure time, ranging from 73% D-Ser (i.e., [D-Ser] > [L-Ser]) at 2 min to 44% (i.e., [D-Ser] < [L-Ser]) at 14 min, clearly indicating a stereochemical preference for D-Ser in Ser release from neuronal cells evoked by Glu-receptor activation.



KEYWORDS: *D-Serine, uptake, release, PC-12 cells, chiral analysis, microchip electrophoresis-mass spectrometry*

D-Serine (D-Ser) is a putative glial neurotransmitter in mammalian central nervous system (CNS).[1−4] It binds glutamatergic *N*-methyl D-aspartate receptors (NMDARs) as an obligatory coagonist, thus modulating their functional activation. In the brain, a deficiency of D-Ser contributes to NMDAR hypofunction involved in neurological conditions such as schizophrenia.[5] Therefore, D-Ser replenishment serves as an effective therapeutic strategy.[5,6] Many studies have shown that D-Ser in adult rat brain is principally enriched in glial cells, particularly in type-2 astrocytes. Serine racemase (SR) is believed to be the enzyme responsible for D-Ser synthesis from L-Ser. SR was initially found in astrocytes and microglia.[7,8] Further studies showed that SR was actually expressed at a significantly higher level in neurons than in astrocytes.[4,9,10] These findings support the theory that D-Ser is synthesized in neurons, and then transported both to other neurons and to surrounding astrocytes where it is stored. However, recent studies comparing SR and D-Ser levels in cell type-specific SR knockout mice found that, despite a significant reduction of SR in neuronal SR knockout mouse brains, D-Ser levels were only minimally reduced, indicating that neurons are not the sole source of D-Ser.[11,12] Another study also revealed that SR was present in glial vesicles isolated from astroglial cells.[13] Further, it has been shown that intraperitoneal (i.p.) administration of D-Ser can cause a significant increase of D-Ser level in the rat brain, confirming D-Ser is able to pass through the blood-brain barrier.[14,15] Exogenous D-Ser intake may, therefore, also have an influence on its level in the brain.

Studies on D-Ser uptake and release in C6 glioma cells, cortical astroglia cells, primary neuronal cultures, and rat brain have been reported. Cultured C6 glioma cells were found to uptake and accumulate both [^{3}H]D- and [^{3}H]L-Ser from culture media in a temperature-dependent and saturable manner. The affinities (k_m values) were measured to be 2.5 mM for [^{3}H]D-Ser and 0.11 mM for [^{3}H]L-Ser.[16] An alanine-serine-cysteine transporter (ASCT2) mediated mechanism was proposed for D-Ser uptake in these cells.[17] A recent study with cultured cortical astroglia cells prepared from Wistar pups showed that astrocytes contained specific vesicles capable of releasing D-Ser by Ca^{2+}-dependent exocytosis.[13] In contrast with the notion that D-Ser is exclusively released from astrocytes, studies with primary neuronal cultures showed that D-Ser was released by neuronal depolarization both in vitro and in vivo. Veratridine (50 μM) or depolarization by 40 mM KCl elicited a significant release of endogenous D-Ser from the cells while controls with astrocyte cultures showed that glial cells were insensitive to veratridine. Since no internal or external Ca^{2+} presence required for the release, it was believed that D-Ser was released from these cells by a nonvesicular release mechanism.[18,19] Using an

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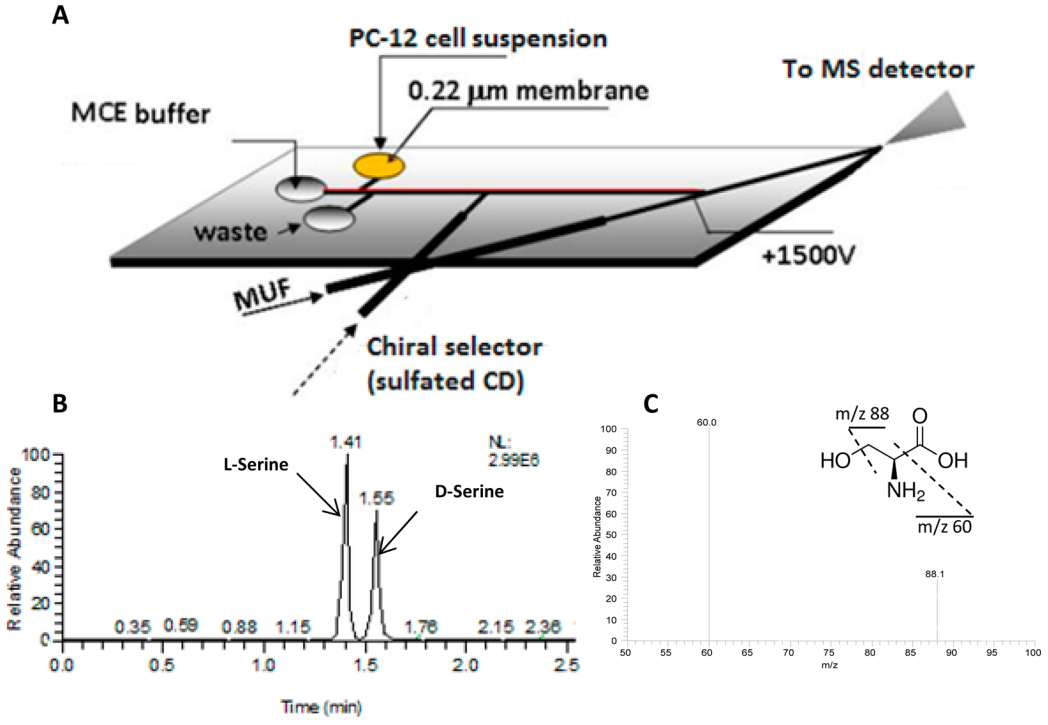

Figure 1. Study of D-Ser uptake and release in PC-12 cells with a chiral microchip electrophoresis-mass spectrometric platform: (A) lab-on-chip experimental setup; (B) electropherograms showing L-Ser and D-Ser peaks separated by chiral microchip electrophoresis and detected by selected reaction monitoring (SRM) m/z 88 → 60; and (C) MS^2 spectrum confirming the peak identity.

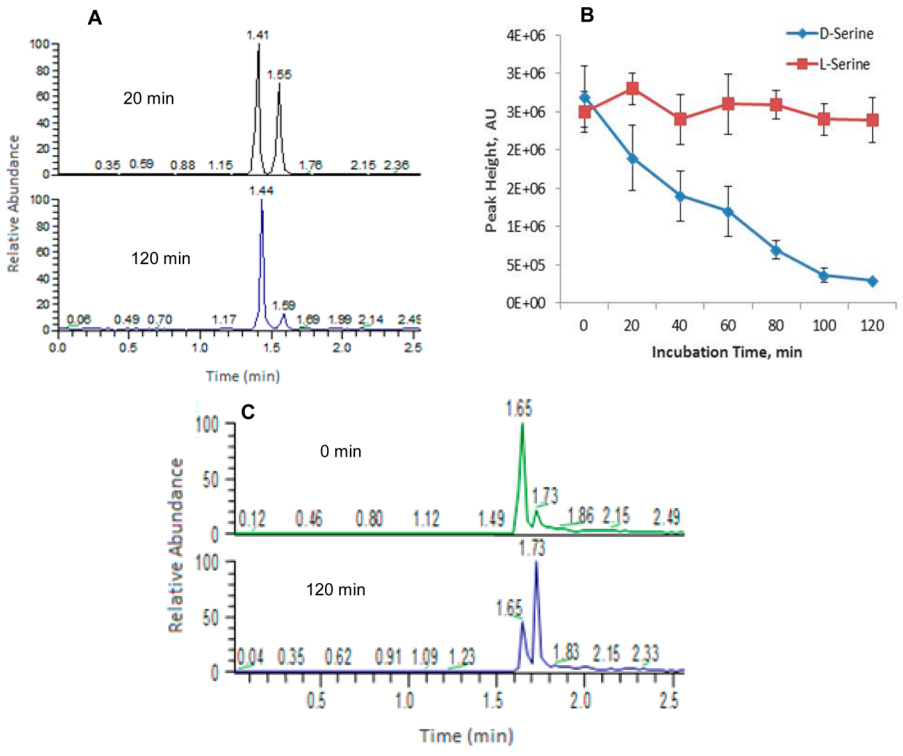

Figure 2. Uptake of Ser by PC-12 neuronal cells: (A) electropherograms obtained from analysis of an incubation solution of PC-12 cells with racemic Ser (at 100 μM each enantiomer) at 20 and 120 min to determine extracellular levels of D-Ser and L-Ser; (B) concentration trends for L-Ser and D-Ser with incubation time; and (C) electropherograms obtained from quantification of intracellular L-Ser and D-Ser enantiomers before (0 min) and after incubation (120 min), respectively.

online microdialysis-CE-LIF method to measure the D- and L-Ser levels in the rat striatum, in vivo studies found that extracellular D-Ser level increased in response to application of high-K^+ aCSF. NMDA also induced increases in D-Ser, L-Ser, glutamate, and GABA concentrations in the striatum. The increases were attenuated by the NMDA receptor antagonist

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MK-801.[20] D-Ser release and uptake were also investigated in vivo using an enzyme-based D-Ser microelectrode implanted in rat brain.[21] It was found that small L-amino acids, including L-Ala and L-Thr, were able not only to block D-Ser reuptake, but also to evoke release of endogenous D-Ser. These findings suggested that small neutral amino acid transporters, including Asc-1 and ASCT-2 might be involved in both release and uptake of D-Ser through an amino acid heteroexchange mechanism.

In this study, D-Ser uptake and release in PC-12 neuronal cells were investigated for the first time. Pheochromocytoma-derived PC-12 cell line is a well-established in vitro model for studies of regulated secretion in neuronal cells.[22] The cell line has been shown to express both monomeric- and dimeric forms of SR, and their biological activity in the cells has been reported.[23,24] An advantageous analytical method based on chiral microchip electrophoresis with mass spectrometric detection was used to quantify L- and D-Ser simultaneously. In this method, L-Ser and D-Ser were separated from each other by electrophoresis and then detected by mass spectrometry with specificity. This allowed exploring the stereochemical preference of the pathways for uptake and release of D-Ser in the neuronal cells. Effects of small amino acids, including L-Thr and L-Arg on D-Ser uptake, were studied to verify the involvement of Asc-1 transporter in the uptake. Cellular release of D-Ser under various chemical stimulations was studied using PC-12 cells that were preloaded with D-Ser. Particular attention was given to comparing stereoisomeric compositions of Ser released due to KCl-induced neuronal polarization with that caused by Glu-induced receptor activation in searching for a better understanding of the mechanism for D-Ser release from neuronal cells.

RESULTS AND DISCUSSION

To study D-Ser uptake in PC-12 neuronal cells, the cells were incubated with racemic Ser at a concentration of 100 μM each enantiomer. After incubation, intracellular and extracellular levels of both L-Ser and D-Ser were simultaneously quantified by using a chiral microchip electrophoresis-mass spectrometric (MCE-MS) method recently developed in our lab.[25,26] To study cellular release of D-Ser, the cells preloaded with D-Ser were incubated with PBS containing various stimulants, and then extracellular levels of both L-Ser and D-Ser were determined. Figure 1A illustrates the MCE-MS analytical platform. Incubation tests were carried out in the sample reservoir on the microfluidic chip. The reservoir was equipped with a piece of 0.22 μm membrane, preventing cells from getting into microfluidic channels. The incubation solution could be injected into the system at selected time intervals for quantifying L-Ser and D-Ser. Under the selected MCE conditions, L-Ser and D-Ser were well separated, and very importantly, the separation was completed within a short time (<2 min) as shown in Figure 2B. The peak identification was verified by MS^2 spectrum (Figure 1C). Various analytical methods based on HPLC,[18,27,28] CE,[17,29−31] biosensors,[21,32] and HPLC-MS[33,34] techniques were previously reported for quantification in study of D-Ser uptake and release. Compared with the methods previously reported, the present MCE-MS method offers advantages of high assay selectivity due to the deployment of mass spectrometric detection and an integration of cell treatment, sample injection, and Ser enantiomeric quantification into a single microfluidic platform, which shortens the analysis time and improves assay accuracy and repeatability.

Stereochemical Preference for D-Ser over L-Ser in the Uptake. D-Ser uptake in PC-12 cells was investigated by incubating the cells with racemic Ser (100 μM each enantiomer) in the sample reservoir on the MCE-MS platform (Figure 1A). Racemic Ser instead of D-Ser was used for the incubation because the stereochemical aspects of the uptake pathways could be investigated. At different time intervals, the incubation solution was injected into the MCE-MS analytical platform to simultaneously quantify L-Ser and D-Ser. Typical electropherograms obtained are shown in Figure 2A. The D-Ser peak decreases as the incubation time increases while the L-Ser peak almost remains the same, indicating D-Ser disappears from the incubation medium and L-Ser remains intact during the incubation. The concentration trends measured for the two enantiomers are shown in Figure 2B. There are two possible causes for these results. One is that PC-12 cells selectively metabolize D-Ser while keeping L-Ser intact, and another is they uptake D-Ser preferably over L-Ser. To clarify this point, intracellular levels of D-Ser and L-Ser were determined before and after incubation with racemic Ser. The results (shown in Figure 2C) suggest that the cells uptake D-Ser while leaving L-Ser alone under the incubation conditions selected. After 120 min incubation with racemic Ser at 100 μM each enantiomer, intracellular level of D-Ser increases substantially. Ser enantiomeric composition inside the cells changed from 13% to 67% D-Ser (calculated by [D-Ser]/([L-Ser] + [D-Ser]). These findings clearly indicate that PC-12 cells uptake D-Ser preferably over L-Ser and accumulate it in the cells for future use.

Asc-1 Transporter Involvement and Other Unknown Pathways Involved in D-Ser Uptake. We have shown that PC-12 neuronal cells preferably uptake D-Ser over its antipode, L-Ser from culture media. Small neutral amino acid transporters (i.e., Asc-1 and ASCT2) are known to transport D-Ser in the nervous system. Asc-1 is primarily expressed in neuronal cells and has a high affinity for Ser, Cys, and Thr.[21,35−37] To assess Asc-1's role in D-Ser uptake by PC-12 cells, the cells were incubated with racemic Ser (at 100 μM each enantiomer) in the presence of amino acids at 80 mM. After 120 min incubation, the culture medium was analyzed to determine L-Ser and D-Ser. For each incubation test, the relative concentration of D-Ser (i.e., [D-Ser]/[L-Ser]) was used to assess the effects of the respective amino acid on D-Ser uptake. Four amino acids, including two neutral (Thr and Ala) and two basic (Lys and Arg) were studied. The results are summarized in Figure 3. In the presence of Ala or Thr, very minor decreases in D-Ser concentration are observed as compared with those in the control or Arg- or Lys-containing solutions. Both of the neutral amino acids tested, that is, Ala and Thr, effectively impaired D-Ser uptake by the cells while the basic amino acids, that is, Arg and Lys, almost showed no impairing effects. These findings firmly indicate that Asc-1 participates in D-Ser uptake by PC-12 neuronal cells, which is in consistence with the previously reported studies. Further, the present study demonstrates that Asc-1 mediated amino acid exchange is not the solo pathway of D-Ser uptake in PC-12 cells. This is because through the amino acid exchange pathway alone, the intracellular D-Ser level should not be higher than that of L-Ser when their extracellular levels are the same or reversed. Nevertheless, as shown above, incubating the cells with racemic Ser (at 100 μM each enantiomer) for 120 min caused a change in intracellular percentage of D-Ser from 13% to 67% D-Ser. These results

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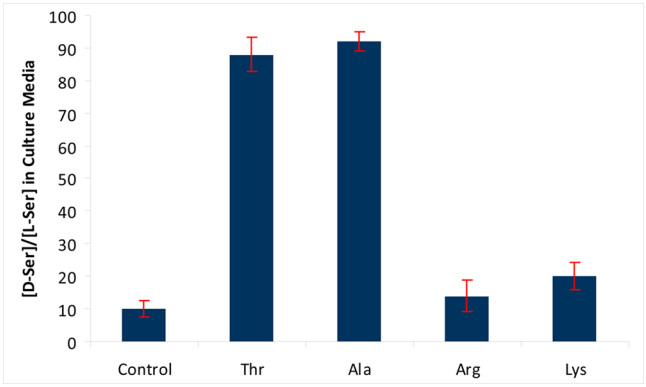

Figure 3. D-Ser concentrations in PC-12 cells. Racemic Ser incubation solutions, with or without amino acids, showing the effects of small neutral amino acids on D-Ser uptake by the cells.

suggest that other mechanisms may participate in D-Ser uptake by PC-12 cells. Based on the intracellular levels of D-Ser and L-Ser after incubation with racemic Ser, PC-12 cells accumulate both D-Ser and L-Ser, but with a much higher affinity for D-Ser. It is worth noting that [3H]D-Ser is accumulated into C6 glioma cells only ~5% relative to L-Ser (K_m value of L-Ser uptake was approximately 1/20 of that for the D-Ser uptake in the C6 glioma cells).[15,18] Further study is needed to elucidate the unknown mechanisms in D-Ser uptake by PC-12 neuronal cells.

Stereochemical Aspects in Ser Release from PC-12 Cells Evoked by KCl and Glu. To study D-Ser release from PC-12 cells, the cells were preloaded with D-Ser through incubation with racemic Ser for 120 min. The cells were then washed and suspended in PBS solution. The cell suspension was transferred to the sample reservoir in the MCE-MS platform. After a stimulant was added, the suspension was injected into the MCE-MS system at different time intervals for analysis to determine D-Ser and L-Ser levels. KCl, glutamate, and glycine were tested as stimulants. Figure 4 shows the electropherograms obtained from the analysis. From the peak heights, exposure to KCl causes a 3−5-fold increase in Ser release compared with stimulation by Gly or Glu. This indicates that neuronal depolarization is a major pathway for D-Ser release. It is worth noting that the amounts of L-Ser and D-Ser released by KCl depolarization are essentially the same, that is, the release caused by depolarization has no stereochemical preference. Gly evokes Ser release, too. Since Gly is a small neutral amino acid, the release is likely through Asc-1 transporter mediated amino acid exchange mechanism. The two enantiomers of Ser released are again at the same level. Glutamate-induced D-serine release from glial cells[38] and neuronal cells[4,19] through activation of glutamate receptors was reported previously. The results from the present study indicate that Glu evokes Ser release from PC-12 neuronal cells and the release exhibits a stereochemical preference for D-Ser over L-Ser (Figure 4, third panel). To further study Glu-induced Ser release, we monitored the Ser enantiomeric levels in the media against exposure time. It was found that the stereochemical composition of Ser released changed with the exposure time (Figure 5). At 2 min, about 73% of Ser released is D-Ser, and the percentage of D-Ser decreases as the exposure time increases. At 14 min, more L-Ser is present in the media than D-Ser. Taking into account of the low basal Glu concentration in the synaptic space in vivo (in the range of 0.5−1.0 mM), we also performed the stimulation tests with Glu at lower concentrations (i.e., 0.25 and 2.5 mM). The data obtained showed the same trend as that with 25 mM Glu, but the amounts of Ser released was significantly smaller and the

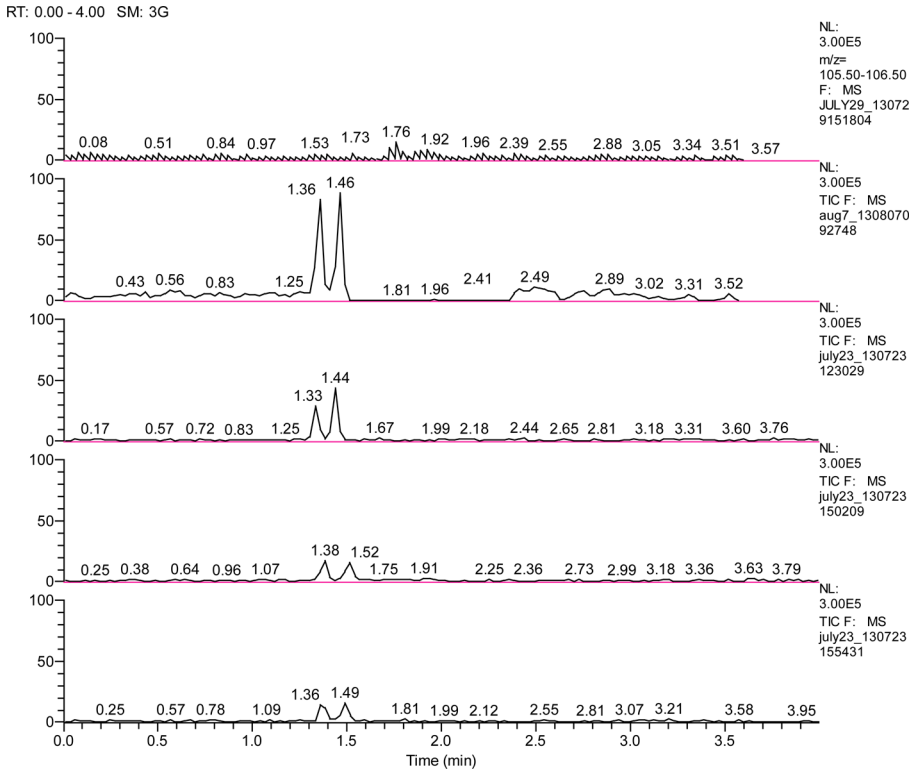

Figure 4. D-/L-Ser released from PC-12 cells exposed to various stimulants for 5 min. [KCl] = 15 mM, [Gly] = 83 mM, and [Glu] = 25 mM.

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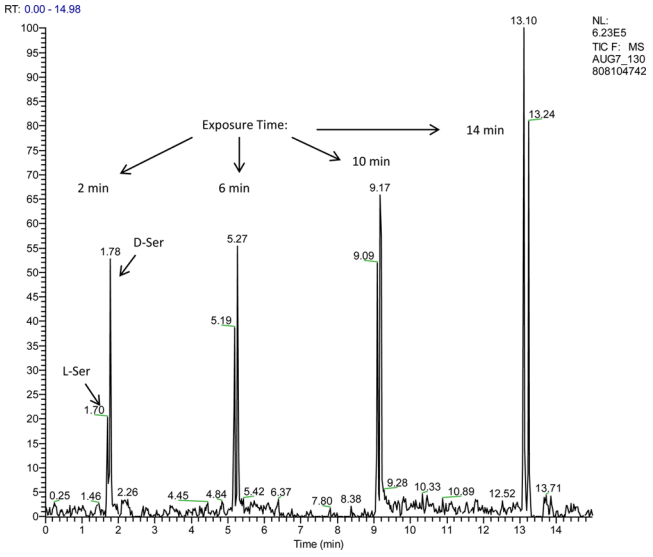

Figure 5. Monitoring D-Ser and L-Ser released from PC-12 cells exposed to 25 mM glutamic acid. Results of extracellular D-/L-Ser ratio indicate that Glu-induced D-/L-Ser release exhibits a stereochemical preference for D-Ser.

stereochemical changes in Ser release occurred at a much slower pace. In the case of stimulation by 0.25 mM Glu, it took >5 h to detect more L-Ser than D-Ser in the media.

CONCLUSION

PC-12 neuronal cells uptake and accumulate Ser with a stereochemical preference for D-Ser over L-Ser. This is confirmed by quantifying extracellular and intracellular levels of D-Ser and L-Ser after incubating the cells with racemic Ser. This stereochemical preference cannot be explained by Acs-1 mediated amino acid exchange mechanism alone. An unknown pathway is, therefore, speculated for D-Ser transport. Ser release from PC-12 cells is evoked by KCl, Gly, and Glu. While equal amounts of D-Ser and L-Ser are released in both KCl depolarization and Gly-induced amino acid exchange, Glu-evoked Ser release through Glu receptor activation exhibits a stereochemical preference for D-Ser.

METHODS

Cell Culture and Incubation Tests. PC-12 cells (obtained from ATCC) were cultured in complete RPMI medium supplemented with 10% heat inactivated horse serum and 5% FBS. Cells were routinely subcultured every 4−5 days. To investigate Ser uptake, portions (450 μL) of a cell suspension at 2×10^6 cells/mL were transferred to respective wells of a plate, and to each well 50 μL Ser racemate solution (1×10^{-3} M each enantiomer prepared in PBS at pH 7.4) was added. The plate was kept in an incubator of 5% CO_2. At selected time intervals, the incubation solution was stirred by gentle pipetting and a portion (25 μL) was transferred to the sample reservoir in the MCE-MS platform for analysis. To study the effects of amino acids on Ser uptake, the respective amino acid was added to Ser racemate solution and the Ser solution was used as described above. To investigate Ser release from PC-12 cells, 450 μL cell suspension (at 2×10^6 cells/mL) was transferred to a plate and then 50 μL Ser racemate solution (1×10^{-3} M each enantiomer prepared in PBS at pH 7.4) was added. The plate was kept in an incubator of 5% CO_2 for 90 min. The cells were collected by spinning at 100 rpm, washed twice with PBS buffer, and suspended in 500 mL PBS. To the sample reservoir in the MCE-MS platform 20 μL cell suspension was transferred and 5 μL stimulant solution prepared in PBS was added. The solution was well mixed by gentle pipetting and injected into the chiral MCE-MS system for analysis at selected time intervals.

Microchip Electrophoresis-Mass Spectrometric System. The MCE-MS system was recently developed in the group.[25,26] It consisted of an ion trap mass spectrometer (LCQ Deca, ThermoFinnigan, San Jose, CA), a microchip prepared in-house, a multichannel high voltage power supply, and two syringe pumps (Figure 1A). One syringe pump was used for delivering the sulfated β-CD-containing buffer solution, and the other for MUF delivery. The microchip was fixed on a XYZ-translational stage and so positioned that the nanoESI emitter tip was about ∼1.0 mm away from the MS orifice. Xcalibur software (ThermoFinnigan) was used to control the mass spectrometer and process MS data. House-written software was used for controlling the potentials applied to the microchip for MCE and nanoESI operations.

Chiral CE-MS Assay. The two syringes were filled with the respective solutions and then connected to the respective capillaries assembled into the microchip. MCE running buffer was added to the buffer reservoir on the chip. Make-up flow (MUF) pump was tuned on to generate a MUF. The sulfated β-cyclodextrin (β-CD) solution (about 35 nL) was infused to partially fill the MCE separation channel by turning on and off the syringe pump set at 200 nL/min. To inject a sample, potentials of 350, 900, 0, and 1500 V were applied at sample reservoir, running buffer reservoir, waste reservoir, and Pt wire for 20 s, respectively. After sample injection, the potentials were changed to float, 3950 V, float, and 1500 V, respectively, to start the MCE-MS assay. At the same time, MS data acquisition was started. When completed, the potentials were removed. MUF delivering syringe pump remained on for some time to clean the nanoESI emitter.

MCE conditions: MCE running buffer, 15 mM ammonium acetate/acetic acid buffer (pH 5.5) and methanol (1:1); MUF, the MCE running buffer at 100 nL/min; sulfated β-CD solution, 15 mM sulfated β-CD in MCE running buffer.

MS conditions: ion source voltage, 0 V; relative collision energy, 25% with an isolation width of 2.0 u and 30 ms activation time; auxiliary gas, 0 unit, sample capillary temperature, 250 °C.

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Author Contributions

X.L., S.Z., H.H., and Y.-M.L. designed the experiments and analyzed the data. X.L. and C.McC. performed the experiments. X.L. and Y.-M.L. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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