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SHORT COMMUNICATION

Increasing effects of *S*-methyl-L-cysteine on the extracellular D-serine concentrations in the rat medial frontal cortex

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Abstract In an in vivo dialysis experiment, the intra-medial frontal cortex infusion of a system A and Asc-1 transporter inhibitor, *S*-methyl-L-cysteine, caused a concentration-dependent increase in the dialysate contents of an endogenous coagonist for the *N*-methyl-D-aspartate (NMDA) type glutamate receptor, D-serine, in the cortical portion. These results suggest that these neutral amino acid transporters could control the extracellular D-serine signaling in the brain and be a target for the development of a novel threapy for neuropsychiatric disorders with an NMDA receptor dysfunction.

Keywords Asc-1 transporter · In vivo microdialysis · Medial frontal cortex · *S*-Methyl-L-cysteine · D-Serine

Introduction

A wealth of evidence has been accumulated indicating that D-serine may play a pivotal role in the control of the *N*-methyl-D-aspartate (NMDA) type glutamate receptor as its intrinsic coagonist (see Nishikawa 2011 for a review). The synthesis, storage, extracellular release, uptake, and degradation processes for D-serine have been found in mammalian brains (Nishikawa 2011). Because the disturbed NMDA receptor functions have been implicated in the pathophysiology of a wide variety of neuropsychiatric disorders, such as brain ischemia and schizophrenia, finding the tools to modulate the extracellular D-serine signaling may contribute to the development of novel therapeutic approaches (Nishikawa 2011). Consequently, the molecules that participate in the above metabolic processes of D-serine would be the candidate targets for these tools. From this viewpoint, it is worthwhile to note that *S*-methyl-L-cysteine (Bracy et al. 1986; Thomsen et al. 2003) exhibits a low to moderate affinity for the amino acid transport system A (Mackenzie and Erickson, 2004) and a sodium-independent neutral amino acid transporter Asc-1 (Fukasawa et al. 2000) that are capable of taking up alanine and serine. Interestingly, Thomsen et al. (2003) demonstrated that *S*-methyl-L-cysteine inhibited the [^{3}H]D-serine uptake into the HEK293 cells expressing human Asc-1, and revealed using an in vivo dialysis technique in which local infusion of this compound into the hippocampus augmented the extracellular contents of substrates of Asc-1 including serine and alanine. However, there are so far no reports that investigated the changes in the extracellular D-serine contents by *S*-methyl-L-cysteine in vivo. Such an in vivo study is important to clarify not only the effects of *S*-methyl-L-cysteine but also the physiological regulation of the extracellular D-serine release in intact mammalian brains because brain D-serine has been suggested to function by interactions with both the neuronal and glial cells (Nishikawa 2011). Therefore, we have monitored the extracellular concentrations of D-serine following local

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perfusion of *S*-methyl-L-cysteine in the medial frontal cortex of freely moving rats by in vivo microdialysis.

Materials and methods

Animals and drugs

The present animal experiments were performed in strict accordance with the guidance of the Tokyo Medical and Dental University and were approved by the Animal Investigation Committee of the Institution. Male Wistar rats (ST strain, Clea Japan, Inc., Japan) at postnatal days 56, weighing 200–230 g, were used. The animals were housed at 23.0 ± 0.5 °C in a humidity-controlled room under a 12-hour light/dark cycle and had free access to food and water. *S*-Methyl-L-cysteine and D-alanine were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA), and dissolved in Ringer solution for the intra-brain perfusion. The control animals received only the Ringer solution.

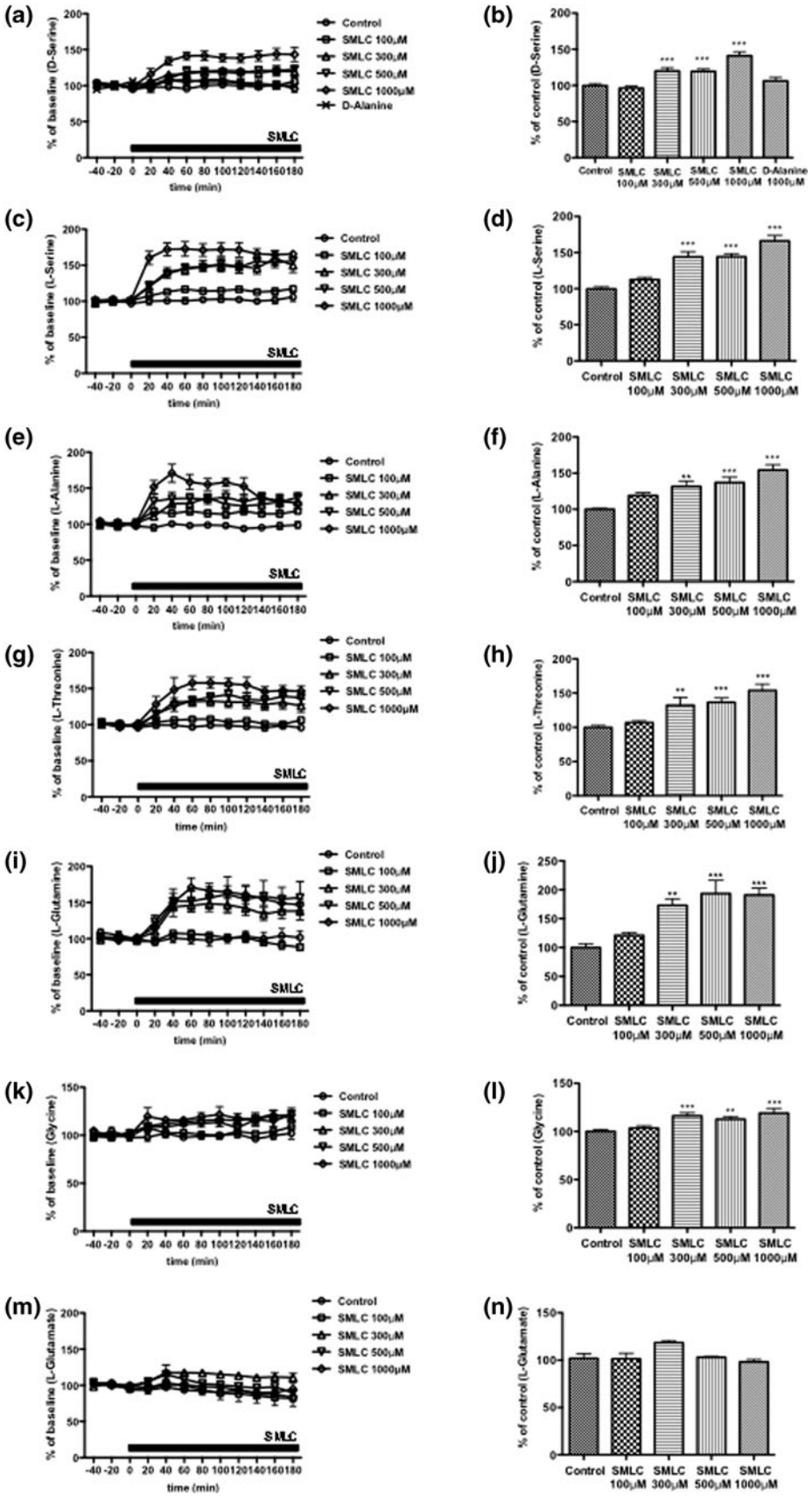
Microdialysis technique

The in vivo microdialysis was performed as previously reported with some modifications (Nishijima et al. 1996; Hashimoto et al. 1995; Kanematsu et al. 2006). The rats were anesthetized with pentobarbital (40 mg/kg, intraperitoneally) and mounted on a stereotaxic frame. A straight cellulose dialysis tube (3.0 mm in length, 0.16 mm internal diameter, molecular weight cutoff 50,000, EICOM Co., Ltd., Japan) was then implanted into the medial prefrontal cortex (mPFC: A +2.2 mm, V +5.0 mm, L −0.7 mm) according to the atlas of Paxinos and Watson (2005). Two days after surgery, the dialysis probe was perfused with a Ringer solution (NaCl, 147 mM; KCl, 4 mM; $CaCl_2$, 1.3 mM; pH 7.4) at the flow rate of 2 µl/min in a freely moving rat. After stabilizing for at least 80 min, the dialysate samples were collected every 20 min. The first three samples were used to determine the basal release of each amino acid, and then various experiments were started by the third sampling of the dialysate as time 0. A 180-min application of *S*-methyl-L-cysteine was performed by perfusing the Ringer solution containing different concentrations of *S*-methyl-L-cysteine from time 0 to 180 min.

Biochemical assay and experimental procedure

The collected samples were stored at −80 °C until derivatization following the addition of D-homocysteic acid as the internal standard. For quantification of the amino acids by HPLC with fluorometric detection (Hashimoto et al. 1993), an aliquot of each sample was derivatized with *N-tert*-butyloxycarbonyl-L-cysteine and *o*-phthaldialdehyde for 2 min at room temperature. The derivatized sample was immediately applied to the HPLC system and then analyzed on a 4-µm Nova-Pak C18 column (300 mm × 3.9 mm) (Waters, Japan). The column was operated at the constant flow rate of 0.8 ml/min at 35 °C. Mobile phase A was 0.1 M acetate buffer (pH 6.4) containing 12 % acetonitrile and mobile phase B was the acetate buffer containing 20 % acetonitrile. The separation of the amino acid derivatives was performed using a linear gradient from mobile phase A to B for 53 min. The fluorescent amino acid derivatives were detected using a Waters 2475 Multi 1 fluorescence detector spectrofluorometer (Waters Co., Ltd., Japan). The

Fig. 1 Effect of intra-medial frontal cortex infusion of *S*-methyl-L-cysteine on the extracellular contents of D-serine and other amino acids in the cortical portion of freely moving rats. As indicated by the closed bars, *S*-methyl-L-cysteine (SMLC: 100, 300, 500 and 1000 µM) or D-alanine at 1000 µM was infused into the medial frontal cortex of freely moving rats via dialysis tube for 180 min from the start of the present experiments (time 0). The concentrations of D-serine (Panels a, b), L-serine (c, d), L-alanine (e, f), L-threonine (g, h), L-glutamine (i, j), glycine (k, l) and L-glutamate (m, n) in the cortical dialysates collected every 20 min were quantitatively determined using HPLC with fluorometric detection. Each point represents the mean with SE mean of data obtained from three to nine animals and expressed as a percentage of the average of the respective basal contents during the period preceding the drug treatment. Absolute values of the average baseline contents of various amino acids in all the animals used for the present study were (µM): D-serine, 1.264 ± 0.064, $n = 36$; L-serine, 4.779 ± 0.231, $n = 31$; L-alanine, 6.719 ± 0.454, $n = 31$; L-threonine, 5.754 ± 0.336, $n = 27$; L-glutamine, 22.84 ± 1.227, $n = 22$; glycine, 6.240 ± 0.434, $n = 29$; L-glutamate, 4.063 ± 0.3068, $n = 24$. Maximal concentrations of amino acids after infusion of 1000 µM SMLC were (µM): D-serine, 1.450 ± 0.1393, $n = 4$, 142 % of basal contents, 1.026 ± 0.057, $n = 4$; L-serine, 7.823 ± 0.6981, $n = 4$, 174 % of basal contents, 4.677 ± 0.355, $n = 4$; L-alanine, 10.95 ± 1.957, $n = 4$, 170 % of basal contents, 6.992 ± 1.029, $n = 4$; L-threonine, 8.695 ± 1.104, $n = 4$, 157 % of basal contents, 5.809 ± 0.635, $n = 4$; L-glutamine, 44.59 ± 5.129, $n = 4$, 172 % of basal contents, 26.88 ± 1.647, $n = 4$; glycine, 7.604 ± 2.361, $n = 4$, 121 % of basal contents, 6.511 ± 1.179, $n = 4$; L-glutamate, 3.836 ± 1.168, $n = 4$, 105 % of basal contents, 3.671 ± 0.699, $n = 4$. The area under the curve (AUC) is also calculated by adding the areas under the graph of the concentration of the respective amino acid between each pair of every 20-min consecutive observation from 20 to 180 min of treatment (panels b, d, f, h, j, l and n) and expressed as a percentage of the respective control values which were: D-serine, 15832 ± 406.9, $n = 9$; L-serine, 16274 ± 465.8, $n = 8$; L-alanine, 15596 ± 278.8, $n = 8$; L-threonine, 15748 ± 480.2, $n = 7$; L-glutamine, 16080 ± 1069, $n = 4$; glycine, 15920 ± 299.8, $n = 8$; L-glutamate, 14796 ± 522.5, $n = 8$. The statistical significance of the data was evaluated using Dunnett's test for comparison among six (for D-serine) or five (for the other amino acids represented in this figure) groups in the present experiments. $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$ compared to the Ringer solution-infused controls. AUC of D-serine value which was D-alanine-infused is 16711 ± 783.4, $n = 4$ (not significant as compared to control D-serine values). The AUC values of D-serine after D-alanine infusion (16711 ± 783.4, $n = 4$) are not significantly different compared to the control D-serine values


(a)
% of baseline (D-Serine)
Control
SMLC 100μM
SMLC 300μM
SMLC 500μM
SMLC 1000μM
D-Alanine
SMLC
time (min)
(b)
% of control (D-Serine)
Control
SMLC 100μM
SMLC 300μM
SMLC 500μM
SMLC 1000μM
D-Alanine 1000μM
(c)
% of baseline (L-Serine)
(d)
% of control (L-Serine)
(e)
% of baseline (L-Alanine)
(f)
% of control (L-Alanine)
(g)
% of baseline (L-Threonine)
(h)
% of control (L-Threonine)
(i)
% of baseline (L-Glutamine)
(j)
% of control (L-Glutamine)
(k)
% of baseline (Glycine)
(l)
% of control (Glycine)
(m)
% of baseline (L-Glutamate)
(n)
% of control (L-Glutamate)

excitation and emission wavelengths were 344 and 443 nm, respectively.

Data analysis

The average concentration of each substance during the period preceding the drug treatment (three measurements were performed every 20 min) was used as the baseline control value (= 100 %). The individual data are expressed as percentages of this baseline period. The means with SEM of the results obtained from 3 to 9 determinations were calculated using the corresponding periods. The areas under the curves (AUC) of the concentration versus time plots for the dialysate amino acids at 20–180 min post-injection were calculated and used as the overall measures of the treatment effects (Matthews et al. 1990). Statistical differences among more than three groups in this dose-dependent study were estimated by Dunnet's test.

Results

The efficiency of the dialysis across the membrane was found to be approximately 15 % (14.62 ± 0.14, $n = 3$) for *S*-methyl-L-cysteine, suggesting that the estimated *S*-methyl-L-cysteine concentrations outside the dialysis probe are from $10^{-6} \sim 10^{-4}$ M in the present experiments.

The acute local infusion of *S*-methyl-L-cysteine at 300, 500 and 1000 μM, but not 100 μM, into the medial frontal cortex via an in vivo dialysis probe caused a concentration-dependent increase in the dialysate contents of D-serine (Fig. 1a, b) up to approximately 140 % of the baseline levels, and those of L-serine (Fig. 1c, d), L-alanine (Fig. 1e, f), L-threonine (Fig. 1g, h), and L-glutamine (Fig. 1i, j) at the maximum magnitude around 150–220 % of the Ringer solution control values by comparing the values at each time point and AUCs in the cortical portion of freely moving rats. The *S*-methyl-L-cysteine infusion elicited a much smaller, but significant augmentation of the extracellular glycine levels up to roughly 115–120 % of those of the controls (Fig. 1k, l), but failed to alter the extracellular L-glutamate contents (Fig. 1m, n).

The facts that ASC-type transporters function as amino acid exchangers in a sodium ion-coupled manner (Kanai and Hediger 2003) raise the possibility that the aforementioned increase in the D-serine contents might reflect the release of the intracellular D-serine exchanged with *S*-methyl-L-cysteine taken up into the brain cells by Asc-1. However, an intra-cortical perfusion of D-alanine at 1000 μM, which can be incorporated by Asc-1, failed to influence on the extracellular D-serine levels (Fig. 1a, b), arguing against this possibility.

Discussion

In the present study, for the first time we have demonstrated using an in vivo dialysis technique that an intramedial frontal cortex application of *S*-methyl-L-cysteine produces an elevation of the extracellular D-serine contents in the cortical portion. Larger increments are observed in the dialysate levels of L-serine, L-alanine, L-threonine, and glutamine than in the D-serine contents, while a slight but significant augmentation and no changes were seen in the glycine and L-glutamate, respectively. The differential effects of *S*-methyl-L-cysteine on the various extracellular amino acids deny the possibility that the alterations in the D-serine contents are due to a non-specific phenomenon.

The *S*-methyl-L-cysteine induced increase in the extracellular D-serine levels is likely to result from inhibition of D-serine uptake, at least, by Asc-1 and ASCT2 because these transporters, but not ASCT1, have been well-documented to accumulate D-serine as well as L-serine, L-alanine and L-threonin without eliciting L-glutamate uptake (Fukasawa et al. 2000; Utsunomiya-Tate et al. 1996; Shafqat et al. 1993). The rank-order of potency of the increasing effects of *S*-methyl-L-cysteine on glutamine and glycine resembles that of the affinity of the two amino acids for ASCT-2 (Utsunomiya-Tate et al. 1996), further supporting the involvement of ASCT-2 in the augmented levels of D-serine. In contrast, Thomsen et al. (2003) observed by in vivo dialysis that *S*-methyl-L-cysteine markedly upregulated the extracellular contents glycine in the hippocampus. This inconsistency could result from the possible regional differences in the effects of *S*-methyl-L-cysteine and/or in the expression of Asc-1 or ASCT2. Moreover, the possible involvement of exchange mechanism by these neutral amino acid transporters appears to be denied by the lack of effects of D-alanine, an exchangeable substance with D-serine, on the prefrontal extracellular D-serine contents (see Fig. 1a, b).

In conclusion, these findings are consistent with the view that ASC-type transporters, Asc-1 and ASCT2, could participate in the regulation of the extracellular D-serine contents and be appropriate targets for useful substances that control NMDA receptor functions by modulating the D-serine signaling, although further investigations are needed to clarify the exact mechanisms underlying the effects of *S*-methyl-L-cysteine on the D-serine dynamics in the brain tissues.

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Conflict of interest None.

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