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ORIGINAL ARTICLE

Alteration of intrinsic amounts of D-serine in the mice lacking serine racemase and D-amino acid oxidase

Yurika Miyoshi · Ryuichi Konno · Jumpei Sasabe · Kyoko Ueno · Yosuke Tojo · Masashi Mita · Sadakazu Aiso · Kenji Hamase

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Abstract For elucidation of the regulation mechanisms of intrinsic amounts of D-serine (D-Ser) which modulates the neuro-transmission of *N*-methyl-D-aspartate receptors in the brain, mutant animals lacking serine racemase (SRR) and D-amino acid oxidase (DAO) were established, and the amounts of D-Ser in the tissues and physiological fluids were determined. D-Ser amounts in the frontal brain areas were drastically decreased followed by reduced SRR activity. On the other hand, a moderate but significant decrease in D-Ser amounts was observed in the cerebellum and spinal cord of SRR knock-out ($SRR^{-/-}$) mice compared with those of control mice, although the amounts of D-Ser in these tissues were low. The amounts of D-Ser in the brain and serum were not altered with aging. To clarify the uptake of exogenous D-Ser into the brain tissues, we have determined the D-Ser of $SRR^{-/-}$ mice after oral administration of D-Ser for the first time, and a drastic increase in D-Ser amounts in all the tested tissues was observed. Because both DAO and SRR are present in some brain areas, we have established the double mutant mice lacking SRR and DAO for the first time, and the contribution of both enzymes to the intrinsic D-Ser amounts was investigated. In the frontal brain, most of the intrinsic D-Ser was biosynthesized by SRR. On the other hand, half of the D-Ser present in the hindbrain was derived from the biosynthesis by SRR. These results indicate that the regulation of intrinsic D-Ser amounts is different depending on the tissues and provide useful information for the development of treatments for neuronal diseases.

Keywords D-Serine · Serine racemase · D-Amino acid oxidase · 2D-HPLC

Y. Miyoshi · K. Ueno · Y. Tojo · K. Hamase (✉)
Graduate School of Pharmaceutical Sciences,
Kyushu University, 3-1-1 Maidashi, Higashi-ku,
Fukuoka 812-8582, Japan
e-mail: hamase@phar.kyushu-u.ac.jp

R. Konno
Department of Pharmaceutical Sciences, International University of Health and Welfare, 2600-1 Kitakanemaru, Ohtawara, Tochigi 324-8501, Japan

J. Sasabe · S. Aiso
Department of Anatomy, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Y. Tojo · M. Mita
Innovative Science Research and Development Center, Shiseido Co., Ltd., 2-12-1 Fukuura, Kanazawa-ku, Yokohama 236-8643, Japan

Introduction

Almost all free amino acids in the biosphere are present in the L-forms, and the enantiomers, D-amino acids, have been considered to be absent especially in higher animals (Corrigan 1969). However, recent progress in analytical technologies certified the presence of D-amino acids in mammals including humans (Kirschner and Green 2009; Miyoshi et al. 2012), and D-amino acids are now recognized as the candidates for novel physiologically active substances and biomarkers (Barañano et al. 2001; Konno et al. 2007; Katane and Homma 2011). Especially, a large amount of free D-serine (D-Ser) was found in the frontal brain (Hashimoto et al. 1992; Schell et al. 1995). This D-amino acid is believed to bind to the glycine site of *N*-methyl-D-aspartate (NMDA) receptors and is reported to be needed for the potent activation of NMDA receptors (Kleckner and Dingledine 1988; Mothet et al. 2000). In the cerebellum, D-Ser is also reported to be the regulator of

synaptic plasticity via the $\delta 2$ glutamate receptors (Kakegawa et al. 2011). Lines of evidences revealed that D-Ser is involved in neuropsychiatric and neurodegenerative diseases (Nishikawa 2011) such as schizophrenia (Tsai et al. 1998; Hashimoto et al. 2003; Bendikov et al. 2007), amyotrophic lateral sclerosis (ALS) (Sasabe et al. 2007, 2012) and cognitive impairment by aging (Mothet et al. 2006). D-Ser is thus increasingly considered as a novel candidate for a diagnostic marker or a medicine for neuronal diseases, and the regulation mechanisms of the intrinsic D-Ser content in various tissues are expected to be elucidated.

As the enzymes relevant to D-Ser regulation in mammals, D-amino acid oxidase (DAO, EC 1.4.3.3) (Ohide et al. 2011) and serine racemase (SRR, EC 5.1.1.18) (Wolosker et al. 1999a, b) have been reported. DAO is an enzyme which catalyzes the oxidative deamination of neutral and basic D-amino acids including D-Ser (Neims et al. 1966; D'Aniello et al. 1993) and is predominantly localized in the kidney, liver and cerebellum (Ohide et al. 2011). On the other hand, SRR is an enzyme synthesizing D-Ser from L-Ser and is highly expressed in the cerebral cortex and hippocampus (Wolosker et al. 1999b; Miya et al. 2008). Both enzymes are suggested to be related to the neuropsychiatric and neurodegenerative diseases relevant to NMDA receptor abnormality via controlling D-Ser amounts (Bendikov et al. 2007; Mitchell et al. 2010). Therefore, for developing a novel treatment for these diseases by regulating the enzymatic activities of DAO and SRR, elucidation of the alteration of the D-Ser amounts caused by changes in the activities of these enzymes is strongly required. Concerning DAO, the model mice and rats lacking DAO have been established (Konno and Yasumura 1983; Konno et al. 2009), and we have already reported D-Ser amounts in various tissues and physiological fluids of these rodents (Miyoshi et al. 2009, 2011). Concerning SRR, a few knock-out mouse strains ($SRR^{-/-}$) have been established (Miya et al. 2008; Labrie et al. 2009; Basu et al. 2009), and a drastic decrease in D-Ser amounts in the cerebral cortex and hippocampus of these mice was reported (Inoue et al. 2008; Labrie et al. 2009; Basu et al. 2009). However, the detailed distribution of D-Ser in the mice with various SRR activities remains to be clarified.

The present study focuses on SRR, and the aim is to obtain information on the regulation mechanism of the D-Ser amount using $SRR^{-/-}$ mice. In addition to the elucidation of the D-Ser distribution in the mice with various age and SRR activities, the following two points have been investigated: (1) accumulation of exogenous D-Ser in various tissues including the frontal brain regions and (2) contribution of SRR to the tissue content of D-Ser including the cerebellum and spinal cord. Concerning the frontal brain areas, D-Ser treatment has been expected as a potential therapy against schizophrenia, and clinical trials using D-Ser have been performed (Tsai et al. 1998; Heresco-Levy et al. 2005). Therefore, the accumulation of D-Ser in a lesion site such as the cerebral cortex after oral administration of D-Ser is intriguing. However, in previous publications reporting the alteration of the D-Ser amount after oral administration of D-Ser to the mice having normal SRR activity, it was not clear whether the administered D-Ser was transferred to the frontal brain area, because high amounts of endogenous D-Ser are present in these tissues (Morikawa et al. 2007). $SRR^{-/-}$ mice seem to be appropriate for the investigation of D-Ser transport to the frontal brain areas after oral administration of D-Ser, because the intrinsic amounts of D-Ser in these mice are low (Inoue et al. 2008; Labrie et al. 2009; Basu et al. 2009). Therefore, in the present study, we have investigated the alteration of the D-Ser amount in the brain tissues and serum after administration of D-Ser using $SRR^{-/-}$ mice.

Concerning the cerebellum and spinal cord, not only SRR but also DAO is expressed (Horiike et al. 1994; Schell et al. 1995; Wolosker et al. 1999b; Miya et al. 2008) and the D-Ser amounts in these tissues remain extremely low (Schell et al. 1995; Nishikawa 2011). Therefore, the amounts of D-Ser produced by SRR in the cerebellum and spinal cord could not be elucidated by simply comparing the D-Ser amounts in $SRR^{+/+}$ and $SRR^{-/-}$ mice. To clarify the contribution of SRR to the amounts of D-Ser in the cerebellum and spinal cord, the D-Ser amount in the mice lacking both SRR and DAO needed to be determined. Therefore, in this study, we have established mice lacking both SRR and DAO ($SRR^{-}DAO^{-}$ mice) and compared the amount of D-Ser in these mice with those of SRR^{-} mice and DAO^{-} mice.

Materials and methods

Materials

Boric acid, citric acid monohydrate for amino acid analysis, D-Ser, HPLC grade methanol (MeOH), sodium hydroxide and trifluoroacetic acid (TFA) were purchased from Wako (Osaka, Japan). HPLC grade acetonitrile (CH_3CN) and L-Ser were from Nacalai Tesque (Kyoto, Japan). The fluorescence derivatization reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) (Imai and Watanabe 1981), and pentobarbital sodium were obtained from Tokyo Kasei (Tokyo, Japan). Deionized water was used after purification using a Milli-Q Elix3 and Gradient A10 system (Millipore, Billerica, MA, USA). All other reagents used were of the highest reagent grade.

Establishment of serine racemase knock-out ($SRR^{-/-}$) strain

Mouse genomic library (129SV, Stratagene) was screened for the clones carrying the serine racemase gene. Three positive clones were isolated. DNA was extracted from the phages. The 6-kb upstream of the serine racemase gene was excised with *Xba*I and inserted into pUC18. The 6-kb downstream was digested with *Sac*I and inserted into pBluescript. After the amplification of the plasmids, the inserts were excised and inserted into a targeting vector (pMH003-5) carrying the neomycin-resistant gene and diphtheria toxin gene. The targeting vector was kindly provided by Drs. Akimitsu and Sekimizu (Tokyo University). The orientation of the inserts was verified by restriction enzyme digestion.

The targeting vector was linearized and electroporated into mouse embryonic stem (ES) cells. These cells were cultured on feeder cells in the medium containing G418. Negative selection was carried out using the diphtheria toxin gene. Selected clones were checked for the homologous recombination by Southern hybridization (Fig. 1). Southern blotting was carried out as described in Konno et al. (1995). The 5′-probe used was a fragment amplified by PCR using mouse genomic DNA as a template with the primers (5′-AGG TGG TGA CTA CTA TTT TCC-3′ and 5′-GCG GCG CCG TGG TTG TTC TCC-3′). The 3′-probe was a fragment amplified with the primers (5′-CCA CCT CAA TAC CCC AAG CAT-3′ and 5′-CTG TGA AGG CGA AAG AAG AAT-3′). The clones were also checked for the karyotype. The ES clones heterogeneous for the serine racemase gene were injected into blastocysts of C57BL/6 mice to produce chimeric mice. The male chimeras were mated with female C57BL/6 mice. F_1 progeny heterozygous for the serine racemase gene were interbred to generate F_2 mice. To determine the genotypes, genomic DNAs purified from tails were digested with *Eco*RV and *Hin*dIII and analyzed by Southern hybridization. Male F_2 mice homozygous for the disrupted serine racemase gene were mated with female C57BL/6 mice. The resultant male mice heterozygous for the serine racemase gene were mated with C57BL/6 female mice. The backcross was continued to establish the serine racemase knock-out ($SRR^{-/-}$) strain with the C57BL/6 genetic background.

Following the standard procedures, serine racemase knock-out ($SRR^{-/-}$) mice were generated by disruption of the third exon containing the initiation codon (Fig. 1). These mice were confirmed not to have SRR mRNA by RT-PCR or SRR proteins with western blotting. RT-PCR was carried out as described in Konno et al. (2009). Mouse cerebrum was used for the extraction of total RNA. A forward primer (5′-AGA ACC ATG TGT GCT CAG T-3′) and a reverse primer (5′-TTT CTT CAT TAG GAT TTT G-3′) were used for the amplification of a 1,107-bp fragment of SRR cDNA. Western blotting was conducted as described in Konno et al. (2009). Tissue homogenate was prepared from mouse cerebrum. Monoclonal antibody against SRR (BD Biosciences) was used as the first antibody.

Genotyping procedure for serine racemase

DNA was extracted from the mouse tail tips (approximately 5-mm length) using a GenElute™ Mammalian Genomic DNA Miniprep Kit G1N70 (Sigma-Aldrich, St. Louis, MO, USA). Extracted DNA solution (1 μL) was added to 3 μL of the primer solution, 25 μL of Go taq® Green Master Mix (Promega, Madison, WI, USA) and 21 μL of DNA-free water. The sequences of the primer were 5′-CTTAGAATGTCATGGTAGGAGCTG-3′ and 5′-CCCAAGTGCTGGGATTAAAG-3′ for the wild-type gene, and 5′-CTTAGAATGTCATGGTAGGAGCTG-3′ and 5′-TAGAGCGAGGGAAGCGTCTA-3′ for the knock-out type gene. The following cycling program was used (PC708-R, ASTEC, Fukuoka, Japan): initial denaturation for 4 min at 94 °C, amplification for 40 cycles (denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C), and final extension for 10 min at 72 °C. The DNA fragments were purified using a QIAquick® PCR purification kit (Qiagen, Hilden, Germany). The purified DNA fragment solution (1 μL) was further mixed with 3 μL of the primer solution (5′-GGTTT GAGCCTTAGCATCCA-3′ and 5′-CTGTGTCTTGGGGT GGGTA-3′ for the wild-type gene, and 5′-GGTTTGAGC CTTAGCATCCA-3′ and 5′-CCTCGTTTTCTGTGAGG TTGT-3′ for the disrupted gene), 25 μL of Go taq® Green Master Mix and 21 μL of DNA-free water and amplified as described above. The obtained DNA fragment solution (1 μL) was loaded onto the Agilent DNA 1000 kit chip (Agilent Technologies, Waldbronn, Germany) and the chip was applied to the microchip electrophoresis system (Agilent 2100 Bioanalyzer, Agilent Technologies). The DNA fragments of 328 base pairs (328 bp) and 285 bp were obtained from the wild-type and knock-out genes, respectively.

Animal experiments

Serine racemase knock-out ($SRR^{-/-}$) mice and wild-type ($SRR^{+/+}$) mice (from 7 weeks to 23 months of age, specific-pathogen-free) were raised in the animal center of Kyushu University. For the investigation of the alteration of D-Ser amounts by changing various SRR activities, the littermates ($SRR^{+/+}$, $SRR^{+/-}$ and $SRR^{-/-}$ mice, 3 months of age, specific-pathogen-free) obtained by breeding of $SRR^{+/-}$ mice were used. For the study of oral

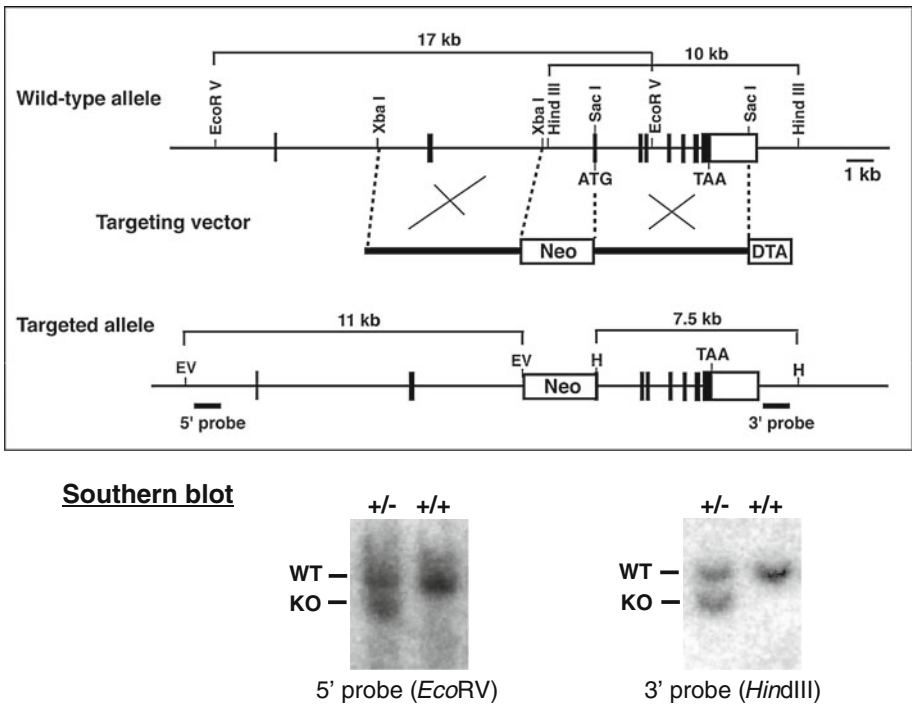

Fig. 1 Strategy for the disruption of the serine racemase gene. The wild-type allele, targeting vector and targeted allele are shown. Untranslated exons were shown in *open boxes* and translated exons are shown in *closed boxes*. Restriction sites are shown with the names of restriction enzymes. ATG and TAA are the initiation codon and termination codon, respectively. *Neo* and *DTA* are neomycin-resistant gene and diphtheria toxin gene, respectively. The probes used for Southern analysis are shown. Southern blots showed that ES cell that succeeded in the homologous recombination (+/−) had both 17- and 11-kb *Eco*RV fragments and both 10- and 7.5 kb *Hin*dIII fragments, whereas ES cells failed of the homologous recombination (+/+) had only a 17-kb *Eco*RV fragment and a 10-kb *Hin*dIII fragment

administration of D-Ser, aqueous solutions containing 10 or 20 mM D-Ser were given to the $SRR^{-/-}$ mice instead of tap water for 1 week, and the diet (NMF diet, Oriental Yeast, Tokyo, Japan) was given ad libitum. The mice lacking both SRR and DAO ($SRR^{-}DAO^{-}$ mice) were obtained by the cross-breeding of $SRR^{-/-}$ mice and ddY/DAO^{-} mice (the ddY/DAO^{-} mouse is a natural mutant strain lacking DAO activity due to a missense mutation (Gly181Arg) (Sasaki et al. 1992)). The genotyping of SRR was performed by the aforementioned procedure, and the genotyping of DAO was performed as reported by Tojo et al. (2009). All experiments were carried out with the permission (A-23-139-0) of the animal care and use committee of Kyushu University.

Sample preparation procedure for mouse tissues and physiological fluids

Tissue and physiological fluid samples were prepared according to the previous reports (Miyoshi et al. 2011) with slight modifications. Briefly, after anesthetization with pentobarbital (100 mg/kg body weight, i.p.), the cerebrospinal fluid (CSF) was obtained from the cerebellomedullary cistern. The mice were then euthanized by exsanguination from the inferior vena cava, and the blood was collected in a 1.5-mL tube and stored at room temperature for an hour followed by centrifugation at 8,000*g* for 15 min to obtain the serum. Urine was obtained from the urinary bladder. Brain tissues (olfactory bulb, cerebral cortex, hippocampus, hypothalamus, cerebellum, medulla oblongata and spinal cord) and peripheral tissues (liver, kidney, heart, lung, pancreas, spleen, testis and ovary) were then excised quickly. All tissues and physiological fluids were stored at −80 °C until use. To the serum, CSF and urine, a 20-fold volume of MeOH was added, mixed for 2 min and centrifuged at 12,100*g* for 10 min to obtain the supernatants. The tissues were homogenized in a 20-fold volume of MeOH (50-fold for the hippocampus and 200-fold for the hypothalamus) using a microhomogenizing system (Micro Smash™ MS-100R, Tomy, Tokyo, Japan), centrifuged at 12,100*g* for 10 min and the supernatants were collected. The supernatant (25 μL for the hippocampus, 100 μL for the hypothalamus, 20 μL for the CSF and 10 μL for other tissues and physiological fluids) was evaporated to dryness under reduced pressure at 40 °C. To the residue, 20 μL of 200 mM sodium borate buffer (pH 8.0) and 5 μL of 40 mM NBD-F in anhydrous CH_3CN were added and heated at 60 °C for 2 min. To the solution, 75 μL (25 μL for CSF) of aqueous 2 % (v/v) TFA was added and 2 μL (5 μL for CSF) of the

reaction mixture was subjected to the 2D-HPLC system described below.

2D-HPLC system for the determination of D-Ser

D-Ser and the enantiomer, L-Ser, were determined using the previously reported 2D-HPLC system (NANOSPACE SI-2 series, Shiseido, Tokyo, Japan) (Miyoshi et al. 2011) with minor improvements. Briefly, NBD-Ser was separated in the first dimension from other compounds as a mixture of the D- plus L-form by a microbore-monolithic ODS column (0.53 mm i.d. × 750 mm, prepared in a fused silica capillary, provided from Shiseido) using 5 % CH_3CN 0.05 % TFA in water (v/v, 30 μL/min) as a mobile phase. The fraction of NBD-Ser (90 μL) was transferred to the loop automatically and introduced to a narrowbore-enantioselective column (Sumichiral OA-2500S, 1.5 mm i.d. × 250 mm, particles were obtained from Sumika Chemical Analysis Service (Osaka, Japan) and self-packed) maintained at 25 °C. Enantiomers of NBD-Ser were then separated using 3 mM citric acid in MeOH-CH_3CN (25:75, v/v) as a mobile phase (flow rate was 150 μL/min), and detected by the fluorescence at 530 nm with excitation at 470 nm.

Results

Determination of D-Ser and L-Ser in various tissues and physiological fluids of serine racemase knock-out ($SRR^{-/-}$) mice

The amounts of D-Ser and L-Ser in seven regions of brain (olfactory bulb, cerebral cortex, hippocampus, hypothalamus, cerebellum, medulla oblongata, spinal cord), eight peripheral organs (liver, kidney, lung, heart, pancreas, spleen, testis, ovary), serum, urine and CSF of $SRR^{-/-}$ mice were determined using a highly sensitive and selective 2D-HPLC system after pre-column fluorescence derivatization of amino acids with NBD-F. The amounts of D- and L-Ser are summarized in Fig. 2a, b, respectively. Relatively large amounts of D-Ser were present in the cerebral cortex and hippocampus (25–35 nmol/g wet tissue) compared with other tissues tested. In the olfactory bulb and hypothalamus, the amounts of D-Ser were 10 and 17 nmol/g wet tissue, respectively, and in the cerebellum, medulla oblongata and spinal cord, the levels of D-Ser were markedly lower compared with those of the cerebral cortex and hippocampus. In the peripheral tissues, the amounts of D-Ser were from 2.5 to 10 nmol/g, and in the serum, urine and CSF, the levels of D-Ser were 1.4, 22 and 0.5 nmol/mL, respectively. Concerning L-Ser, the amounts were from 500 to 2,500 nmol/g wet tissue, and those in the serum, urine and CSF were about 50–100 nmol/mL.

Determination of D-Ser and L-Ser in the brain and serum of aged $SRR^{-/-}$ mice

Dysfunction of the NMDA receptors in the brain areas, especially in the hippocampus, was reported to be involved in age-related cognitive deficits (Wenk et al. 1991). D-Ser is needed to activate NMDA receptors potently (Kleckner and Dingledine 1988; Mothet et al. 2000), and $SRR^{-/-}$ mice are expected to be candidate model mice with reduced intrinsic D-Ser amounts (Inoue et al. 2008; Labrie et al. 2009; Basu et al. 2009). Therefore, the clarification of D-Ser amounts in the brain tissues and serum of aged $SRR^{-/-}$ mice is highly expected, and the amounts of D-Ser in the cerebral cortex, hippocampus, cerebellum, spinal cord and serum of 3-, 5- and 23 month-old-$SRR^{-/-}$ mice were investigated. The results are shown in Fig. 3. In the serum and all tested brain tissues including the hippocampus, the amounts of D-Ser were not drastically different depending on their ages, and intrinsic D-Ser levels in the cerebral cortex and hippocampus remained low continuously (30 nmol/g wet tissue, approximately), and in the cerebellum, spinal cord and serum, the amounts of D-Ser were only trace (1–5 nmol/g wet tissue or mL). Concerning L-Ser, the levels in the brain tissues and serum were also almost unchanged regardless of their age.

Determination of D-Ser and L-Ser in the mice with various SRR activities

To clarify the alteration of D-Ser amounts followed by a change in SRR activity, D-Ser was determined in the brain tissues (cerebral cortex, hippocampus, cerebellum, spinal cord), serum and CSF of $SRR^{-/-}$ and $SRR^{+/-}$ mice and compared with those in the control $SRR^{+/+}$ mice. The results are shown in Fig. 4. Though the amounts of D-Ser in the cerebral cortex and hippocampus of $SRR^{+/+}$ mice were high (300–320 nmol/g wet tissue), the D-Ser levels were significantly reduced in the $SRR^{+/-}$ and $SRR^{-/-}$ mice (210–230 nmol/g in the $SRR^{+/-}$ mice, and 30 nmol/g in the $SRR^{-/-}$ mice). In the cerebellum and spinal cord, the drastic alteration of the D-Ser amount along with change in SRR activity was not observed. In the serum, the D-Ser amounts of $SRR^{+/+}$, $SRR^{+/-}$ and $SRR^{-/-}$ mice were almost the same (1–2 nmol/mL). Small amounts of D-Ser were present in the CSF of all strains of mice, and the amounts in $SRR^{+/+}$, $SRR^{+/-}$ and $SRR^{-/-}$ mice were 1.5, 0.8 and 0.5 nmol/mL, respectively. Concerning L-Ser, the amounts in the tissues and physiological fluids of $SRR^{+/+}$, $SRR^{+/-}$ and $SRR^{-/-}$ mice were not changed by the alteration of SRR activity.

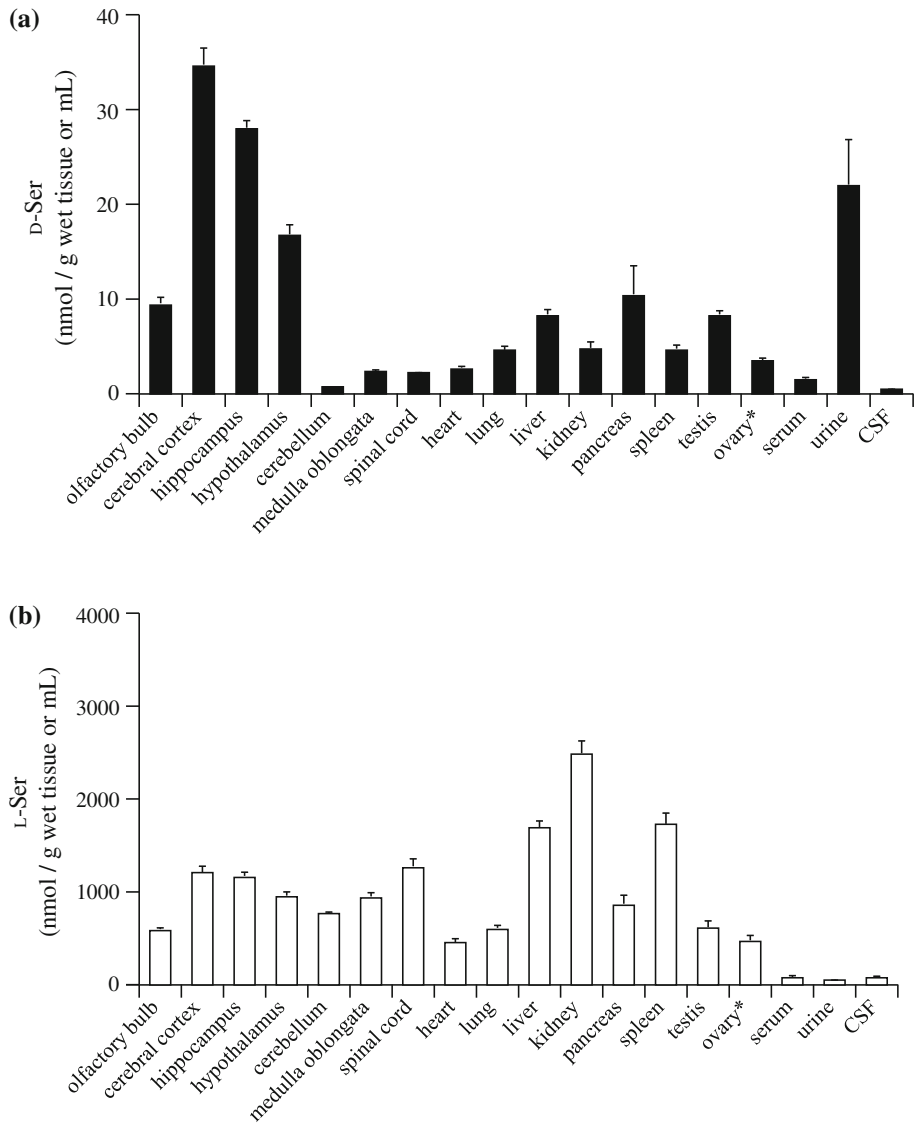

Fig. 2 Determination of D-Ser (**a**) and L-Ser (**b**) in various tissues and physiological fluids of serine racemase knock-out mice ($SRR^{-/-}$ mice). Values represent mean ± SE (nmol/g wet tissue or mL) of 3–4 mice (7–15 weeks of age, male, SPF). *CSF* cerebrospinal fluid. *: Ovary was obtained from female $SRR^{-/-}$ mice of the same age

Alteration of D-Ser amounts in the tissues and serum of $SRR^{-/-}$ mice after oral administration of D-Ser

The alteration of D-Ser amounts in the brain tissues and serum was investigated using $SRR^{-/-}$ mice after oral administration of D-Ser. The $SRR^{-/-}$ mice were given tap water or water containing D-Ser (10 or 20 mM) for 1 week, and then the D-Ser amounts in the cerebral cortex, hippocampus, cerebellum, spinal cord and serum were analyzed. As a result, the amounts of D-Ser in all the tested tissues were increased after administration of D-Ser (Fig. 5a). Especially, a drastic increase in D-Ser amount was observed in the serum, and the amounts of D-Ser after administration of 10 or 20 mM D-Ser were about 20 and 60 times that of the control group (control, 1.2 nmol/mL; 10 mM D-Ser-administered group, 26 nmol/mL; 20 mM D-Ser-administered group, 71 nmol/mL). In the cerebral cortex and hippocampus of the 10 mM D-Ser-administered mice, the D-Ser amounts were 150–200 nmol/g, five to six times those of the control group. After the administration of 20 mM D-Ser, the D-Ser amounts in the cerebral cortex and hippocampus were 310–360 nmol/g and were about ten times those of control mice. Concerning the cerebellum, the D-Ser amounts were also five and ten times higher after 10 and 20 mM D-Ser administration, respectively, and in the spinal cord of the 10 and 20 mM D-Ser-administered groups, the amounts of D-Ser were 34 and 59 nmol/g wet tissue, respectively (10–20 times those of the control). Concerning L-Ser, the amounts were almost unchanged after administration of D-Ser in all the tested tissues and serum (Fig. 5b).

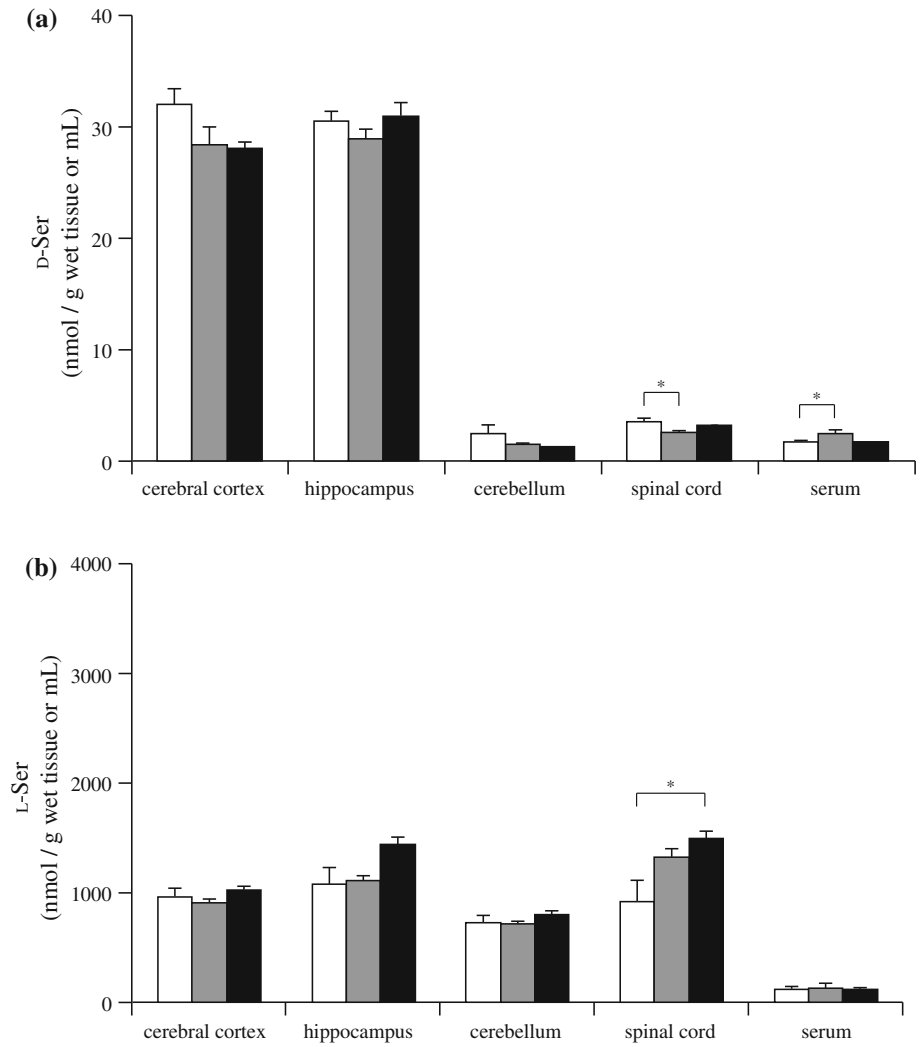

Fig. 3 Age-dependent change in D-Ser (**a**) and L-Ser (**b**) in the brain and serum of $SRR^{-/-}$ mice. Values represent mean ± SE (nmol/g wet tissue or mL) of three mice. *Open bars* 3 months, *gray bars* 5 months, *closed bars* 23 months of age. $*P < 0.05$, significant difference determined by one-way ANOVA followed by Bonferroni's multiple comparison test

Determination of D-Ser in the brain and serum of mice lacking both SRR and DAO

In the hindbrain areas, strong DAO activities are observed (Horiike et al. 1994; Schell et al. 1995). Therefore, to clarify the contribution of SRR to the tissue amounts of D-Ser in the cerebellum and spinal cord, the use of mice lacking both SRR and DAO ($SRR^{-}DAO^{-}$ mice) is desirable. Therefore, we created $SRR^{-}DAO^{-}$ mice by crossing $SRR^{-/-}$ mice and ddY/$DAO^{-/-}$ mice, and the D-Ser amounts in the cerebral cortex, hippocampus, cerebellum, spinal cord and serum of these mice were determined. The results are shown in Fig. 6. In the cerebral cortex and hippocampus, the D-Ser amounts of the $SRR^{-}DAO^{-}$ mice were around 50 nmol/g wet tissue and were drastically reduced compared with those of the $SRR^{+}DAO^{-}$ mice (D-Ser amounts in the $SRR^{+}DAO^{-}$ mice were almost the same as that of $SRR^{+}DAO^{+}$ mice, 250–350 nmol/g wet tissue). On the other hand, compared with the D-Ser amount in the cerebral cortex and hippocampus of the $SRR^{-}DAO^{+}$ mice lacking only SRR, the D-Ser amounts in $SRR^{-}DAO^{-}$ mice were increased. Concerning the cerebellum and spinal cord, the D-Ser amounts of the $SRR^{-}DAO^{-}$ mice (39 and 31 nmol/g wet tissue, respectively) were significantly decreased compared with those of the $SRR^{+}DAO^{-}$ mice (50–150 nmol/g wet tissue) and were 10–20 times those of the $SRR^{-}DAO^{+}$ mice. The D-Ser amount in the serum of the $SRR^{-}DAO^{-}$ mice was almost the same as that of the $SRR^{+}DAO^{-}$ mice ($SRR^{-}DAO^{-}$, 5.8 nmol/mL; $SRR^{+}DAO^{-}$, 5.9 nmol/mL) and was higher than that of the $SRR^{-}DAO^{+}$ mice. Concerning L-Ser, a drastic alteration in the amounts by changing the activities of SRR and DAO was not observed.

Discussion

In the present investigation, we have determined D-Ser in various tissues and physiological fluids of $SRR^{-/-}$ mice. Until now, a few mouse strains lacking SRR have been

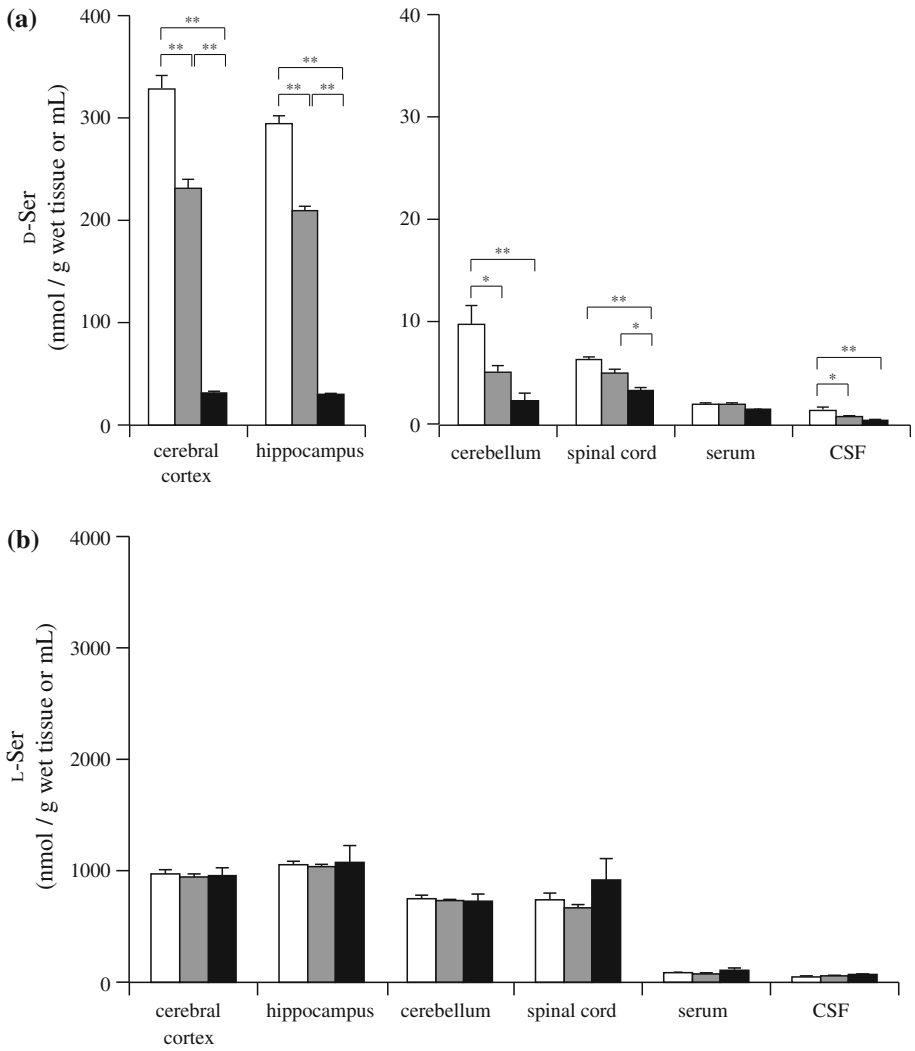

Fig. 4 The amounts of D-Ser (**a**) and L-Ser (**b**) in the brain, serum and CSF of $SRR^{+/+}$ (*open bars*), $SRR^{+/-}$ (*gray bars*) and $SRR^{-/-}$ mice (*closed bars*). Values represent mean ± SE (nmol/g wet tissue or mL) of 3–4 mice. *CSF* cerebrospinal fluid. $*P < 0.05$, $**P < 0.01$, significant difference determined by one-way ANOVA followed by Bonferroni's multiple comparison test

established by different research groups (Miya et al. 2008; Labrie et al. 2009; Basu et al. 2009), and the D-Ser amounts in the frontal brain areas and some peripheral tissues were reported (Horio et al. 2011). The reported amounts of D-Ser in the cerebral cortex and hippocampus were 35–45 nmol/g, which were consistent with those obtained in the present study. Concerning peripheral tissues, the reported D-Ser amounts (2–12 nmol/g) were also in good agreement with those of the present $SRR^{-/-}$ mice (2.5–10 nmol/g). In addition to the reported tissues, the D-Ser amounts in various brain tissues (olfactory bulb, hypothalamus, medulla oblongata, spinal cord), ovary, urine and CSF of $SRR^{-/-}$ mice were determined for the first time in the present study. In the olfactory bulb and hypothalamus, 10–20 nmol/g of D-Ser was present. In the medulla oblongata and spinal cord, the D-Ser amount was quite small as that in the cerebellum. Because high DAO activity was observed in hindbrain areas (Horiike et al. 1994; Schell et al. 1995), the present results that small amounts of D-Ser were observed in these tissues are conceivable. Concerning the mouse CSF, a highly sensitive and selective analytical method is essential for the determination of intrinsic D-Ser, because the sample volume is limited (a few μL) and the D-Ser amount is extremely low. In the present study, we have used a highly selective and sensitive 2D-HPLC system for the precise determination of small amounts of D-Ser with improved sample preparation procedure. As a result, a small amount of D-Ser in the CSF of $SRR^{-/-}$ mice (0.5 nmol/mL) could be determined for the first time. Regarding L-Ser, the amounts in the frontal brain areas and serum of $SRR^{-/-}$ mice were reported to be 500–1,000 nmol/g and 80 nmol/mL (Inoue et al. 2008; Horio et al. 2011), respectively, consistent with those obtained in the present paper.

The aging profiles of D-Ser amounts have also been investigated for the first time in the $SRR^{-/-}$ mice, and a drastic alteration in the D-Ser amount with aging was not observed in all the tested tissues. In the previous reports using rodents having normal SRR activity, the D-Ser amounts were not drastically altered from 7 to 86 weeks of

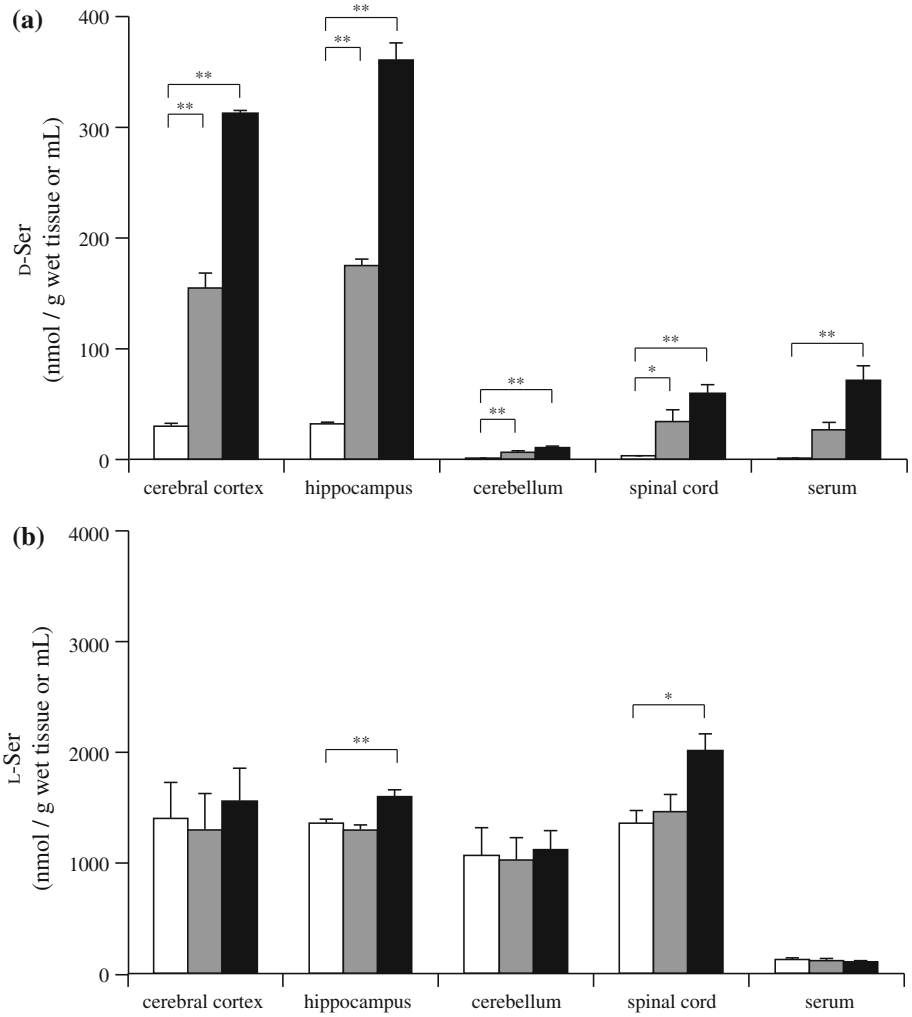

Fig. 5 The accumulation of D-Ser (**a**) and L-Ser (**b**) in the brain and serum of $SRR^{-/-}$ mice after oral administration of D-Ser. Values represent mean ± SE (nmol/g wet tissue or mL) of four mice. *Open bars* control mice ($SRR^{-/-}$ mice given tap water instead of water containing D-Ser), *gray bars* 10 mM D-Ser-administered mice, *closed bars* 20 mM D-Ser-administered mice. $*P < 0.05$, $**P < 0.01$, significant difference from the values of control mice determined by one-way ANOVA followed by Bonferroni's multiple comparison test

age in the cerebral cortex and cerebellum (Hashimoto et al. 1993; Nagata et al. 1994). In the hippocampus, a significant decrease in the D-Ser amount in aged rats was reported compared with that of young rats (Mothet et al. 2006; Turpin et al. 2011). On the other hand, the D-Ser amount in the hippocampus of aged $SRR^{-/-}$ mice was not altered drastically in the present study. Because alteration of the D-Ser amount in the aged rat hippocampus was reported to be due to the decrease in the expression of SRR mRNA and protein (Turpin et al. 2011), the present result using $SRR^{-/-}$ mice is reasonable. Moreover, $SRR^{-/-}$ mice were reported to have an impairment in learning and memory (Mori and Inoue 2010; Basu et al. 2009); therefore, the present result provides useful information for wide utilization of $SRR^{-/-}$ mice in the pathological studies of neuronal diseases, including age-associated impairment.

The changes in D-Ser amounts in the cerebral cortex, hippocampus and cerebellum followed by the alteration of SRR activity have already been reported (Labrie et al. 2009; Basu et al. 2009). In the cerebral cortex and hippocampus, the D-Ser amounts in $SRR^{+/-}$ and $SRR^{-/-}$ mice were reported to be 50–70 and 10–20 % of that of $SRR^{+/+}$ mice. In the present study, a decrease in the D-Ser amount by reducing SRR activity was also observed ($SRR^{+/-}$, 70 %; $SRR^{-/-}$, 10 % of that in the $SRR^{+/+}$ mice), quite similar to the previous reports. So, these results strongly indicate that SRR catalyzes the biosynthesis of D-Ser from L-Ser in the frontal brain area, although SRR has racemization and also β-elimination activities in vitro (Stříšovský et al. 2003). In the cerebellum, the D-Ser amount was reported not to be affected by the decrease in SRR activity (Labrie et al. 2009). On the other hand, the D-Ser amount was slightly reduced in the present study following a decrease in SRR activity. This inconsistency might be due to the difference in the way of producing $SRR^{-/-}$ mice or the breeding conditions, and further studies are needed. In addition to the reported tissues, the D-Ser amounts in the spinal cord, serum and CSF of $SRR^{+/-}$ mice were determined for the first time in the present study. As a result, the D-Ser amount in the spinal cord of $SRR^{+/-}$ mice was decreased compared with that in the $SRR^{+/+}$ mice, while the D-Ser amounts in the serum and CSF of $SRR^{+/-}$ mice

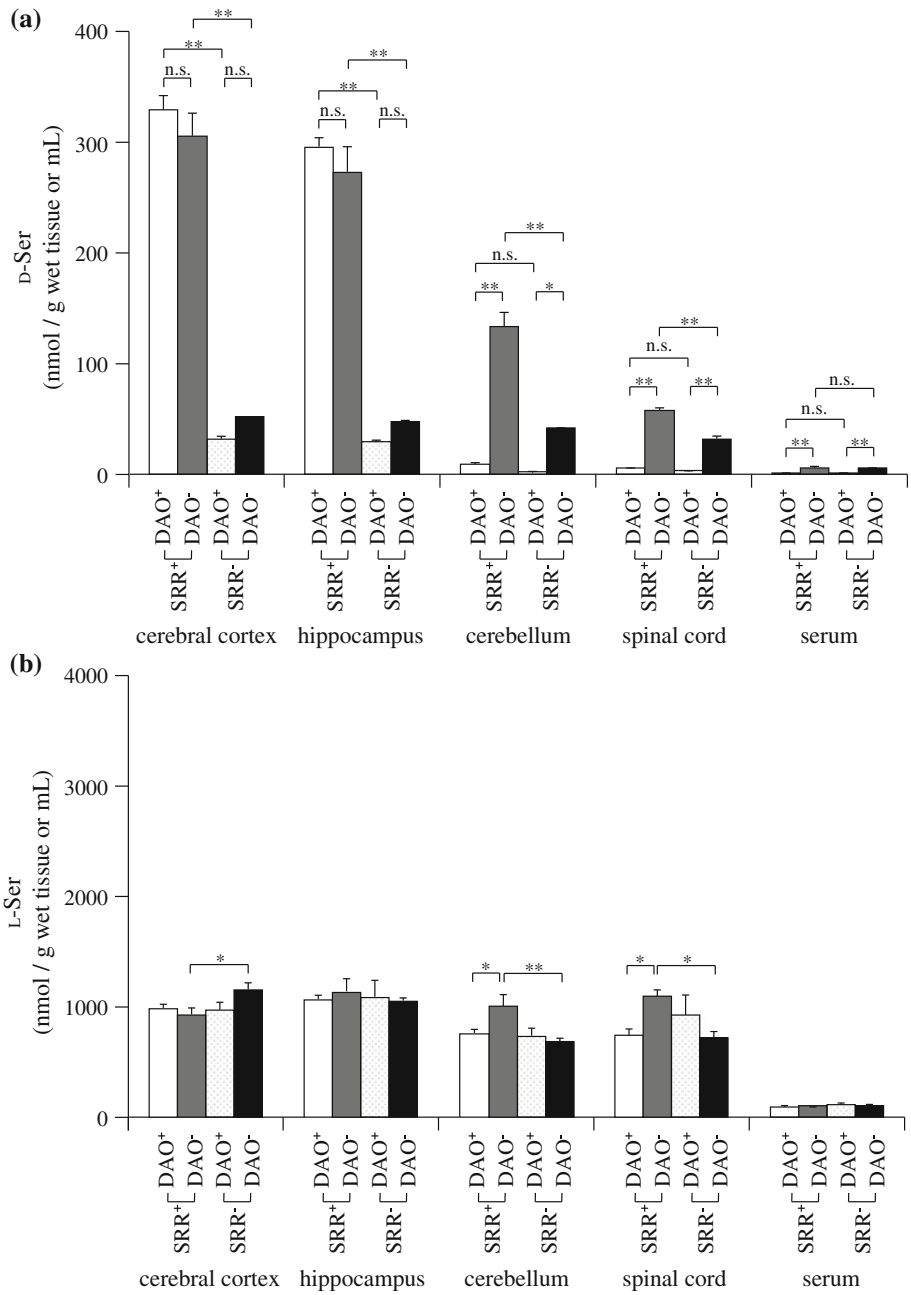

Fig. 6 The amounts of D-Ser (**a**) and L-Ser (**b**) in the brain and serum of $SRR^{+}DAO^{+}$, $SRR^{+}DAO^{-}$, $SRR^{-}DAO^{+}$ and $SRR^{-}DAO^{-}$ mice. Values represent mean ± SE (nmol/g wet tissue or mL) of 3–4 mice. $*P < 0.05$, $**P < 0.01$, significant difference determined by two-way ANOVA followed by Bonferroni's multiple comparison test. *n.s.* not significant. The values in the cerebral cortex, hippocampus, cerebellum and serum of $SRR^{+}DAO^{-}$ mice are the results described in our previous reports (Miyoshi et al. 2009)

were not altered. SRR is reported to be localized in the cerebral cortex and hippocampus (Wolosker et al. 1999b; Miya et al. 2008); therefore, the D-Ser amount in these tissues is likely to be influenced largely by the change in SRR activity. In the cerebellum and spinal cord, the DAO activity is high (Horiike et al. 1994; Schell et al. 1995), and the D-Ser amount is regulated to be low even in the mice having normal SRR activity (Nagata et al. 1994; Miyoshi et al. 2009). DAO is also expressed in the kidney (Ohide et al. 2011) and choroid plexus (Ono et al. 2009); the D-Ser in serum and CSF is considered to be regulated by DAO in these tissues. Therefore, the alterations of the D-Ser amounts in the cerebellum, spinal cord and physiological fluids following the change in SRR activity might not be drastic compared with those in the cerebral cortex and hippocampus. However, the D-Ser amounts in the cerebellum and spinal cord of $SRR^{+/-}$ and $SRR^{-/-}$ mice were decreased in the present study. It is thus suggested that the D-Ser amounts in not only the cerebral cortex and hippocampus but also the cerebellum and spinal cord are regulated by SRR.

In the present study, an increase in the D-Ser amount was observed in all the tested tissues of $SRR^{-/-}$ mice following the oral administration of D-Ser (the estimated daily doses

of D-Ser are 525 and 1,050 mg/kg, respectively, when a mouse drank 10 mL of 10 and 20 mM D-Ser solution). These results indicated that D-Ser is accumulated in these tissues including the cerebral cortex and hippocampus. On the other hand, using the control mice having normal SRR activity, the D-Ser amount was reported to be constant in the cerebral cortex and hippocampus after administration of D-Ser (Morikawa et al. 2007). According to these results, it is suggested that the D-Ser levels in the cerebral cortex and hippocampus remain around 300–400 nmol/g in the normal mice by unknown mechanisms when D-Ser is orally administered. In the cerebellum, the D-Ser amount was also reported to be constant after administration of D-Ser to the mice having normal SRR activity (Morikawa et al. 2007). However, an increase in the D-Ser amount could be observed in the $SRR^{-/-}$ mice. This discrepancy might be due to the differences in the intrinsic amount of D-Ser in the cerebellum (the D-Ser amount of $SRR^{-/-}$ mice is significantly decreased compared with $SRR^{+/+}$ mice) and the dose of D-Ser (10 and 20 mM D-Ser in the present study, 10 mM D-Ser in the previous report). In serum, the D-Ser amount in 10 mM D-Ser-administered mice having normal SRR activity was reported to be higher (22 nmol/mL) than that of the control group (2 nmol/mL) (Morikawa et al. 2007). In the present study, an increase in the D-Ser amount following the administration of D-Ser was also observed (control, 1.2 nmol/mL; 10 mM D-Ser administration, 26 nmol/mL). The increase in D-Ser amount is greater in the cerebral cortex and hippocampus than that in the cerebellum and spinal cord, because D-Ser is likely to be metabolized by DAO expressed in the hindbrain tissues (Horiike et al. 1994; Schell et al. 1995). These results clearly indicate that exogenous D-Ser is transferred and accumulated in the brain tissues including the cerebral cortex and hippocampus, which is useful information for the development of novel treatments for neuronal diseases using D-Ser.

In the present investigation, the contribution of SRR to the amounts of D-Ser in the brain tissues including the cerebellum and spinal cord is also clarified. For this aim, we established the mice lacking both SRR and DAO and determined the D-Ser in the tissues and physiological fluids for the first time. A drastic decrease in the D-Ser amount was observed in the cerebral cortex and hippocampus of the $SRR^{-}DAO^{-}$ mice (which is 15 % of that of the $SRR^{+}DAO^{-}$ mice), indicating that most of the D-Ser present in these tissues is biosynthesized by SRR. In the cerebellum and spinal cord, the D-Ser amount in $SRR^{-}DAO^{-}$ mice was also drastically decreased to about half of that of the $SRR^{+}DAO^{-}$ mice. These results indicate that half of the D-Ser present in the cerebellum and spinal cord of the $SRR^{+}DAO^{-}$ mice originate from the biosynthesis by SRR. On the other hand, the D-Ser amount in the serum of $SRR^{-}DAO^{-}$ mice was not altered compared with that of the $SRR^{+}DAO^{-}$ mice; therefore, it is indicated that most of the D-Ser in the serum of $SRR^{+}DAO^{-}$ mice was derived from sources other than SRR. According to these results, it was represented that the contribution of SRR to the D-Ser amount was different depending on the tissues. When the D-Ser amounts in $SRR^{-}DAO^{-}$ mice and $SRR^{-}DAO^{+}$ mice were compared, the D-Ser amounts in the $SRR^{-}DAO^{-}$ mice were higher than those in the $SRR^{-}DAO^{+}$ mice in all the tested tissues. Because DAO activity is high in the kidney, cerebellum and spinal cord, the D-Ser amounts in the serum, cerebellum and spinal cord were drastically influenced by the lack of DAO (Ohide et al. 2011; Miyoshi et al. 2009). On the other hand, the DAO activity is low in the cerebral cortex and hippocampus where D-Ser amounts are not regulated by DAO directly. Therefore, the increase in the D-Ser amount in these tissues of $SRR^{-}DAO^{-}$ mice is considered to be due to the increase in D-Ser amounts in the serum. Interestingly, an appreciable amount of D-Ser remained in the brain tissues and serum of $SRR^{-}DAO^{-}$ mice (30–50 nmol/g in brain tissues, 6 nmol/mL in serum), indicating that a part of D-Ser is not derived from the biosynthesis by SRR. The origin of D-Ser other than the SRR is still not clear; however, diet and intestinal bacteria are reported to contain D-amino acids (Asakura and Konno 1997; Brückner and Schieber 2001). Some other pathway of D-Ser synthesis such as a glycine cleavage enzyme system, serine hydroxymethyl transferase and phosphoserine phosphatase should be also considered (Iwama et al. 1997; Wood et al. 1996). For investigation of the dietary origin, we determined the D-Ser amounts in the cerebral cortex and hippocampus of $SRR^{-/-}$ mice that fasted for 4 days. As a result, a moderate but significant decrease in the D-Ser amount in the fasted $SRR^{-/-}$ mice was obtained (cerebral cortex, 23.2 nmol/g, hippocampus, 22.4 nmol/g). Therefore, it is suggested that a part of residual D-Ser is likely to be derived from their diet; however, further investigations are necessary.

Conclusion

In the present study, we have elucidated the distribution of D-Ser in the young and aged mice having various SRR activities, and the effect of D-Ser administration on the intrinsic D-Ser amount in the brain tissues and serum of $SRR^{-/-}$ mice has also been clarified. The contribution of SRR and DAO to the D-Ser amounts in brain tissues including the cerebellum and spinal cord has been investigated using the $SRR^{-}DAO^{-}$ mice first established in the present study, and the results indicate that an appreciable amount of D-Ser is synthesized by SRR also in the

hindbrain areas similar to the frontal brain areas. The results described in the present paper are useful for the design of treatments for neuronal disorders via regulating D-Ser and also for understanding of physiological D-Ser regulation in the brain. Further utilization of the $SRR^{-/-}$ mice and $SRR^{-}DAO^{-}$ mice established in the present study is expected for the pathological and physiological study of SRR, DAO and D-Ser.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Asakura S, Konno R (1997) Origin of D-serine present in urine of mutant mice lacking D-amino-acid oxidase activity. Amino Acids 12:213–223

Barañano DE, Ferris CD, Snyder SH (2001) Atypical neural messengers. Trends Neurosci 24:99–106

Basu AC, Tsai GE, Ma C-L, Ehmsen JT, Mustafa AK, Han L, Jiang ZI, Benneyworth MA, Froimowitz MP, Lange N, Snyder SH, Bergeron R, Coyle JT (2009) Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. Mol Psychiatry 14:719–727

Bendikov I, Nadri C, Amar S, Panizzutti R, De Miranda J, Wolosker H, Agam G (2007) A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. Schizophr Res 90:41–51

Brückner H, Schieber A (2001) Ascertainment of D-amino acids in germ-free, gnotobiotic and normal laboratory rats. Biomed Chromatogr 15:257–262

Corrigan JJ (1969) D-Amino acids in animals. Science 164:142–149

D'Aniello A, Vetere A, Petrucelli L (1993) Further study on the specificity of D-amino acid oxidase and of D-aspartate oxidase and time course for complete oxidation of D-amino acids. Comp Biochem Physiol 105B:731–734

Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T, Takahashi K (1992) The presence of free D-serine in rat brain. FEBS Lett 296:33–36

Hashimoto A, Nishikawa T, Oka T, Takahashi K (1993) Endogenous D-serine in rat brain: *N*-methyl-D-aspartate receptor-related distribution and aging. J Neurochem 60:783–786

Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, Nakazato M, Kumakiri C, Okada S, Hasegawa H, Imai K, Iyo M (2003) Decreased serum levels of D-serine in patients with schizophrenia. Arch Gen Psychiatry 60:572–576

Heresco-Levy U, Javitt DC, Ebstein R, Vass A, Lichtenberg P, Bar G, Catinari S, Ermilov M (2005) D-Serine efficacy as add-on pharmacotherapy to risperidone and olanzapine for treatment-refractory schizophrenia. Biol Psychiatry 57:577–585

Horiike K, Tojo H, Arai R, Nozaki M, Maeda T (1994) D-Amino-acid oxidase is confined to the lower brain stem and cerebellum in rat brain: regional differentiation of astrocytes. Brain Res 652:297–303

Horio M, Kohno M, Fujita Y, Ishima T, Inoue R, Mori H, Hashimoto K (2011) Levels of D-serine in the brain and peripheral organs of serine racemase (*Srr*) knock-out mice. Neurochem Int 59:853–859

Imai K, Watanabe Y (1981) Fluorimetric determination of secondary amino acids by 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole. Anal Chim Acta 130:377–383

Inoue R, Hashimoto K, Harai T, Mori H (2008) NMDA- and β-amyloid$_{1\text{-}42}$-induced neurotoxicity is attenuated in serine racemase knock-out mice. J Neurosci 28:14486–14491

Iwama H, Takahashi K, Kure S, Hayashi F, Narisawa K, Tada K, Mizoguchi M, Takashima S, Tomita U, Nishikawa T (1997) Depletion of cerebral D-serine in non-ketotic hyperglycinemia: possible involvement of glycine cleavage system in control of endogenous D-serine. Biochem Biophys Res Commun 231:793–796

Kakegawa W, Miyoshi Y, Hamase K, Matsuda S, Matsuda K, Kohda K, Emi K, Motohashi J, Konno R, Zaitsu K, Yuzaki M (2011) D-Serine regulates cerebellar LTD and motor coordination through the δ2 glutamate receptor. Nat Neurosci 14:603–611

Katane M, Homma H (2011) D-Aspartate—an important bioactive substance in mammals: a review from an analytical and biological point of view. J Chromatogr B 879:3108–3121

Kirschner DL, Green TK (2009) Separation and sensitive detection of D-amino acids in biological matrices. J Sep Sci 32:2305–2318

Kleckner NW, Dingledine R (1988) Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. Science 241:835–837

Konno R, Yasumura Y (1983) Mouse mutant deficient in D-amino acid oxidase activity. Genetics 103:277–285

Konno R, Sasaki M, Enami J, Niwa A (1995) Loss of *Hin*dIII cleavage sites in the D-amino acid oxidase gene in some inbred strains of mice. Amino Acids 8:97–107

Konno R, Brückner H, D'Aniello A, Fisher G, Fujii N, Homma H (eds) (2007) D-Amino acids: a new frontier in amino acids and protein research—practical methods and protocols. Nova Science Publishers, New York

Konno R, Okamura T, Kasai N, Summer KH, Niwa A (2009) Mutant rat strain lacking D-amino-acid oxidase. Amino Acids 37:367–375

Labrie V, Fukumura R, Rastogi A, Fick LJ, Wang W, Boutros PC, Kennedy JL, Semeralul MO, Lee FH, Baker GB, Belsham DD, Barger SW, Gondo Y, Wong AHC, Roder JC (2009) Serine racemase is associated with schizophrenia susceptibility in humans and in a mouse model. Hum Mol Genet 18:3227–3243

Mitchell J, Paul P, Chen H, Morris A, Payling M, Falchi M, Habgood J, Panoutsou S, Winkler S, Tisato V, Hajitou A, Smith B, Vance C, Shaw C, Mazarakis ND, Belleroche J (2010) Familial amyotrophic lateral sclerosis is associated with a mutation in D-amino acid oxidase. Proc Natl Acad Sci USA 107:7556–7561

Miya K, Inoue R, Takata Y, Abe M, Natsume R, Sakimura K, Honggou K, Miyawaki T, Mori H (2008) Serine racemase is predominantly localized in neurons in mouse brain. J Comp Neurol 510:641–654

Miyoshi Y, Hamase K, Tojo Y, Mita M, Konno R, Zaitsu K (2009) Determination of D-serine and D-alanine in the tissues and physiological fluids of mice with various D-amino-acid oxidase activities using two-dimensional high-performance liquid chromatography with fluorescence detection. J Chromatogr B 877:2506–2512

Miyoshi Y, Hamase K, Okamura T, Konno R, Kasai N, Tojo Y, Zaitsu K (2011) Simultaneous two-dimensional HPLC determination of free D-serine and D-alanine in the brain and periphery of

mutant rats lacking D-amino-acid oxidase. J Chromatogr B 879:3184–3189

Miyoshi Y, Koga R, Oyama T, Han H, Ueno K, Masuyama K, Itoh Y, Hamase K (2012) HPLC analysis of naturally occurring free D-amino acids in mammals. J Pharm Biomed Anal 69:42–49

Mori H, Inoue R (2010) Serine racemase knockout mice. Chem Biodivers 7:1573–1578

Morikawa A, Hamase K, Inoue T, Konno R, Zaitsu K (2007) Alterations in D-amino acid levels in the brains of mice and rats after the administration of D-amino acids. Amino Acids 32:13–20

Mothet JP, Parent AT, Wolosker H, Brady RO Jr, Linden DJ, Ferris CD, Rogawski MA, Snyder SH (2000) D-Serine is an endogenous ligand for the glycine site of the *N*-methyl-D-aspartate receptor. Proc Natl Acad Sci USA 97:4926–4931

Mothet JP, Rouaud E, Sinet P-M, Potier B, Jouvenceau A, Dutar P, Videau C, Epelbaum J, Billard J-M (2006) A critical role for the glial-derived neuromodulator D-serine in the age-related deficits of cellular mechanisms of learning and memory. Aging Cell 5:267–274

Nagata Y, Horiike K, Maeda T (1994) Distribution of free D-serine in vertebrate brains. Brain Res 634:291–295

Neims AH, Zieverink WD, Smilack JD (1966) Distribution of D-amino acid oxidase in bovine and human nervous tissues. J Neurochem 13:163–168

Nishikawa T (2011) Analysis of free D-serine in mammals and its biological relevance. J Chromatogr B 879:3169–3183

Ohide H, Miyoshi Y, Maruyama R, Hamase K, Konno R (2011) D-Amino acid metabolism in mammals: biosynthesis, degradation and analytical aspects of the metabolic study. J Chromatogr B 879:3162–3168

Ono K, Shishido Y, Park HK, Kawazoe T, Iwana S, Chung SP, El-Magd RMA, Yorita K, Okano M, Watanabe T, Sano N, Bando Y, Arima K, Sakai T, Fukui K (2009) Potential pathophysiological role of D-amino acid oxidase in schizophrenia: immunohistochemical and in situ hybridization study of the expression in human and rat brain. J Neural Transm 116:1335–1347

Sasabe J, Chiba T, Yamada M, Okamoto K, Nishimoto I, Matsuoka M, Aiso S (2007) D-Serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. EMBO J 26:4149–4159

Sasabe J, Miyoshi Y, Suzuki M, Mita M, Konno R, Matsuoka M, Hamase K, Aiso S (2012) D-Amino acid oxidase controls motoneuron degeneration through D-serine. Proc Natl Acad Sci USA 109:627–632

Sasaki M, Konno R, Nishio M, Niwa A, Yasumura Y, Enami J (1992) A single-base-pair substitution abolishes D-amino-acid oxidase activity in the mouse. Biochim Biophys Acta 1139:315–318

Schell MJ, Molliver ME, Snyder SH (1995) D-Serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release. Proc Natl Acad Sci USA 92:3948–3952

Stříšovský K, Jirásková J, Bařinka C, Majer P, Rojas C, Slusher BS, Konvalinka J (2003) Mouse brain serine racemase catalyzes specific elimination of L-serine to pyruvate. FEBS Lett 535:44–48

Tojo Y, Hamase K, Konno R, Koyanagi S, Ohdo S, Zaitsu K (2009) Simple and rapid genotyping of D-amino acid oxidase gene recognizing a crucial variant in the ddY strain using microchip electrophoresis. J Sep Sci 32:430–436

Tsai G, Yang P, Chung L, Lange N, Coyle JT (1998) D-Serine added to antipsychotics for the treatment of schizophrenia. Biol Psychiatry 44:1081–1089

Turpin FR, Potier B, Dulong JR, Sinet PM, Alliot J, Oliet SHR, Dutar P, Epelbaum J, Mothet JP, Billard JM (2011) Reduced serine racemase expression contributes to age-related deficits in hippocampal cognitive function. Neurobiol Aging 32:1495–1504

Wenk GL, Walker LC, Price DL, Cork LC (1991) Loss of NMDA, but not GABA-A, binding in the brains of aged rats and monkeys. Neurobiol Aging 12:93–98

Wolosker H, Sheth KN, Takahashi M, Mothet JP, Brady RO Jr, Ferris CD, Snyder SH (1999a) Purification of serine racemase: biosynthesis of the neuromodulator D-serine. Proc Natl Acad Sci USA 96:721–725

Wolosker H, Blackshaw S, Snyder SH (1999b) Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-*N*-methyl-D-aspartate neurotransmission. Proc Natl Acad Sci USA 96:13409–13414

Wood PL, Hawkinson JE, Goodnough DB (1996) Formation of D-serine from L-phosphoserine in brain synaptosomes. J Neurochem 67:1485–1490