Spatiotemporal relationships among *D*-serine, serine racemase, and *D*-amino acid oxidase during mouse postnatal development[1]

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KEY WORDS *D*-amino acid oxidase; serine; serine racemase; inbred C57BL mice

ABSTRACT

AIM: To elucidate the spatiotemporal relationships among *D*-serine, serine racemase, and *D*-amino acid oxidase (EC 1.4.3.3; DAO) in mouse cortex, striatum, cerebellum, heart, lung, liver, spleen, kidney, and skeletal muscle during mouse postnatal development. **METHODS:** The transcription levels of serine racemase and DAO were assayed by reverse transcription-polymerase chain reaction (RT-PCR). The protein levels of serine racemase were examined by Western blot. DAO activities were assayed by colorimetric method. *D*-serine was measured by HPLC. **RESULTS:** In cortex, striatum, and cerebellum, free *D*-serine increased drastically after birth and coincided well with the increase of serine racemase expression. However, among the 9 tissues examined, DAO activities were detected only in cerebellum and kidney. During the 3rd week, DAO activity in cerebellum and kidney increased dramatically, which concurred with the drastic decline of *D*-serine content in these tissues. On the other hand, while *D*-serine and serine racemase fall to trace level in cerebellum and kidney at the 3rd weekend, DAO activities in these tissues increased continuously. **CONCLUSION:** The free *D*-serine is mainly synthesized by serine racemase. However, novel mechanisms might be involved in *D*-serine deposition in mouse tissues with high level of *D*-serine and no detectable DAO activity such as cortex and striatum. DAO in cerebellum and kidney might have other physiological functions in addition to degrading *D*-amino acid.

INTRODUCTION

It has long been assumed that free *D*-amino acids are unnatural in mammalian. However, recent advances in analytical methodologies for separating chiral amino acids have shown that *D*-aspartate and *D*-serine occur in the mammalian central nervous system and periphery at unexceptionally high concentrations[1-3]. These high level free *D*-amino acids suggest that they may have important physiological function in mammalian. Evidences have shown that *D*-serine play an important role in the activation of *N*-methyl-*D*-aspartate (NMDA) receptor. In fact, *D*-serine is a selective and potent agonist for glycine site of NMDA receptor[4]. And the potency of *D*-serine in activating the NMDA receptor is at least similar to glycine[5,6], another endogenous agonist for glycine site of the NMDA receptor. However, the distributions of glycine and *D*-serine are different. *D*-serine is confined to the forebrain of adult mammalian with a high concentration[7,8], while glycine is the highest in the hindbrain and spinal cord[9]. In the striatum and medial prefrontal cortex, extracellular concentrations of *D*-serine are comparable to or even more than glycine[10]. Immunohistochemistry and biochemi-

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cal assays have revealed that *D*-serine is associated with protoplasmic astrocytes, a subtype of glial cells that closely apposed NMDA synapses[7,9]. Activation of non-NMDA glutamate receptor, the kainite subtype, invokes the release of *D*-serine from these astrocytes[7]. Selective destruction of the endogenous *D*-serine by applying DAO markedly decreases NMDA neurotransmission[11].

The biosynthetic pathway for *D*-serine has been established *in vitro*. *D*-serine is synthesized from *L*-serine by serine racemase and degraded by DAO[11-13]. Serine racemase, a pyridoxal 5'-phosphate-dependent enzyme, can selectively catalyze racemization of *L*-serine to *D*-serine and shows no activity to any other amino acid[12]. Mouse as well as human serine racemase has recently been cloned[13,14]. Mouse serine racemase contains 339 amino acids with a predicted molecular weight of 36.3 kDa. The overall identity of the enzyme in these two species is 88 %. DAO, a flavo-enzyme can selectively catalyze the oxidative deamination of neutral *D*-amino acid such as *D*-serine, *D*-proline and *D*-alanine at physiologic pH, but *D*-proline and *D*-alanine are not present in the mammalian tissue in significant levels[15].

Since NMDA receptor plays an important role in many functions, including learning, memory, development, and other forms of synaptic plasticity[16-18], *D*-serine, as an endogenous co-agonist of NMDA receptor, may take part in several physiological and pathological processes related to NMDA receptor function. Studies on *D*-serine and the enzymes involved in its metabolism during mammalian development and various pathological conditions would help to clarify the biological role of *D*-serine. Some researches have shown that serine racemase is most concentrated in the forebrain of adult rat and greatly enriched in protoplasmic astrocytes, revealing a close parallel between the localization of serine racemase and *D*-serine[13]. Whereas others have shown that DAO is particularly concentrated in astrocytes of the adult hindbrain and cerebellum, which is inversely correlated to the levels of *D*-serine[7]. Distributions of *D*-serine and DAO during postnatal development have been studied respectively in rat by several groups[2,19], however, the anatomical distribution of serine racemase during development has not yet been documented. In addition, there is no report about anatomical distributions of DAO in mouse during different postnatal developmental stages.

In the present studies, we investigated the developmental profiles of free *D*-serine and expressions of serine racemase and *D*-amino acid oxidase simultaneously in mouse, to elucidate further the spatiotemporal relationships among *D*-serine, serine racemase, and DAO.

MATERIALS AND METHODS

Material Keyhole Limpet hemocyanin (KLH), complete Freund's adjuvant, incomplete Freund's adjuvant, IgepalCA-630, phenylmethylsulfonyl fluoride (PMSF), aprotinin, pepstatin, leupeptin and aprotinin, catalase, *D*-alanine, FAD, *N*-*tert*-butyloxycarbonyl-*L*-cysteine (*L*-Boc-cys), *o*-phthaldialdehyde (OPA), and *L*-homocysteic acid were purchased from Sigma Chemicals Co (USA). DEAE Sephadex A-50 was purchased from Pharmacia Co (Sweden). Horseradish peroxidase (HRPO)-conjugated goat anti-rabbit antibody was purchased from Calbiochem Co (USA). BCA protein assay kit and ECL chemiluminescence system were purchased from Pierce Co (USA). Prestained proteins were purchased from Life Technologies (USA). TRI-REAGENT-LS extraction kit was purchased from Molecular Research Center Inc (USA). RNasin, dNTP, oligo $(dT)_{18}$ primer, and *Taq* DNA polymerase were obtained from Sangon Biotechnology Co (Canada). M-MuLV reverse transcriptase was from Fermentas Inc (Lithuania).

Animals C57BL/6N male mice were provided by the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Grade II, Certificate No 003), and were housed at a room temperature of 22 ºC, with food and water *ad libitum* and a 12-h light-dark cycle. Postnatal distributions of *D*-serine, serine racemase, and DAO were investigated from postnatal day (PD) 1 through PD 49.

Preparation of antibody against serine racemase A peptide corresponding to the mouse serine racemase amino acid sequence, 72-85 (*N*'-EEKPKAVVTHSSGN-C') was synthesized (Genemed Synthesis Inc, South San Francisco, USA) as immunogen. Peptide was conjugated to KLH essentially as described[20] using 0.1 % glutaraldehyde. Rabbit was immunized with KLH conjugates 1 mg in complete Freund's adjuvant for the first injection and 0.8 mg in incomplete Freund's adjuvant for subsequent boosts on d 21 and then every two weeks thereafter for 4 times in subcutaneous injection. After 6 times injection, the antiserum was finally collected. The immunoglobulin was purified by ammonium sulfate fractionation and ion-

exchange chromatography[21]. DEAE Sephadex A-50 was used for separating IgG from the majority of serum proteins. The specificity of the antibody was tested by immunoblotting.

Tissue preparation Mouse was decapitated, followed by removal of brain, heart, lung, liver, spleen, kidney, and skeletal muscle. Then the cortex, striatum, and cerebellum were immediately dissected. These tissue samples were frozen in liquid nitrogen and stored at -80 ºC. In DAO activity studies, tissues were kept frozen in liquid nitrogen until use. Six to eight samples for PD 1 group and five to six striatum, cerebellum, and skeletal muscle samples for PD 7 groups were pooled together to provide a sufficient amount of tissues for each analysis in the experiment. Individual tissues were tested in all other sample groups.

Measurement of serine racemase by Western blot Tissues were homogenized with RIPA (NaH_2PO_4 9.1 mmol/L, Na_2HPO_4 1.7 mmol/L, NaCl 150 mmol/L, pH 7.4, 1 % IgepalCA-630, 0.5 % sodium deoxycholate, 0.1 % SDS) supplemented with PMSF, aprotinin, pepstatin, leupeptin, and aprotinin, and incubated on ice for 40 min. The homogenate was centrifuged at 20 000×*g* for 30 min at 4 ºC. Protein concentration in the supernatant solution was determined by BCA protein assay kit. Protein sample (90 mg) in Tris-buffer loading solution (Tris-HCl 20 mmol/L, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, and 0.002 % bromophenol blue) was electrophoresed in 12 % polyacrylamide gels according to Laemmli[16]. The prestained proteins (80.9 kDa, 63.8 kDa, 49.5 kDa, 37.4 kDa, 26 kDa, 19. 6 kDa) (Life Technologies, Grand Island, NY, USA) were used as molecular weight (Mr) standards. Proteins were then electrically transferred onto polyvinylidene fluoride membranes in a semidry blotting apparatus. The transfer of protein to membranes was assessed by Ponceau S staining. Membranes were blocked with 5 % non-fat dried milk in TBS-T (Tris-HCl 20 mmol/L, pH 7.6, NaCl 137 mmol/L, 0.1% Tween-20) overnight at 4 ºC, then with rabbit anti-mouse serine racemase antibody (1:5000 in TBS-T containing 2 % BSA) for 1 h at room temperature (RT), and finally with horseradish peroxidase (HRPO)-conjugated goat anti-rabbit antibody (1:10 000 dilution) for 1 h at RT. Signal detection was carried out using the ECL chemiluminescence system. Extensive washing with TBS-T was carried out between each step. The specificity of the antibody of serine racemase were tested on the cortex samples from PD 49 using the primary antibody after incubation with excess corresponding peptide overnight at 4 ºC. The relative densities of the serine racemase protein bands were analyzed using Smart View Analysis program (Electrophoresis Image Analysis System FR-980, Furi Company, Shanghai, China).

Measurement of mRNA level of serine racemase and DAO by RT-PCR Total RNA was isolated from tissues of mouse during the different postnatal developmental stage with Trizol-Reagent (Life Technologies), according to manufacturer's instructions. Aliquots of total RNA (2 mg) were reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (M-MLV) (Promega, Madison, WI) and oligo (dT) primer (18-mer). Aliquots of the obtained cDNAs were then amplified by PCR performed in Tris 15 mmol/L (pH 8.3), $MgCl_2$ 1.5 mmol/L, KCl 50 mmol/L, dNTPs 200 μmol/L, DNA polymerase 2.5 U, and 10 pmol of each oligonucleotide. PCR conditions were as follows: warm up period of 5 min at 95 ºC, cycles of PCR [95 ºC for 45 s, 61 ºC for 50 s (for serine racemase and β-actin) or 60 ºC for 50 s (for DAO), 72 ºC for 1 min] and a final elongation period of 10 min at 72 ºC. The cycles were performed 29 times for serine racemase, 33 times for DAO and 22 times for β-actin, respectively. Sequences of primer are described in Tab 1.

Assay of DAO activity Tissues were thawed by the addition of pyrophosphate buffer 7 mmol/L (pH 8.3) and homogenized in a glass-Teflon homogenizer. The homogenates were centrifuged at 550×*g* for 5 min at 4 ºC. The supernatant solutions were used for the assay of DAO activity. The enzyme activity was measured by the colorimetric method of Watanabe *et al*[22] and described by Konno *et al*[23]. Protein concentration in the supernatant solution was determined by BCA reagent using bovine serum albumin as a standard. DAO activity was finally expressed as the amount of *D*-alanine oxidase per min per milligram of protein. Pig kidney DAO was used as a control.

Measurement of *D*-serine by HPLC Amino acid enantiomers were separated by HPLC using a carbon 18 reverse-phase column (250 mm) (Inertsil, GL Sciences Inc, Tokyo, Japan) with fluorimetric detection after derivatization with *L*-Boc-cys and OPA , as described[3]. In brief, the tissue sample was homogenized in 10 volume of 5 % trichloroacetic acid (TCA) after addition of *L*-homocysteic acid as internal standard, and the homogenate was centrifuged at 18 000×*g* at 4 ºC for 30 min. To remove TCA, the supernatant was

Tab 1. Primer sequences used for PCR.

cDNA	Primer sequences		Product size
Serine Racemase	Forward	5'-GTATACTGTGACCCAAGTGACG-3'	288 bp
	Reverse	5'-TAGACTGGTAGCAGTCATCTGC-3'	
DAO	Forward 1	5'-GCAGTTCTGGGATTCCGGAAG-3'	399 bp
	Reverse 1	5'-ACCTCCGAGTGTAACTGTCTT-3'	
	Forward 2	5'-AGCAGTCCTGCTGGAACCTGC-3'	447 bp
	Reverse 2	5'-ATCAGGGAACAGGTCCATCTC-3'	
β-Actin	Forward	5'-GGTGTGATGGTGGGAATGGGTC-3'	
	Reverse	5'-CTTCTCCAGGGAGGAAGAGGATG-3'	594 bp

washed three times with water-saturated diethyl ether. The resultant sample was derivatized with Boc-*L*-Cys and OPA at RT for 2 min. The Boc-*L*-Cys/OPA derivatives were immediately applied to the HPLC system (Bioanalytical Systems, West Lafayette, IN, USA). Mobile phase A was MeCN-0.1 mol/L sodium acetate buffer (pH 6.0) (9:91, v/v), and mobile phase B was MeCN-0.1 mol/L sodium acetate buffer (pH 6.0) (16: 84, v/v). A linear gradient was applied for 35 min from mobile phase A to B; then the elution was carried out with mobile phase B alone. The flow-rate was 1.4 mL/ min. Fluorescence detection of the derivative of each amino acid was carried out at 443 nm with excitation at 344 nm[24].

Data analysis Serine racemase protein measurements were normalized to total protein and expressed as a percentage of the respective PD 7 density in cortex, striatum or kidney and PD 1 density in cerebellum located on the same membrane. All data were represented as Mean±SD. Data were subjected to statistical analysis with the Student's *t*-test. Data analysis was performed using Origin 6.1 software (Originlab Corporation, Northampton, MA, USA).

RESULTS

Antibody specific to serine racemase By Western blot analysis, purified antibodies recognized a band of about 37 kDa selectively in cytosolic protein preparations from PD 49 mouse cortex. Immunoreactivity of antisera could be blocked completely by preadsorption with immunogenic peptide (Fig 1A). The results indicated that the antibody specifically recognized the serine racemase.

Serine racemase expression in developing mouse In order to investigate the ontogenetic distribution of serine racemase *in vivo*, we examine the mRNA and protein level of serine racemase in nine tissues of mouse from different developmental stages, with RT-PCR and Western blot analysis. As shown by RT-PCR analysis (Fig 2A), serine racemase transcript was widespread in mouse tissues including the cortex, striatum, cerebellum, heart, lung, liver, spleen, kidney, and skeletal muscle, with low expression level at born. The expression level of serine racemase gradually increased to adult level in the most tissues during the first three weeks, except for the lung and spleen in which the transcript level increased in the first week and then declined to a trace level during the late developmental stages. To further analyze whether these transcripts are indeed translated, 90 μg cytosolic protein preparations of different mouse tissues from different developmental stages were subjected to Western blot analysis using serine racemase specific antibody (Fig 1B-D). In the cortex and striatum, serine racemase was low at PD 7, gradually increased to high levels by PD 21, and then maintained this level until adult (Fig 1C). But in cerebellum, immunoreaction intensity increased during the first week after birth, remained relatively constant in the late three weeks, and thereafter decreased to undetectable level in PD 49 (Fig 1B). However, the protein levels in heart, lung, liver, spleen, kidney, and skeletal muscle were so low that the immune signal was not detected in these tissues when the exposure time of autoradiography film was about 1 min, as it was when the immune signals were detected in the cortex, striatum, and cerebellum. When the exposure time was prolonged to 3 min, a weak immune signal was observed in mouse

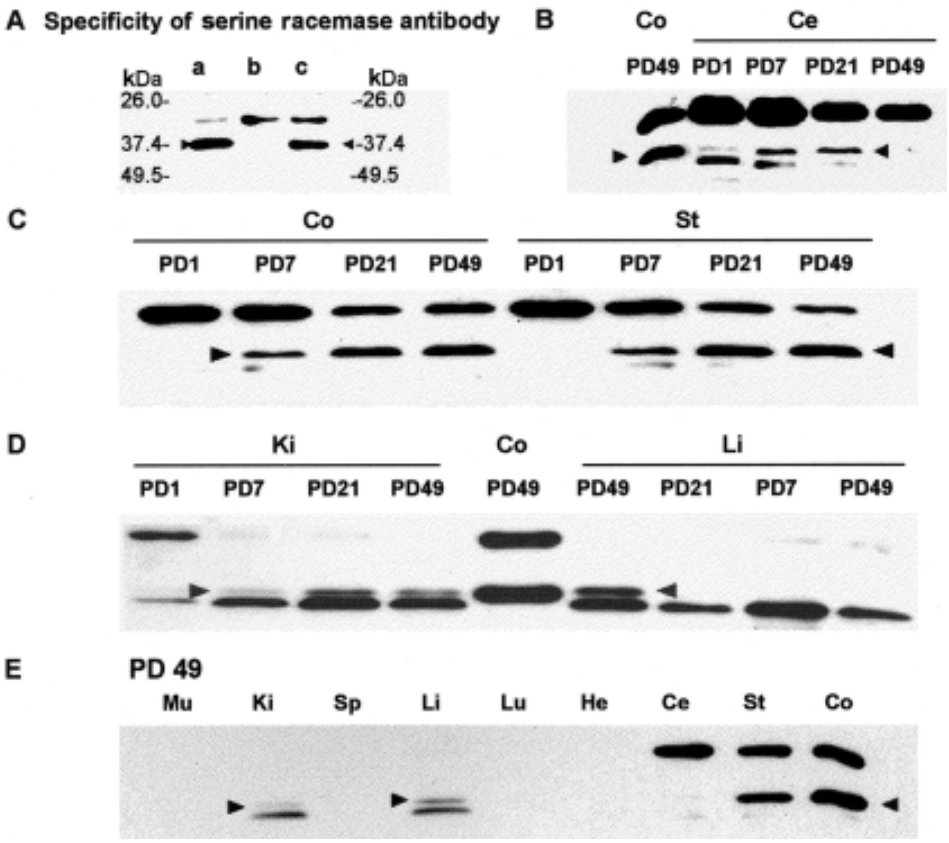

Fig 1. Western blot analysis of serine racemase protein expression. (A) Characterization of purified serine racemase antibodies by Western blot analysis. Antibodies against mouse serine racemase specifically recognized serine racemase as a band of about 37 kDa in cytosolic protein preparations of mouse cerebral cortex. Signal could be blocked by overnight preincubation with corresponding excess antigenic peptide (lane b). Lane a and c represent that the antibodies were overnight preincubated with PBS. (B) The protein levels of serine racemase in mouse cerebellum (Ce) from PD 1 to 49. Cytosolic protein preparation (90 μg/lane for cerebellum or cortex PD 49 as control). (C) The protein levels of serine racemase in mouse cerebral cortex (Co) and striatum (St) from PD 1 to 49. Cytosolic protein preparation (90 μg/lane). (D) The protein levels of serine racemase in mouse kidney (Ki) and liver (Li) from PD1 to 49. Cytosolic protein preparation (90 μg/lane for kidney and liver, 30 μg for cortex PD 49 as control). (E) The protein levels of serine racemase in mouse tissues at PD 49. Cytosolic protein preparations (30 μg/lane for cortex and striatum, 90 μg/lane for other tissues). The arrow indicates serine racemase.

kidney and liver (Fig 1D,E). In the kidney, the immune signal appeared in PD 7, reached maximal intensity in PD21 and then slightly decreased in PD49, while in liver, a clear immune signal was obtained only in PD 49 (Fig 1D). No immune signal was detected in mouse heart, lung, spleen, and skeletal muscle at any developmental stages even when the exposure time of autoradiography film was prolonged to 3 min (data not shown).

DAO expression and activity in developing mouse To investigate the expression pattern of DAO mRNA in postnatal developmental stages of mouse, two independent pairs of DAO-specific oligonucleotides were used to amplify the DAO transcription in mouse tissues. In the cortex, striatum, cerebellum, heart, lung, liver, spleen, kidney, and skeletal muscle obtained from mouse at PD1, low expressions of DAO were only found in the kidney and skeletal muscle (Fig 2B). The expressions of DAO in the kidney and skeletal muscle increased dramatically within the first week after birth, and then reached the adult level at PD 21. In cerebellum, the expression of DAO was failure to be found at PD 7, but at PD 21 the DAO transcription was quite high. In contrast, no DAO signal could be detected in the cortex, striatum, heart, lung, liver and spleen of the mouse during the postnatal development (Fig 2B).

In mouse cerebellum, DAO activity was undetectable until 12 d after birth and then increased drastically during the 3rd week after birth, from 0.1 μmol·min^{-1}·g^{-1}

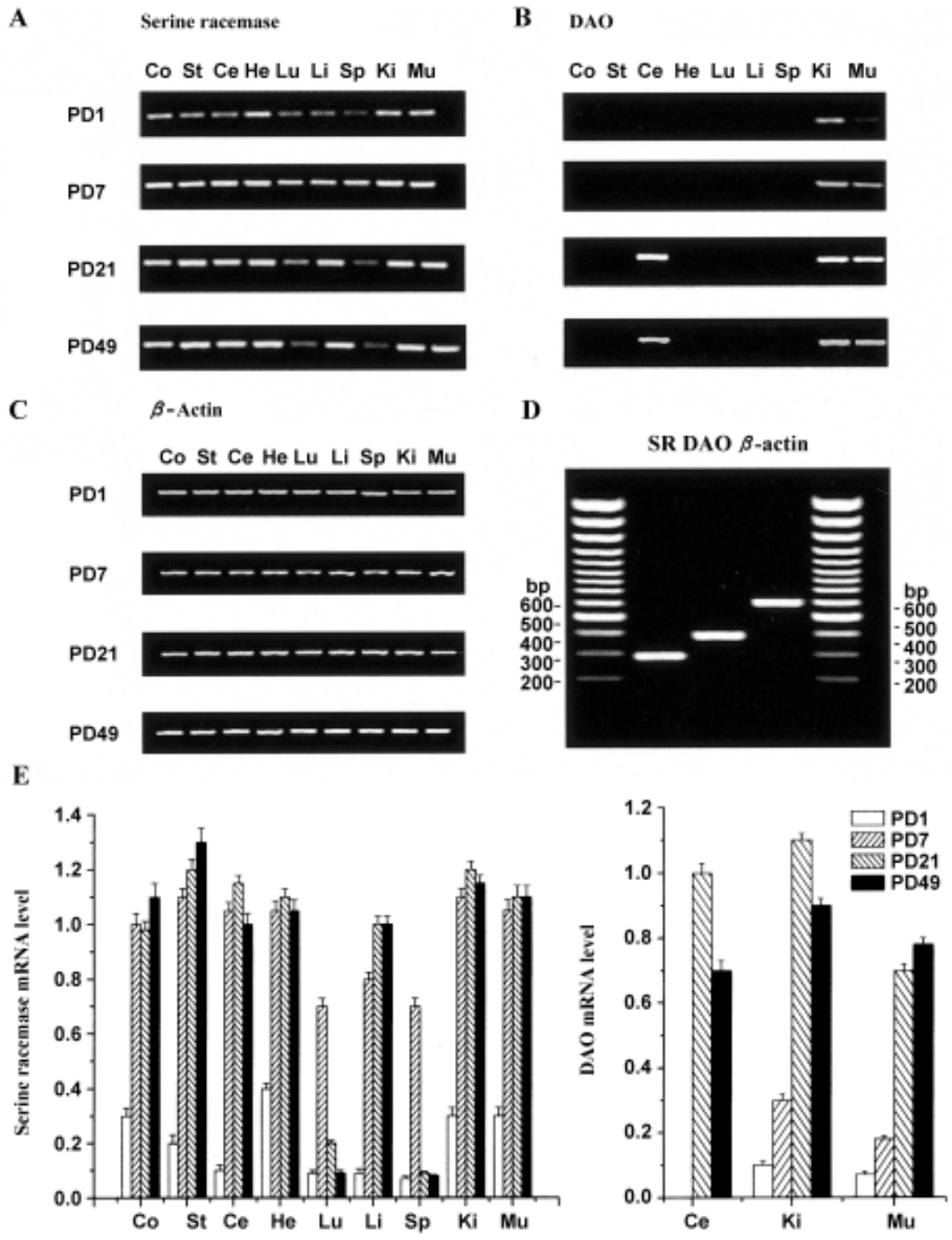

Fig 2. Developmental expression of serine racemase and *D*-amino acid oxidase mRNA. (A) Amplification of serine racemase (29 cycles); (B) Amplification of *D*-amino acid oxidase (33 cycles); (C) Amplification of β-actin (22 cycles) used as internal control for RNA/complementary DNA input. (D) Size of the RT-PCR products of serine racemase (SR, 289 bp), *D*-amino acid oxidase (DAO, 399 bp) and β-actin (594 bp). (E) Densitometric scanning for serine racemase and *D*-amino acid oxidase DNA bands of various tissues at indicated postnatal stages. *n*=6. Mean±SD. Co, Cortex; St, Striatum; Ce, Cerebellum; He, Heart; Lu, Lung; Li, Liver; Sp, Spleen; Ki, Kidney; Mu, Skeletal muscle.

protein in PD12 to 1.5 μmol·min^{-1}·g^{-1} protein in PD 18, and reached the adult level of 2.58 μmol·min^{-1}·g^{-1} protein at PD 49 (Tab 2). In contrast, substantial DAO activity was found in mouse kidney at the day after birth and reached the adult level of 8.78 μmol·min^{-1}·g^{-1} at PD 49. The maximal activity attained in mouse kidney was three times higher than that of cerebellum (Tab 2). The skeletal muscle did not have any detectable DAO activity at all developmental stages, although significant amount of DAO transcription was

Tab 2. *D*-serine concentration (nmol·g^{-1} wet weight) and DAO activity (μmol·min^{-1}·g^{-1} protein) in mouse organ from various postnatal development stages. *n*=6 to 8 mice in each group. Mean±SD.

Postnatal		Cortext	Striatum	Cerebellum	Lung	Heart	Liver	Spleen	Kidney	Skeletal muscle
PD 1	*D*-serine	33.3±1.7	7±4	36±6	17.5±1.1	10.3±0.6	17.2±2.4	11.4±1.4	27.8±2.4	19.3±0.7
	DAO								1.50±0.14	
PD7	*D*-serine	130±8	103±3	158±6	17.4±1.2	16.9±1.5	16.8±1.1	15.4±1.4	34.0±2.3	20.5±0.7
	DAO								2.57±0.04	
PD12	*D*-serine			107.3±1.1					47.9±1.3	
	DAO			0.10±0.02					3.20±0.05	
PD18	*D*-serine			7.5±0.7					34±4	
	DAO			1.69±0.03					4.00±0.21	
PD21	*D*-serine	311±26	327±26	9.9±1.1	6.3±0.5	5.3±0.8	13.2±1.1	8.9±0.1	14.8±0.7	7.2±0.3
	DAO			1.95±0.05					7.00±0.13	
PD49	*D*-serine	332±17	318±14	7.5±0.2	6.1±0.2	2.9±0.2	13.2±0.5	9.4±1.9	10.4±0.9	3.5±0.6
	DAO			2.58±0.07					8.78±0.08	

observed in this tissue with RT-PCR analysis. When the homogenates of kidney at PD 49 were mixed with skeletal muscle homogenates of mouse from the same stage, no decrease of DAO activity was found in the homogenates mixture (data not shown). This result suggests that there is not dissociable inhibitor in mouse skeletal muscle. The DAO activities in cortex, striatum, heart, lung, liver, and spleen of mouse were also measured. However, corresponding well with the results of RT-PCR analysis, no measurable DAO activity was obtained in all these tissues.

Distribution of *D*-serine in developing mouse Free *D*-serine level in nine tissues of mouse of various ages, ranging from 1 d to 49 d, were presented in Tab 2. On the day of birth, a substantial amount of *D*-serine was observed in mouse cortex, striatum, and cerebellum, and was quite comparable in all these brain areas. The *D*-serine concentrations in the cortex and striatum increased dramatically by PD 21 and then remained rather constant, whereas the cerebellar *D*-serine concentration increased by PD 7 and declined drastically to only a trace level from PD 12 to PD 17. In contrast to the CNS, substantial concentrations of *D*-serine were found in peripheral organs within the first week after birth, and then declined rapidly to trace levels by PD 21.

DISCUSSION

Spatiotemporal relationships among serine racemase, DAO, and *D*-serine in mouse In present study, we have investigated the development profiles of serine racemase and DAO expression, at both the mRNA and protein levels, in brain and periphery of mouse. With RT-PCR, serine racemase were dectected to be low expressed in most tissue of infant mouse but high expressed in adult cortex, striatum, cerebellum, heart, liver, kidney, and skeletal muscle. While with Western blot analysis, we found that the protein levels of serine racemase were quite high in cortex and striatum, but low in liver and kidney and even not detectable in other tissues (Fig 1). The apparent discrepancy between the relative quantities of serine racemase protein and mRNA in mouse cerebellum, heart, skeletal muscle and kidney could have several explanations. It could be that the translation of its mRNA is inefficient, or that the turnover rate of this protein is fast and therefore the protein levels are low in these regions. With RT-PCR analysis and colorimetric method, we examined DAO mRNA level and its activity in mouse during various developmental stages. In mouse kidney and cerebellum, high levels of DAO mRNA were detected during the late developmental stages (Fig 2B), corresponding well with high enzyme activity in both tissues (Tab 2). In contrast, no measurable DAO activity was detected in skeletal muscle of mouse across its whole life span although significant amount of DAO transcription was observed in this tissue (Fig 2 B).

When the spatiotemporal relationships among *D*-serine, serine racemase and DAO were evaluated during mouse postnatal development, a parallel increase in the amount of *D*-serine and serine racemase protein lev-

els were found in mouse cortex and striatum (Fig 3A). A transient emergence of high level of *D*-serine concomitant with an increase of serine racemase expression was also observed in cerebellum during early developmental stage (Fig 3B). From PD 12-18, a drastic decline in cerebellar *D*-serine content was observed. The decline of *D*-serine was coincided well with the dramatic increase in the DAO activity in cerebellum during this period (Fig 3B). Parallel increases in the amount of *D*-serine and serine racemase and inverse changes in *D*-serine and DAO were also found in mouse kidney (Fig 3B). In mouse kidney, *D*-serine content began to increase in PD 7 and kept at high level until PD18 when the protein level of serine racemase was also relatively high. However, DAO activity did not attain the highest level at this developmental stage. The relationship among serine racemase, DAO and *D*-serine in adult mouse was also revealed by the patterns of their anatomical distribution. *D*-serine was confined preferentially to the forebrain, as was serine racemase [13], whereas DAO was selectively accumulated in hindbrain, cerebellum and kidney[25]. Overall, our results of the spatiotemporal relationships among *D*-serine, serine racemase and DAO strongly support the notion that the free *D*-serine *in vivo* is mainly synthesized by serine racemase and that the *D*-serine in cerebellum and kidney are preferentially degraded by DAO.

Disposition of *D*-serine in mouse forebrain and liver In our studies, we found that in cortex and striatum of adult mouse, the protein levels of serine racemase were quite high and the concentration of *D*-serine was rather constant (Fig 1C, Tab 2 and Fig 3A). However, no measurable DAO activity was detected in these regions. Similar results were also obtained in adult

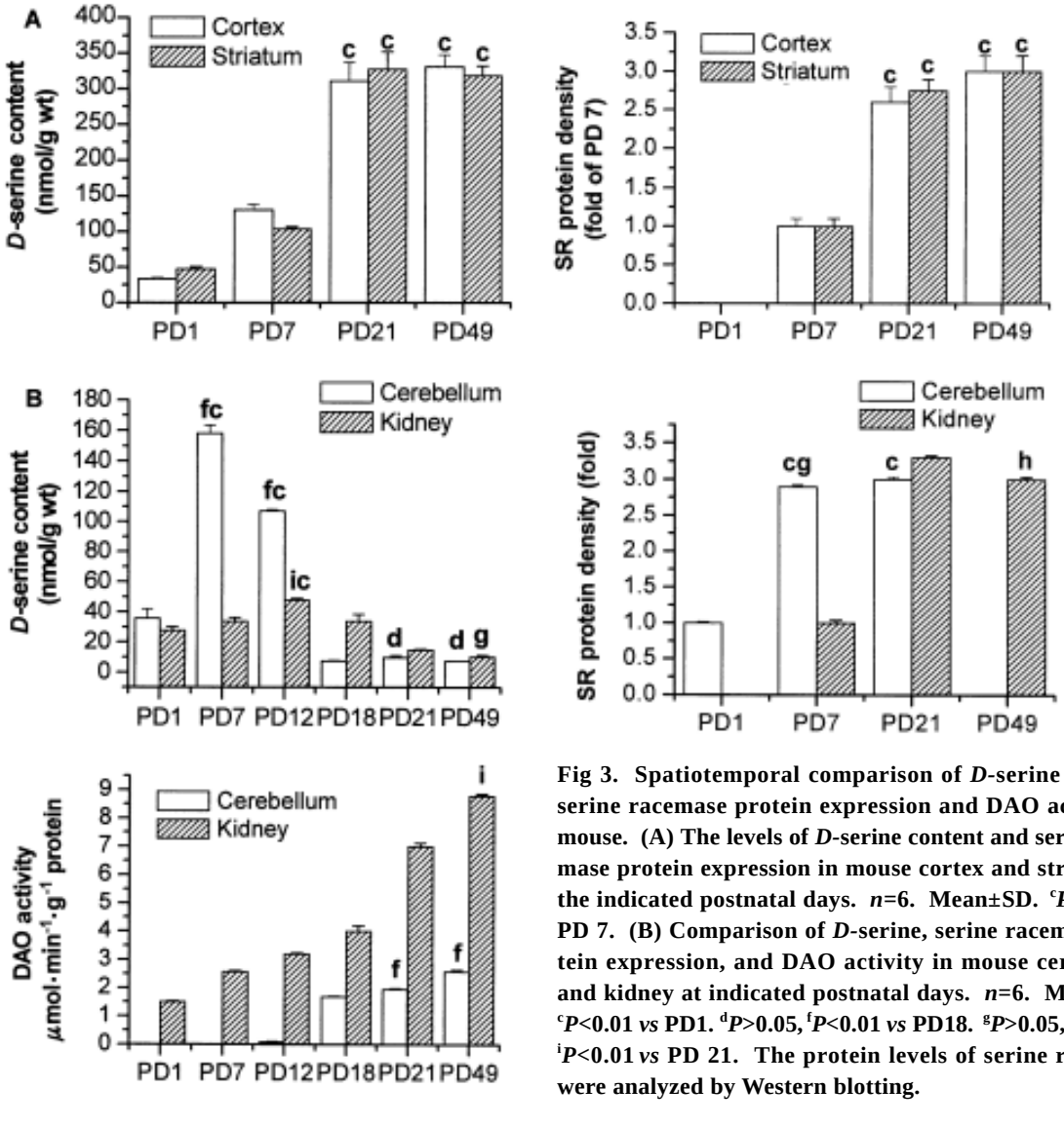

Fig 3. Spatiotemporal comparison of *D*-serine content, serine racemase protein expression and DAO activity in mouse. (A) The levels of *D*-serine content and serine racemase protein expression in mouse cortex and striatum at the indicated postnatal days. *n*=6. Mean±SD. $^{c}P<0.01$ *vs* PD 7. (B) Comparison of *D*-serine, serine racemase protein expression, and DAO activity in mouse cerebellum and kidney at indicated postnatal days. *n*=6. Mean±SD. $^{c}P<0.01$ *vs* PD1. $^{d}P>0.05$, $^{f}P<0.01$ *vs* PD18. $^{g}P>0.05$, $^{h}P<0.05$, $^{i}P<0.01$ *vs* PD 21. The protein levels of serine racemase were analyzed by Western blotting.

mouse liver. The protein level of serine racemase in mouse liver was much higher than that in other peripheral tissues such as lung, heart, kidney, spleen and skeletal muscle (Fig 2D), whereas levels of free *D*-serine in these peripheral tissues were quite comparable (Tab 2). Our results demonstrated that no measurable DAO activity was present in mouse liver. This result is in agreement with previous reports by several other groups[23]. Since no significant change of *D*-serine levels in *ddY*/DAO$^-$ mouse forebrain[24], it can exclude the possibility that free *D*-serine in forebrain is delivered to and then degraded by DAO in other tissues. It seems that the constant concentration of *D*-serine in mouse cortex and striatum or liver might be regulated by some other mechanisms. These may include some endogenous inhibitors regulating *D*-serine synthesis or some other enzymes degrading *D*-serine.

More physiological roles of DAO besides the function of degrading *D*-serine in mouse kidney and cerebellum It is worth to notice that, in mouse kidney, while *D*-serine declined to the adult level at PD 21, the DAO activity kept increasing until PD 49 (Fig 3B). Similar result was also obtained in mouse cerebellum in which DAO activity kept raising till PD 49, while *D*-serine declined to adult level at PD17 (Fig 3B). It is unlikely that the increase of DAO in cerebellum and kidney after PD 21 is due to increased turnover of *D*-serine, since the protein levels of serine racemase, the enzyme for *D*-serine production, in cerebellum and kidney were quite low at PD 21. Thus it seems that the activity of DAO in mouse kidney and cerebellum is more than enough to degrade *D*-serine. Although *D*-serine, *D*-aspartate, *D*-alanine, *D*-proline, and *D*-leucine have also been detected in mammalian tissues[26], only *D*-serine is observed with a large amount in adult forebrain[27], the others are present in a trace level in higher organisms. Even in the DAO-knock out mouse, the concentrations of *D*-proline and *D*-leucine are still rather low[26]. However, obvious increases of *D*-serine and *D*-alanine contents were found in these mice[24]. Unlike *D*-serine which markedly increased in *ddY*/DAO$^-$ mouse cerebellum with no significant change in forebrain, the *D*-alanine contents in all brain regions and serum of *ddY*/DAO$^-$ mouse concurrently increased at same extent. Moreover, in control *ddY*/DAO$^+$ mouse, the contents of *D*-alanine among various brain areas were quite comparable and very close to the level of *D*-alanine in serum. The results suggest that *D*-alanine in tissues is mainly derived from exogenous *D*-alanine. *D*-Alanine in peptideglycan of bacterial cell walls might be the main origin[28]. This hypothesis was supported by the findings that no *D*-alanine was observed in germ-free rat serum lacking intestinal bacteria[29]. Based on the findings that exogenous alanine is the main origin of *D*-alanine in mammalian tissues and the trace amount of *D*-alanine is rather constant in the body during the whole postnatal developmental stages, it seems that the magnitude of DAO activity has already been enough to dispose exogenous *D*-alanine in mammalian during its early postnatal developmental stages. Thus, the degrading role of DAO for *D*-serine, *D*-alanine, *D*-proline, and *D*-leucine could not completely explain the physiological function of such high level of DAO activity in mouse kidney and cerebellum during its late postnatal developmental stage. Our results favor the idea that DAO might have other physiological functions in addition to degrading *D*-amino acids. To confirm this speculation, further studies will be needed.

Functional implications of *D*-serine in mouse ontogeny Recently, Schell *et al*[9] mapped the localization of *D*-serine and NMDAR2A/B in rat brain, showing a particularly close relationship between *D*-serine and NMDAR2A/B. During PD14, parallel ontogeny of *D*-serine in Bergmann glia and protoplasmic astrocytes surrounding glomeruli and NMDAR2A/2B in Purkinjie cell and migrating granule cell were observed in cerebellum. In our studies, we found that transient emergence of high level cerebellar *D*-serine occurred during PD 14 (Tab 2). Many dynamic development events occurred in cerebellum within this period, including proliferation of cerebellar cells, granule cell migration and proper synapse formation and elimination[30,31]. NMDA receptors are involved in these events since blocking NMDA receptor in critical period would effect on the normal ontogeny of cerebellum[31-33]. Thus, as a selective and potent agonist for glycine site of NMDA receptor, *D*-serine might play an important role in NMDA-mediated development of the cerebellum.

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