Brief Communications

NMDA- and β-Amyloid$_{1-42}$-Induced Neurotoxicity Is Attenuated in Serine Racemase Knock-Out Mice

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D-Serine is detected in the brain and acts as a coagonist at the "glycine-site" of the NMDA-type glutamate receptor. Although D-serine can be directly produced from L-serine by serine racemase (SR), the relative contribution of SR in D-serine formation *in vivo* is not known. Pathological roles of brain D-serine mediating NMDA receptor overactivation are suggested in studies using *in vitro* culture systems. However, we have recently demonstrated the differential SR protein expression *in vivo* and in culture. Here, we reported an ~90% decrease in forebrain D-serine content in SR knock-out (KO) mice. We also found a reduced neurotoxicity induced by NMDA- and Aβ$_{1-42}$-peptide injections into the forebrain in SR KO mice. These results suggest that SR is the major enzyme for D-serine production in the brain, D-serine is the predominant endogenous coagonist of the NMDA receptor in the forebrain, and D-serine may be involved in controlling the extent of NMDA receptor-mediated neurotoxic insults observed in disorders including Alzheimer's disease. The control of SR activity and D-serine level in the brain may lead to a novel strategy for neuroprotection against various neurodegenerative diseases.

Key words: **D-Serine; serine racemase; NMDA receptor; neurotoxicity; Alzheimer's disease; gene knock-out mice**

Introduction

The mammalian brain contains high levels of D-serine, which acts as a coagonist at the "glycine-site" of the NMDA-type glutamate receptor (GluR). The origin of brain D-serine was speculative before the discovery of serine racemase (SR) in the mammalian brain (Wolosker et al., 1999a,b). SR catalyzes racemization and dehydration of serine (De Miranda et al., 2002). Enzymatic characterizations of purified SR suggest that the catalytic constant (k_{cat}) for D-serine in the racemization by mouse SR is ~1/400 of prokaryotic racemase, and the k_{cat}/K_m of SR in the dehydration of L-serine is higher than those in the racemization for serine (Yoshimura and Goto, 2008). The activity of SR is decreased by glycine at a physiological concentration (Dunlop and Neidle, 2005) and by modification with *S*-nitrosylation (Mustafa et al., 2007). Thus, the contribution of SR to D-serine production in the brain should be evaluated *in vivo*.

NMDA-type GluR plays key roles in neural network formation during development, synaptic plasticity, and neurodegenerative disorders including Alzheimer's disease (AD) (Bliss and Collingridge, 1993; Komuro and Rakic, 1993; Lancelot and Beal, 1998). In cultured hippocampal neurons, degradation of D-serine by treatment with D-amino acid oxidase (DAAO) decreases NMDA-receptor-mediated currents (Mothet et al., 2000) and diminishes long-term potentiation in cultured hippocampal neurons (Yang et al., 2003). In the forebrain, there are sufficient amounts of free D-serine and glycine for activation of the NMDA receptor (Hashimoto et al., 1995). D-Serine and glycine may control the extent of NMDA receptor-mediated neurotoxicity. A study using D-serine deaminase demonstrated that D-serine, but not glycine, mediates NMDA receptor-elicited cell death in organotypic hippocampal slices (Shleper et al., 2005). These reports using culture systems strongly support the hypothesis that D-serine is a major endogenous ligand for the NMDA receptor. However, we have recently reported the differential expression of the SR protein in the brain and in culture (Miya et al., 2008). Thus, the relative importance of two coagonists, D-serine and glycine, in NMDA receptor activation in the brain remains unclear.

AD is a major neurodegenerative disorder in which the excitotoxic effect of D-serine may be involved. The β-amyloid peptide (Aβ) is proposed as the main pathological factor for AD. Some extent of calcium-mediated neurotoxicity exerted by Aβ can be mediated by the NMDA receptor (Suh and Checler, 2002). DAAO protects neurons against Aβ-induced Ca^{2+} overload and neurotoxicity, providing evidence that D-serine may be a death signal induced by Aβ (Wu et al., 2004). As mentioned above, most of the studies on D-serine with respect to the regulation of various brain functions have been limited in culture systems and enzymatic degradation of D-serine. To investigate the roles of SR and endogenous D-serine *in vivo*, we have recently established SR knock-out (KO) mice (Miya et al., 2008). In the present study, we found that SR KO mice showed ~90% decrease in brain D-serine content and attenuation of NMDA- and Aβ-elicited neurotoxicity.

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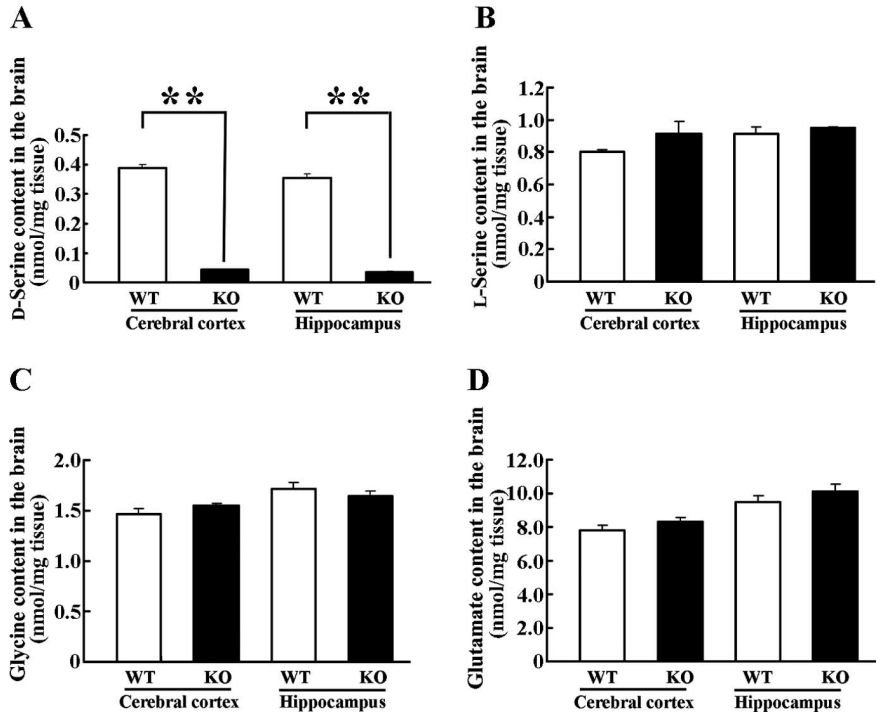

Figure 1. Serine racemase is a major enzyme for D-serine production in the brain. ***A***, HPLC revealed that D-serine contents in the cerebral cortex and hippocampus of serine racemase knock-out (SR KO, KO) mice were ~10-fold lower than those of WT mice. ***B–D***, The contents of L-serine, glycine, and glutamate in the cerebral cortex and hippocampus of SR-KO mice were comparable with those of WT mice. The data represent mean ± SEM from six mice. $^{**}p < 0.001$; two-tailed Student's *t* test.

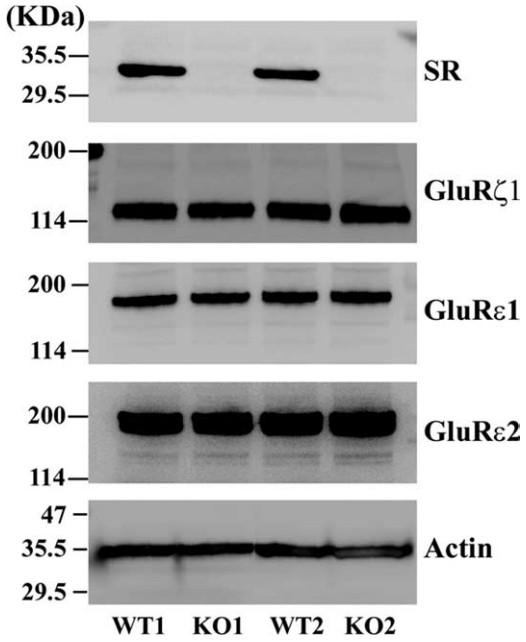

Figure 2. Expression level of NMDA receptor subunits in SR KO mice is comparable with that in WT mice. Western blot analyses of the forebrain proteins of two WT and SR KO (KO) mice by use of anti-SR, anti-GluRζ1, anti-GluRε1, anti-GluRε2, and anti-actin antibodies. The positions of protein size markers are indicated on the left side. The SR protein was detected in WT mice, but not in SR KO mice. There were no marked differences in the expression levels of the three NMDA receptor subunits between WT and SR KO mice.

Materials and Methods

Mice. Animal care and experimental protocols were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at University of Toyama. The SR KO mice were generated from C57BL/6-derived embryonic stem cells transfected with the gene-targeting vector containing C57BL/6 mouse genomic DNA, and were expanded by crossing with C57BL/6 mice (Miya et al., 2008). Generation and genotyping of the SR KO and wild-type (WT) control mice with pure C57BL/6 genetic background have been reported previously (Miya et al., 2008). The WT and SR KO mice at the age of 2–3 months were used for analyses in a genotype blind manner.

High-performance liquid chromatography analysis. Mice were starved overnight. Measurement of D-serine, L-serine, glycine, and glutamate levels in the brain homogenate was performed according to established methods using a column-switching high-performance liquid chromatography (HPLC) system (Shimadzu) (Fukushima et al. 2004). A 20 μl aliquot sample was processed and analyzed as described previously (Kanahara et al., 2008).

Antibodies. Goat polyclonal anti-SR antibody, rabbit polyclonal anti-actin antibody, HRP-conjugated donkey anti-goat IgG, and HRP-conjugated donkey anti-mouse IgG were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-GluRε1 and anti-GluRε2 antibodies (Watanabe et al., 1998) were provided by Dr. Masahiko Watanabe (Hokkaido University, Sapporo, Japan). Mouse monoclonal anti-GluRζ1 was purchased from BD Biosciences Pharmigen. HRP-conjugated goat anti-rabbit IgG was purchased from Bio-Rad.

Western blotting. The WT and SR KO mice were deeply anesthetized with pentobarbital sodium [100 mg/kg body weight (b.w.)] by intraperitoneal injection, perfused transcardially with ice-cold PBS, pH 7.4. Brains were quickly removed and forebrain tissue was homogenized in ice-cold mammalian protein extraction reagent (Pierce). Protein extracts (100 μg) was subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking with a solution containing 5% skim milk in PBS, membranes were incubated with primary antibodies of anti-SR (1:500), anti-GluRε1 (NR2A) (0.5 μg/ml), anti-GluRε2 (NR2B) (0.5 μg/ml), and anti-GluRζ1 (NR1) (0.5 μg/ml) overnight at 4°C, then with HRP-conjugated secondary antibody for 1 h. Protein bands were detected using the ECL chemiluminescence detection system (GE Healthcare).

Aβ preparation. Human $A\beta_{1-42}$ peptide and control human $A\beta_{42-1}$ peptide (Peptide Institute) were dissolved in 2 mM DMSO at a concentration of 4 μg/μl and were stored at −80°C until use. The $A\beta_{1-42}$ or $A\beta_{42-1}$ peptide was diluted with same volume of 200 mM DMSO immediately before Aβ injection.

Intracerebral drug administration. The WT and SR KO mice were deeply anesthetized with 3.6% chloral hydrate in PBS by intraperitoneal injection (10 ml/kg b.w.) and mounted in a stereotaxic frame. The skull was exposed and 0.5 μl of 100 mM NMDA or 100 mM NMDA combined with 100 mM D-serine (Tocris) was injected into right parietal cortex at a site, 1.6 mm caudal to bregma, 1 mm right from the midline, and 0.8 mm below the dural surface. Mice were decapitated 24 h after the injection under the deep anesthesia with pentobarbital sodium.

For Aβ injection, 1 μl of $A\beta_{1-42}$ or $A\beta_{42-1}$ peptide at the concentration 2 μg/μl was injected into right hippocampus at a site, 2 mm caudal to bregma, 1.5 mm right from the midline, and 1.9 mm below the dural surface. Dizocilpine maleate (MK-801, 0.8 mg/kg b.w.) was intraperitoneally administered 2 h before $A\beta_{1-42}$ injection. Mice were decapitated 48 h after the injection under the deep anesthesia with pentobarbital sodium.

Histology. The WT and SR KO mice were deeply anesthetized with pentobarbital sodium by intraperitoneal injection and perfused transcardially with ice-cold PBS followed by 4% paraformaldehyde. Brains were

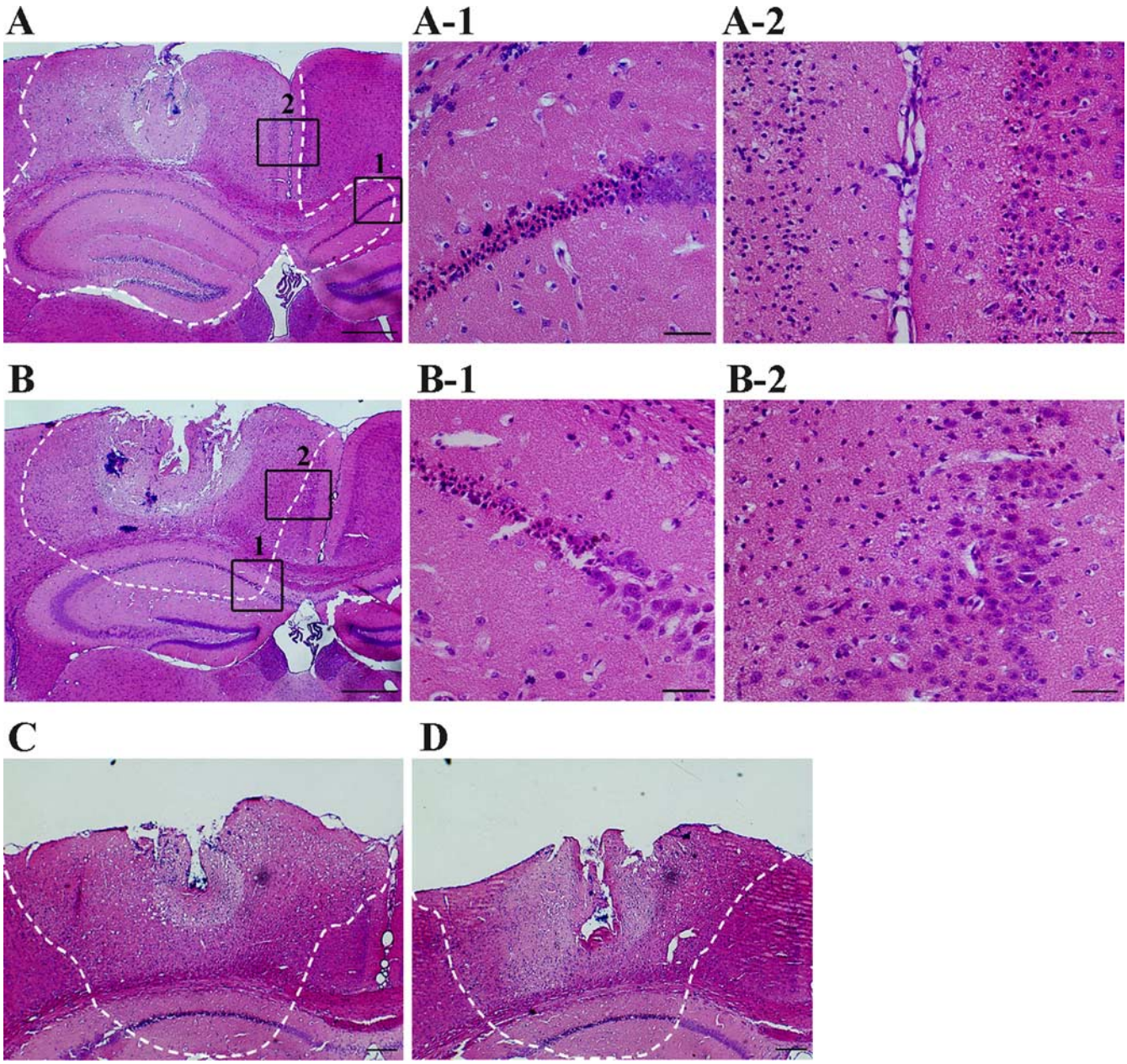

Figure 3. NMDA-induced neuronal damage is attenuated in SR KO mice. ***A***, ***B***, HE-stained brain sections of WT (***A***) and SR KO (***B***) mice 24 h after NMDA injection. ***A-1***, ***A-2***, High-magnification images corresponding to rectangles 1 and 2 in ***A***, respectively. ***B-1***, ***B-2***, High-magnification images corresponding to rectangles 1 and 2 in ***B***, respectively. ***C***, ***D***, HE-stained brain sections of WT (***C***) and SR KO (***D***) mice 24 h after coinjection of NMDA and D-serine. White dotted lines in ***A–D*** delineate the regions of neurodegeneration. Scale bars: ***A***, ***B***, 500 μm; ***A-1***, ***A-2***, ***B-1***, ***B-2***, 50 μm; ***C***, ***D***, 200 μm.

removed and fixed with same fixative for overnight. The fixed brains were then embedded in paraffin and cut into 10 μm thick coronal sections at intervals of 100 μm. These sections were glass-mounted and stained for hematoxylin/eosin (HE). Digital image of HE-stained sections were taken with an Olympus AX80 microscope (Olympus). In HE-stained brain sections, the area damaged after drug injection showed decreased stainability that probably correspond to the edematous change and cellular degeneration. In accordance with these areas, the high-magnification view revealed that the degenerated neurons were distributed with shrunken and pyknotic nuclei. Neuronal death was further confirmed in these damaged areas by the Nissl staining conducted on the adjacent sections. After these observations, the damaged areas were delineated on the digital images of HE-stained sections and were quantified using MetaMorph software (Universal Imaging). Then, the injury volume was calculated as a pile of columns, each with an evaluated area and a height of 100 μm as reported by Ishii et al. (2006).

Statistical analysis. All values are represented as mean ± SEM. Statistical significance between WT and SR KO mice was determined by two-tailed Student's *t* test. Values of $p < 0.05$ were considered significant.

Results

D-Serine content in SR KO mice brain

To examine whether SR is responsible for the production of D-serine in the brain, D-serine content was measured in the brain homogenate of SR KO and WT mice. HPLC revealed that D-serine content in the cerebral cortex and hippocampus of SR KO mice (0.043 ± 0.004 and 0.037 ± 0.001 nmol/mg tissue, respectively; $n = 6$) were ~10-fold lower than those in WT mice (0.388 ± 0.011 and 0.355 ± 0.012 nmol/mg tissue, respectively; $n = 6$) (Fig. 1*A*). In contrast, the levels of L-serine, glycine, and glutamate in the cerebral cortex of SR KO mice (0.917 ± 0.073,

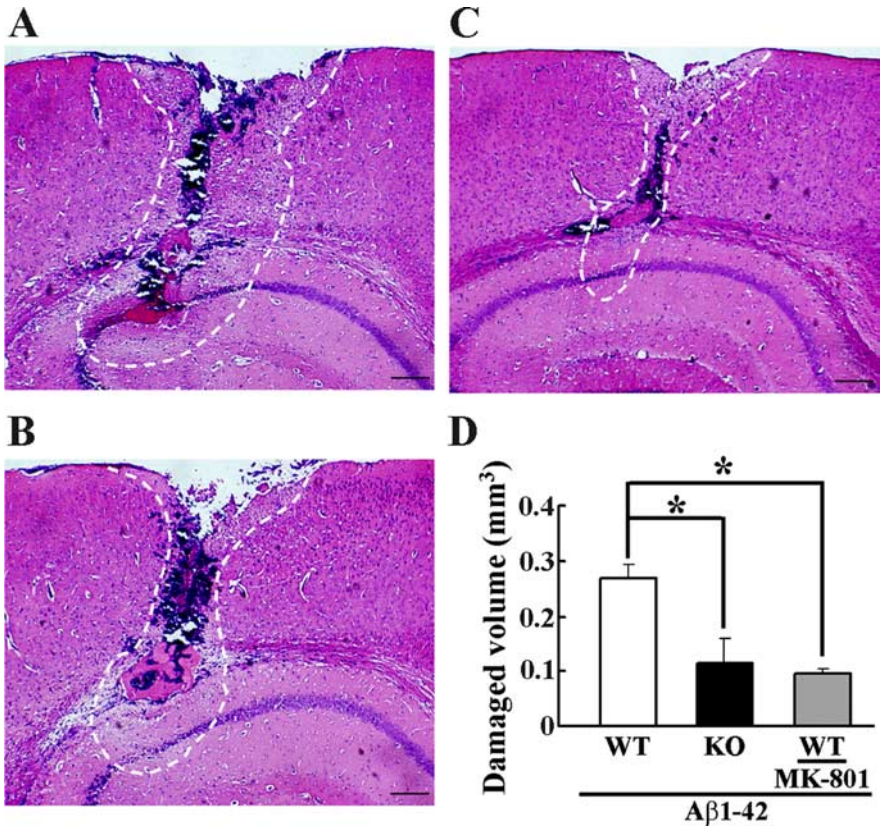

Figure 4. $A\beta_{1-42}$-induced neurotoxicity is inhibited in SR KO mice. ***A***, ***B***, HE-stained brain sections of WT (***A***) and SR KO (***B***) mice 48 h after $A\beta_{1-42}$ injection. ***C***, HE-stained brain section of WT mice intraperitoneally administered with MK-801 2 h before $A\beta_{1-42}$ injection. White dotted lines in ***A–C*** delineate the regions of neurodegeneration. Scale bars, 200 μm. ***D***, Lesion volume in WT (open bar, $n = 3$) and SR KO (KO, closed bar, $n = 3$) mice after $A\beta_{1-42}$ injection, and in WT mice intraperitoneally administered with MK-801 before $A\beta_{1-42}$ injection (gray bar, $n = 4$). The data represent mean ± SEM; *$p < 0.05$; two-tailed Student's *t* test.

1.552 ± 0.026, and 8.292 ± 0.282 nmol/mg tissue, respectively; $n = 6$) were equivalent to those in WT mice (0.804 ± 0.011, 1.473 ± 0.045, and 7.815 ± 0.296 nmol/mg tissue, respectively; $n = 6$) (Fig. 1*B–D*). In the hippocampus, the levels of these amino acids were also comparable between SR KO and WT mice (Fig. 1*B–D*). These results indicate that D-serine production in the mouse forebrain is predominantly catalyzed by SR.

NMDA-induced neurotoxicity is attenuated in SR KO mice

D-Serine is an important coagonist of NMDA receptors. Whether the decrease in the level of endogenous D-serine affects the expression of NMDA receptor subunits, we investigated the expression level of NMDA receptor subunits in the WT and SR KO mouse brains. Western blot analysis demonstrated that the targeted mutation of the SR gene resulted in the complete absence of the SR protein in the forebrain homogenate of SR KO mice. However, the expression levels of GluRε1, GluRε2, and GluRζ1 subunits protein were comparable between WT and SR KO mice (Fig. 2).

To examine the role of endogenous D-serine in NMDA receptor activation, we compared the extent of NMDA-induced lesions in WT and SR KO mice. The degenerated neurons were identified with shrunken and pyknotic nuclei in HE-stained sections (Fig. 3*A*,*B*). Neuronal death was further confirmed with Nissl staining conducted on the adjacent sections (data not shown). Excitotoxic lesions produced by direct microinjection of 50 nmol of NMDA into the cerebral cortex in SR KO mice (3.59 ± 0.40 mm^3, $n = 4$) were significantly smaller ($p < 0.05$) than those produced in WT mice (6.63 ± 0.43 mm^3, $n = 3$) (Fig. 3*A*,*B*).

Because the levels of other amino acids, such as glycine, L-serine, and glutamate, were not altered in mutant mice, this difference is possibly caused by the decreased D-serine levels in SR KO mice. We further injected 50 nmol of NMDA plus 50 nmol of D-serine into the cerebral cortex of WT and SR KO mice and found no significant difference in injury volume between SR KO (4.55 ± 0.72 mm^3, $n = 5$) and WT mice (4.95 ± 0.54 mm^3, $n = 6$) (Fig. 3*C*,*D*). These results indicate that endogenous D-serine is the predominant and necessary coagonist for NMDA receptor activation in the forebrain and also in NMDA-induced neurotoxicity *in vivo*.

$A\beta_{1-42}$-induced neuronal damage is inhibited in SR KO mice

NMDA-mediated excitotoxicity is a possible mechanism hypothesized to underlie the pathophysiology of AD (Suh and Checler, 2002). Because D-serine deficiency attenuates NMDA-induced excitotoxicity in SR KO mice, we examined $A\beta_{1-42}$-induced neuronal injury by directly injecting $A\beta_{1-42}$ into the hippocampus of WT and SR KO mice. The volume of $A\beta_{1-42}$-induced neuronal degeneration was significantly smaller in SR KO mice (0.115 ± 0.044 mm^3, $n = 3$) than in WT mice (0.270 ± 0.024 mm^3, $n = 3$) (Fig. 4*A*,*B*,*D*). The control peptide $A\beta_{42-1}$ failed to induce neuronal damage in WT mice (data not shown). Intraperitoneal administration of MK-801, an NMDA receptor antagonist, 2 h before $A\beta_{1-42}$ injection significantly attenuated $A\beta_{1-42}$-induced neurotoxicity in WT mice (0.095 ± 0.008 mm^3, $n = 4$) (Fig. 4*C*,*D*), supporting the view that NMDA receptor plays a central role in $A\beta_{1-42}$-induced toxicity. These findings further indicate that D-serine may control the extent of NMDA receptor-mediated neurotoxicity *in vivo* and may be involved in neuronal death in AD.

Discussion

In this study, we demonstrated that SR is the major enzyme responsible for D-serine production in the forebrain, by showing an ~90% decrease in the content of D-serine in the cerebral cortex and hippocampus of SR KO mice. In addition, we found that NMDA- and $A\beta_{1-42}$-induced neurotoxicity is significantly attenuated in SR KO mice, strongly suggesting that D-serine is the endogenous coagonist of the NMDA receptor in the forebrain and that it controls the extent of NMDA receptor-mediated neurotoxic insults.

In a pioneering study by Hashimoto et al. (1992), the authors reported the presence of significant amounts of D-serine in the rat brain. Although the involvement of the glycine cleavage system (GCS) (Iwama et al., 1997) or the hydrolysis of phosphoserine by phosphoserine phosphatase (Wood et al., 1996) in D-serine formation was suggested, the origin of D-serine in mammalian brain was largely unclear until the discovery of SR (Wolosker et al., 1999a,b). SR is a bifunctional enzyme that catalyzes the formation of D-serine and pyruvate (De Miranda et al., 2002). SR activ-

ity is increased by Mg^{2+}, ATP, and Ca^{2+}, and by protein-protein interactions with the GluR-interacting protein (GRIP), protein interacting with C kinase 1 (PICK1), and Golgin subfamily A member 3 (Golga 3) (Baumgart and Rodríguez-Crespo, 2008). In contrast, SR activity is decreased by amino acids, such as glycine at a physiological level (Dunlop and Neidle, 2005), and by modification with *S*-nitrosylation (Mustafa et al., 2007). Thus, it is important to evaluate the contribution of SR to D-serine synthesis in the brain. In the present study, we verified that SR contributes to ~90% of D-serine biosynthesis in the mouse forebrain. The synthesis of the remaining 10% of D-serine might be regulated by GCS, phosphoserine phosphatase, or an as yet unknown pathway.

D-Serine and glycine are coagonists for the glycine-binding site of the NMDA receptor (Kleckner and Dingledine, 1988). An *in vivo* microdialysis study revealed the presence of free D-serine and glycine sufficient for the activation of the NMDA receptor (Hashimoto et al., 1995). Furthermore, recombinant NMDA receptors showed that the ED_{50} values for D-serine are three to four times lower than those for glycine (Matsui et al., 1995). Thus, the relative importance of the two coagonists remains unclear *in vivo*. To evaluate the role of D-serine in NMDA receptor function in the brain, we directly injected NMDA into the cerebral cortex of WT and SR KO mice. We found that the NMDA-induced excitotoxic lesion was significantly smaller in SR KO mice than in WT mice. These results strongly indicate that D-serine is a predominant endogenous coagonist of the NMDA receptor in the forebrain. Although the content of D-serine in the forebrain in SR KO mice decreased to ~10% of the control, the decrease in the volume of neuronal degeneration induced by NMDA injection in SR KO mice is ~50% of the control. Thus, it is possible that glycine works as a coagonist of the NMDA receptor in the forebrain.

Neuronal damage resulting from overactivation of NMDA receptors contributes to various pathological processes in the CNS, including acute disorders such as ischemia (Dirnagl et al., 1999) and slowly progressive neurodegenerative diseases (Lancelot and Beal, 1998). Shleper et al. (2005) proposed that D-serine is a predominant endogenous glycine site agonist for induction of NMDA cytotoxicity in hippocampal slice cultures. In this slice culture system, despite the high glycine levels in the medium, removal of endogenous D-serine completely abolishes NMDA neurotoxicity. However, another study demonstrated that the cytotoxicity of NMDA on rat cerebrocortical slice cultures is potentiated by addition of both glycine and D-serine (Katsuki et al., 2004). We have shown that SR is predominantly localized in forebrain neurons, and that the expression pattern of SR changes under the culture condition (Miya et al., 2008). These cytotoxicity experiments using culture systems suggest a necessity to investigate the role of the two glycine-binding site coagonists, glycine and D-serine, on NMDA-mediated neurotoxicity *in vivo*. In the present study, we found that the NMDA-induced excitotoxic lesion was significantly smaller in SR KO mice than in WT mice. To exclude other factors that may mediate such neuronal damage, we further examined the expression levels of NMDA receptor subunits and the concentrations of other amino acids. There was no compensatory increase in the protein expression levels of GluRε1, GluRε2, and GluRζ1 subunits of the NMDA receptor in SR KO mice. Moreover, HPLC revealed no alteration in the levels of L-serine, glycine, and glutamate in the cerebral cortex and hippocampus of mutant mice. Furthermore, coinjection of NMDA and D-serine induced similar extents of lesions in WT and SR KO mice. Our findings suggest that the attenuated neuronal damage in SR KO mice is attributed to the decrease in the level of D-serine.

Progressive dementia in AD is associated with selective neuronal degeneration and death (Price, 1986). $A\beta_{1-42}$ is a self-associating peptide whose neurotoxic derivatives are considered to play a role in AD (Lambert et al., 1998). Our present study demonstrated that $A\beta_{1-42}$-induced neurotoxicity is attenuated in D-serine-deficient mice and in WT mice pretreated with MK-801 before $A\beta_{1-42}$ injection, strongly suggesting that D-serine is involved in NMDA-receptor-mediated neurotoxicity induced by $A\beta_{1-42}$. In the microglia culture system, Aβ induces SR expression and stimulates the release of excitotoxic levels of D-serine (Wu et al., 2004). In line with these *in vitro* observations, a marked increase in D-serine level was detected in the CSF of AD patients (Fisher et al., 1998). The contribution of microglia-derived D-serine needs to be clarified in future studies. Inhibitors of SR may be useful for neurodegenerative diseases involving the overactivation of the NMDA receptor. Our findings suggest that SR can be the target for further study of the D-serine role in neuronal damage, and that our mouse model is useful for the development of new therapies for neurodegenerative disorders involving NMDA receptors.

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