Brief Communications

Serine Racemase Deletion Protects Against Cerebral Ischemia and Excitotoxicity

Asif K. Mustafa,[1] Abdullah S. Ahmad,[2] Emil Zeynalov,[2] Sadia K. Gazi,[1] Gautam Sikka,[2] Jeffrey T. Ehmsen,[1] Roxanne K. Barrow,[1] Joseph T. Coyle,[5,6] Solomon H. Snyder,[1,3,4] and Sylvain Doré[2,3]

[1]Solomon H. Snyder Department of Neuroscience and Departments of [2]Anesthesiology and Critical Care Medicine, [3]Pharmacology and Molecular Sciences, and [4]Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and [5]Department of Psychiatry and [6]Laboratory for Psychiatric and Molecular Neuroscience, Harvard Medical School, Belmont, Massachusetts 02478

D-Serine, formed from L-serine by serine racemase (SR), is a physiologic coagonist at NMDA receptors. Using mice with targeted deletion of SR, we demonstrate a role for D-serine in NMDA receptor-mediated neurotoxicity and stroke. Brain cultures of SR-deleted mice display markedly diminished nitric oxide (NO) formation and neurotoxicity. In intact SR knock-out mice, NO formation and nitrosylation of NO targets are substantially reduced. Infarct volume following middle cerebral artery occlusion is dramatically diminished in several regions of the brains of SR mutant mice despite evidence of increased NMDA receptor number and sensitivity.

Introduction

D-Serine, formed by serine racemase (SR), which converts L-serine to D-serine, is a physiologic coagonist with glutamate at NMDA receptors (Wolosker, 2006). D-Serine is primarily localized to glia that ensheathe neurons, whereas SR occurs both in glia and neurons (Wolosker, 2006). SR is dynamically regulated by glutamate. Thus, activation of metabotropic glutamate receptors leads to cleavage of phosphatidylinositol (4,5)-bisphosphate (PIP2) by phospholipase C (PLC), thereby diminishing inhibition by PIP2 of SR (Mustafa et al., 2009). Glutamate receptor interacting protein (GRIP), normally bound to AMPA receptors, dissociates upon neuronal depolarization to bind to and activate SR (Kim et al., 2005). Glutamate/D-serine activation of NMDA receptors leads to formation of nitric oxide (NO), which nitrosylates and inactivates SR, providing a feedback homeostatic regulation (Mustafa et al., 2007). D-Serine is degraded by D-amino acid oxidase, which, along with its associated protein G72, has been linked to schizophrenia (Coyle, 2006).

To elucidate physiologic roles of D-serine, we created SR-deleted mice (Basu et al., 2009). The mutant mice display an 85% reduction in D-serine levels with alterations in NMDA neurotransmission and decreased long term potentiation (LTP). However, both NMDA transmission and LTP are enhanced by exogenous application of D-serine in the mutant mice, suggesting receptor supersensitivity. Male, but not female, SR knock-outs exhibit spatial memory deficits (Basu et al., 2009). The reasons for this gender discrepancy are not entirely clear. In the present study, we have examined pathophysiologic consequences of SR deletion. We show marked diminution in neuronal death following oxygen–glucose deprivation of $SR^{-/-}$ brain cultures and substantially less brain damage following middle cerebral artery occlusion. These changes are accompanied by pronounced declines in NO formation and nitrosylation of its targets. $SR^{-/-}$ mice also display NMDA receptor supersensitivity, as evidenced by increased NR1 receptor protein levels and enhanced NMDA-elicited brain damage.

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Correspondence should be addressed to Solomon H. Snyder or Sylvain Doré, Johns Hopkins University, 725 North Wolfe Street, Baltimore, MD 21205, E-mail: ssnyder@jhmi.edu or sdore@jhmi.edu.

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Materials and Methods

Animals. Biochemical experiments involving animals were performed on brains removed from 8–10-week-old male wild-type, $SR^{-/-}$ (Basu et al., 2009), and neuronal NO synthase $(nNOS)^{-/-}$ animals. Animals were maintained on a 12 h light/dark cycle at a room temperature of 23°C, with *ad libitum* access to food and water. All animal-use procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Johns Hopkins University Animal Care and Use Committee.

Antibodies. Antibodies to SR and nNOS were from BD Biosciences, antibodies to NMDA receptor NR1, β-tubulin, and actin were from Millipore, and antibodies to GAPDH were from Calbiochem.

Oxygen–glucose deprivation. The oxygen–glucose deprivation (OGD) experiments were done as described previously (Eliasson et al., 1997). Briefly, cortical cultures grown for 12–14 d in Neurobasal media supplemented with horse serum at 37°C were treated with 10 mM L-serine for 5 h and then exposed to a gas mixture of 5% CO_2/95% N_2 in an airtight chamber with OGD buffer containing (in mM) 125 NaCl, 3 KCl, 1.6 $CaCl_2$, 0.2 arginine, 25 HEPES, pH 7.4, 1 D-glucose, and 1.25 Na_2HPO_4 for 1 h at 37°C. The OGD solution was then replaced with regular neuronal culture media and the cells grown for 24 h at 37°C. Toxicity was assayed by microscopic examination with computer-assisted cell counting following staining of all nuclei with 1 μg/ml Hoechst 33258 stain and dead cells with 7 μM propidium iodide. Total and dead cells were counted, and the percentage of cell death was determined. Experimenters were blinded.

Middle cerebral artery occlusion. All mice were 8–10-week-old male $SR^{-/-}$ and matched wild-type littermates weighing between 20 and 25 g. Transient focal ischemia was induced by a 90 min occlusion of the middle

cerebral artery (MCA) in wild-type and SR$^{-/-}$ mice, as described previously (Zeynalov and Doré, 2009). Successful occlusion was confirmed by an 87–90% reduction in cerebral blood flow (CBF), as measured by Laser-Doppler flowmetry. After reperfusion was begun and isoflurane anesthesia was discontinued, the animals were kept in a humidified thermoregulated chamber until they became completely awake (usually within 20–30 min). At 24 h of reperfusion, the mice were deeply anesthetized and the brains processed for analysis of infarct volume. Brains were harvested, sliced into 2-mm-thick sections and stained with 1% 2,3,5-triphenyltetrazolium chloride. Infarct volume was calculated as a percentage of the contralateral hemisphere and corrected for swelling.

NMDA excitotoxicity assays. Weight and rectal temperature of each mouse was recorded before the surgical procedure. Anesthesia was induced with 3.0% halothane and thereafter maintained at 1.0% halothane. Each mouse was mounted on a stereotaxic frame, and 0.3 μl of NMDA (67 mM), prepared in PBS, was injected into the right striatum over a 2 min period; the needle was left *in situ* for an additional 5 min to prevent back flow. After injections, mice were placed in a humidified, thermoregulated chamber maintained at 31°C and then returned to their cages after full recovery from anesthesia. Throughout the experimental procedure, mouse rectal temperature was monitored and maintained at 37.0 ± 0.5°C. Forty-eight hours after injection, brains were harvested and immediately frozen in 2-methylbutane (precooled over dry ice); 20 μm sections were cut on a cryostat and stained with cresyl violet to measure lesion volume. Brain sections were photographed and analyzed with SigmaScan Pro 5.0 (Systat Software).

NO generation. NO formation was assessed in cortical neurons cultured for 12–14 d at 37°C or from 6–8-week-old mouse brains. Cultures, treated with 10 mM L-serine for 5 h, were incubated for 5 min with 2 μM 4-amino-5-methylamino-2′7′-difluorofluorescein diacetate (DAF-FM DA; Invitrogen), a specific dye that emits fluorescence intracellularly only upon interaction with NO. The cells were then gently washed with fresh media and subjected to immunofluorescence microscopy with excitation wavelength at 495 nm and emission wavelength at 515 nm for 15 min with continuous signal recording. For measurements from mouse tissue, brains were sliced into 300 μm sections using a McIlwain Tissue Chopper and equilibrated with 95% oxygen/5% CO_2 at 37°C for 30 min in pre-oxygenated artificial CSF (ACSF) buffer containing (in mM) 125 NaCl, 3 KCl, 1.6 $CaCl_2$, 0.2 arginine, 25 HEPES, pH 7.4, 11 D-glucose, and 1.25 Na_2HPO_4. The slices were then incubated with 0.2 mM DAF-FM DA at 37°C for 1 h, following which they were mechanically lysed and centrifuged at 14,000 rpm for 10 min, and the protein concentration was measured with the BioRad protein assay solution. Lysate (0.25 mg of protein), reconstituted in 1 ml of 20 mM ACSF buffer at pH 7.4, was then subjected to fluorescence measurements to detect NO generation as above.

S-nitrosylation biotin switch assay. The assay was carried-out as described previously (Jaffrey and Snyder, 2001) but with minor modifications. Briefly, brain tissue from wild-type, SR$^{-/-}$, and nNOS$^{-/-}$ mice was homogenized in HEN buffer (250 mM HEPES-NaOH, pH 7.7, 1 mM EDTA, 0.1 mM Neocuproine) supplemented with 100 μM deferoxamine (DFO) and centrifuged at 13,000 × *g* for 20 min at 4°C. Lysate (0.24 mg of protein) was added to blocking buffer [HEN buffer plus 25% SDS and 20 mM methymethanethiosulfonate (MMTS)] at 50°C for 20 min with frequent vortexing. The MMTS was then removed by acetone and the proteins precipitated at −20°C for 20 min. After acetone removal, the proteins were resuspended in HENS buffer (HEN + 1% SDS). To the suspension was added 1 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide (biotin-HPDP) in DMSO with 1 mM ascorbic acid. After incubation for 2 h at 25°C, biotinylated proteins were precipitated by streptavidin-agarose beads that were then washed with HENS buffer. The biotinylated proteins were eluted by SDS-PAGE sample buffer and subjected to Western blot analysis. For quantitation of protein *S*-nitrosylation, the signals were densitometrically analyzed using the software EagleSight 3.2 (Stratagene) and Odyssey 2.1 (Li-Cor).

Cell culture. Cortical neuronal cultures were obtained as described previously (Kartvelishvily et al., 2006).

L-Serine measurements. L-Serine from wild-type and SR$^{-/-}$ cortical neuronal cultures, treated with 10 mM L-serine for 5 h, was measured as described earlier (Kartvelishvily et al., 2006).

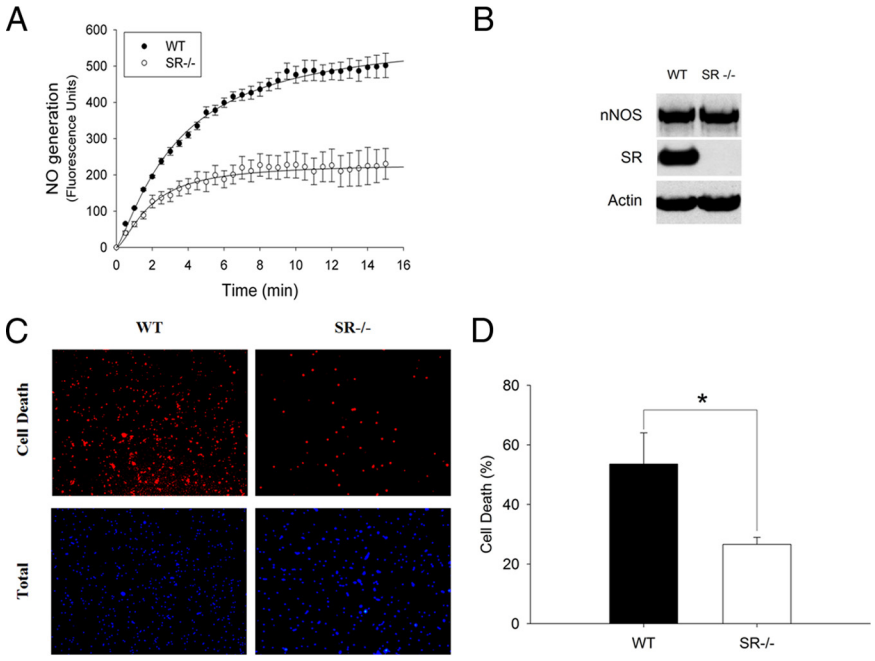

Figure 1. Neurotoxicity is markedly reduced in SR-deleted cerebral cortical cultures. ***A***, NO generation, measured using the NO-specific dye DAF-FM DA, is markedly diminished in SR$^{-/-}$ cultures. ***B***, nNOS protein expression is similar in wild-type (WT) and SR$^{-/-}$ neurons. ***C***, SR$^{-/-}$ neurons display diminished cell death under OGD as indicated by a lower ratio of propidium iodide (label for dead cells) to Hoechst 33258 stain (nuclear label for all cells). ***D***, Quantitation of cell death from OGD shows a 50% reduction in SR$^{-/-}$ neurons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

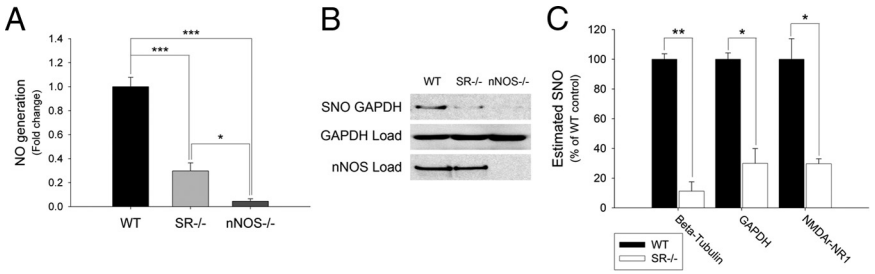

Figure 2. Synthesis of nitric oxide and protein *S*-nitrosylation are decreased in SR$^{-/-}$ brains. ***A***, NO generation in wild-type (WT), SR$^{-/-}$, and nNOS$^{-/-}$ brain slices. NO generation is reduced by ~70% in SR$^{-/-}$ slices compared with wild-type controls. As nNOS is the primary NOS isozyme responsible for NO formation in the brain, nNOS$^{-/-}$ mice display almost complete absence of NO generation. ***B***, *S*-nitrosylation (SNO) of GAPDH is markedly reduced in SR$^{-/-}$ brain and absent in nNOS$^{-/-}$ preparations. nNOS is expressed equally in wild-type and SR$^{-/-}$ brain tissues. ***C***, Relative densitometric quantitation of protein *S*-nitrosylation in wild-type versus SR$^{-/-}$ brains reveals a pronounced reduction (70–90%) in *S*-nitrosylation of GAPDH, β-tubulin, and the NR1 subunit of the NMDA receptor in SR-deleted animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

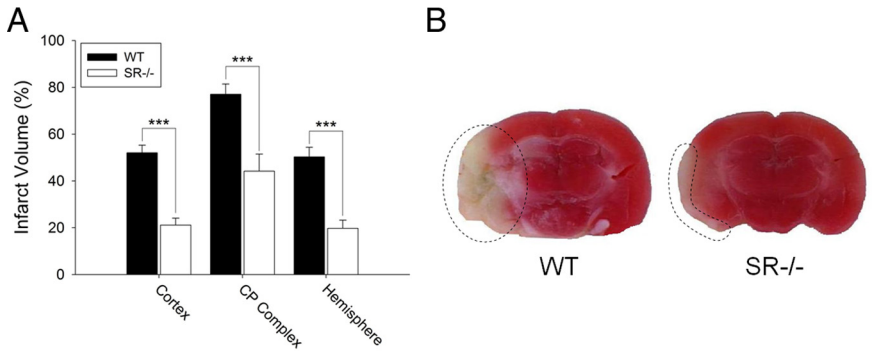

Figure 3. SR deletion protects against focal cerebral ischemia. ***A***, Infarct volume following MCAO is reduced by 61% in the cerebral hemispheres, 60% in the cerebral cortex, and 43% in the caudate–putamen (CP) region of the SR$^{-/-}$ mice compared with wild-type (WT) littermate controls ($n = 9$ animals each). ***B***, Representative images of a wild-type and SR$^{-/-}$ brain 24 h post-MCAO. SR$^{-/-}$ mice display marked reduction in infarct area (contours delineated by dotted lines) compared with wild-type littermates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

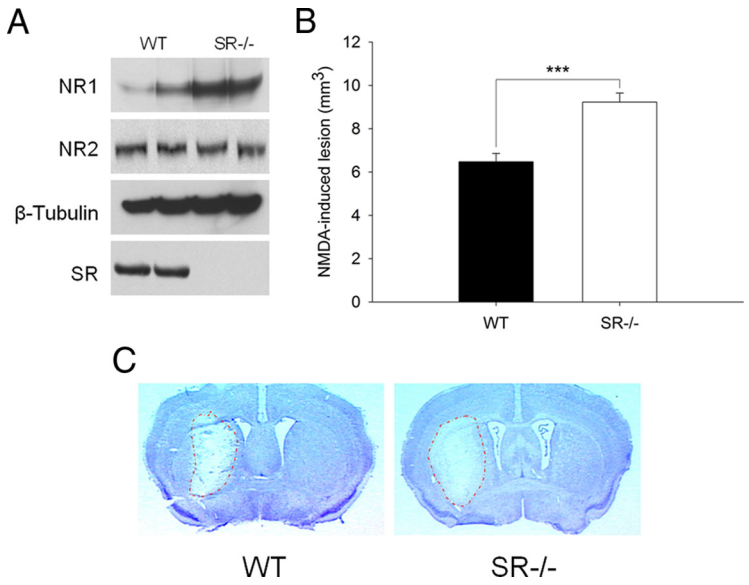

Figure 4. SR$^{-/-}$ mice display NMDA receptor supersensitivity. ***A***, NMDA receptor NR1, but not NR2, subunit expression is increased in SR$^{-/-}$ brains. ***B***, NMDA-elicited lesion volume is increased by nearly 50% in the striatum of SR$^{-/-}$ animals compared with wild-type (WT) littermate controls ($n = 15$ animals each). ***C***, Representative images of a wild-type and SR$^{-/-}$ brain post-NMDA injection into the corpus striatum. SR$^{-/-}$ mice display increased lesion area (contours delineated by dotted lines) compared with wild-type littermates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Statistical analysis. All data are expressed as means ± SEM of three independent experiments, each performed in triplicate unless otherwise indicated. Data were analyzed by unpaired Student's *t* test.

Results

Diminished neurotoxicity in SR$^{-/-}$ cerebral cortical cultures

In SR$^{-/-}$ cerebral cortical cultures, D-serine levels are reduced ~90%, resembling findings in intact mouse brain (Basu et al., 2009). NO generation by the SR$^{-/-}$ cultures is diminished by ~50% despite no change in nNOS protein levels (Fig. 1*A*,*B*). Oxygen–glucose deprivation neurotoxicity is ~50% lower in SR$^{-/-}$ cultures as monitored by propidium staining (Fig. 1*C*,*D*).

NO generation and protein *S*-nitrosylation are reduced in SR$^{-/-}$ brains

We examined the disposition of NO signaling and neurotoxicity in intact animals. NO generation is 70% lower in whole brain of SR$^{-/-}$ animals compared with a 95% reduction in nNOS$^{-/-}$ animals (Fig. 2*A*). NO signals in major part by *S*-nitrosylating a variety of protein targets (Hess et al., 2005). The diminished NO levels are accompanied by reductions in nitrosylation of ~90%, 70%, and 75% of the NO targets β-tubulin, GAPDH, and the NR1 subunit of NMDA receptors, respectively (Fig. 2*B*,*C*), extending our preliminary observations of reduced β-tubulin and GAPDH nitrosylation in SR$^{-/-}$ mice (Basu et al., 2009). This profound reduction in nitrosylation emphasizes the intimate linkage of D-serine-associated NMDA neurotransmission with NO signaling.

SR deletion protects against focal cerebral ischemia

We monitored stroke by assessing infarct volume following transient MCA occlusion (MCAO) (Fig. 3*A*,*B*). In SR$^{-/-}$ animals, infarct volume is reduced by 50–60% in cerebral cortex, caudate–putamen, and cerebral hemisphere. The lesser neurotoxicity of brain cultures and diminished MCAO damage in SR mutants fits with diminished NMDA neurotransmission.

SR$^{-/-}$ mice display NMDA receptor supersensitivity

Neurotransmitter deficiency is often associated with receptor supersensitivity that may be manifested in increased levels of receptor protein, as exemplified for dopamine receptors (Kostrzewa et al., 2008). Because of the extremely high levels of endogenous glutamate derived from multiple sources, relationships of glutamate deficit to receptor sensitivity have not been readily studied. Nonetheless, a study by Nong et al. (2003) demonstrate that the concurrent binding of D-serine (or glycine) and glutamate prime NMDA receptor internalization. It is therefore conceivable that in the absence of D-serine, surface expression of NMDA receptors will likely be increased, leading to receptor supersensitivity. In our earlier study of SR deleted mice, neurophysiologic experiments revealed supersensitivity of NMDA neurotransmission and LTP to D-serine stimulation (Basu et al., 2009). In SR$^{-/-}$ corpus striatum, we observe a fourfold increase in levels of NR1 NMDA receptor protein with no change in NR2 protein (Fig. 4*A*). To assess the functional consequences of increased receptor number, we injected NMDA directly into the striatum of SR$^{-/-}$ animals and detected a 50% augmentation in NMDA-elicited lesion volume (Fig. 4*B*,*C*).

Discussion

In summary, our study of SR-deleted mice reveals a major regulatory influence of SR-generated D-serine upon NO disposition and neurotoxicity. The pronounced decline of NO formation and nitrosylation of its targets in the mutant mice indicates a greater dependence of NO disposition upon D-serine-associated NMDA neurotransmission than has been previously appreciated. Abundant evidence has implicated overproduction of NO in neurotoxicity,

although under some circumstances NO may be neuroprotective (Hara and Snyder, 2007).

The marked reduction in infarct volume of $SR^{-/-}$ animals following MCAO is comparable to protection against stroke damage associated with pharmacologic blockade of NMDA receptors (Lipton, 2006). NMDA receptor antagonists, however, elicit adverse effects that have precluded clinical application in stroke therapy (Vallance and Leiper, 2002; Lipton, 2006). Conceivably, selective inhibition of D-serine formation by SR inhibitors would diminish acute stroke damage with fewer undesirable effects. Thus, SR deleted mice appear generally healthy with minimal neurocognitive abnormalities (Basu et al., 2009), whereas complete genetic deletion of NMDA receptors is lethal (Mohn et al., 1999).

Because of the difficulties in manipulating glutamate levels, their influences upon NMDA receptor supersensitivity have not been examined in depth. Our findings of a 400% increase in numbers of NR1 subunits in $SR^{-/-}$ mice associated with increased NMDA-elicited brain damage provides evidence that receptor occupancy by D-serine is an important determinant of receptor sensitivity. Recently, Inoue et al. (2008) reported no alterations in NR1 levels in the cortex of $SR^{-/-}$ animals and 40% decrease in cortical damage following NMDA injections. Reasons for the discrepancies between these findings and ours may reflect a variety of factors, including their use of the cerebral cortex 24 h following NMDA injection and our use of the striatum 48 h following NMDA injection as well as differences in NMDA dose.

It is striking that $SR^{-/-}$ mice display less stroke damage despite increased NMDA receptor sensitivity. Presumably with MCAO, levels of D-serine are rate limiting so that the increased glutamate release associated with MCAO is less capable of overstimulating receptors. Direct injections of NMDA may expose receptors to overwhelming stimulation. Moreover, needle damage may lead to substantial release of endogenous glycine, which compensates for the loss of D-serine.

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