Serine racemase: A glial enzyme synthesizing D-serine to regulate glutamate-*N*-methyl-D-aspartate neurotransmission

Herman Wolosker*, Seth Blackshaw†, and Solomon H. Snyder‡

Departments of Neuroscience, Pharmacology and Molecular Sciences, and Psychiatry, The Johns Hopkins University, School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205

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Although D amino acids are prominent in bacteria, they generally are thought not to occur in mammals. Recently, high levels of D-serine have been found in mammalian brain where it activates glutamate/*N*-methyl-D-aspartate receptors by interacting with the "glycine site" of the receptor. Because amino acid racemases are thought to be restricted to bacteria and insects, the origin of D-serine in mammals has been puzzling. We now report cloning and expression of serine racemase, an enzyme catalyzing the formation of D-serine from L-serine. Serine racemase is a protein representing an additional family of pyridoxal-5′ phosphate-dependent enzymes in eukaryotes. The enzyme is enriched in rat brain where it occurs in glial cells that possess high levels of D-serine *in vivo*. Occurrence of serine racemase in the brain demonstrates the conservation of D-amino acid metabolism in mammals with implications for the regulation of *N*-methyl-D-aspartate neurotransmission through glia-neuronal interactions.

Recently, free D-serine and D-aspartate have been reported in mammals, especially in the nervous system (1, 2). Using highly selective antibodies we localized D-serine to protoplasmic astrocytes in the gray matter areas enriched in *N*-methyl-D-aspartate (NMDA) receptors for the neurotransmitter glutamate (3, 4). We also showed that glutamate, acting through non-NMDA receptors, releases D-serine from astrocyte cultures (3). NMDA receptors require coactivation at a "glycine site" (5) at which D-serine is up to three times more potent than glycine (6–8), suggesting that D-serine is an endogenous ligand for this site, and is released by glutamate from astrocytic processes that ensheath the synapse. Extracellular levels of endogenous D-serine are comparable to glycine in prefrontal cortex, whereas in the striatum, extracellular D-serine values are more than twice the values for glycine as measured by *in vivo* microdialysis (9). Direct evidence that D-serine normally mediates NMDA transmission comes from experiments showing that destruction of endogenous D-serine selectively by application of D-amino acid oxidase greatly reduces NMDA receptor activation in slices and cell culture preparations (J.P. Mothet, A.T. Parent, H.W., R.O. Brady, Jr., D.J. Linden, C.D. Ferris, M.A. Rogawski, and S.H.S., unpublished observations). Characterization of the physiologic role of D-serine requires identifying its biosynthetic mechanism. Using partial amino acid sequence from our purified preparation of an enzyme from rat brain converting L-serine to D-serine (10), we now report cloning and expression of serine racemase and its localization to astroglia.

Materials and Methods

Cloning. Full-length serine racemase was cloned by reverse transcription–PCR from mouse brain mRNA using primers based on mouse expressed sequence tags (ESTs) 615391 and 464586 (GenBank accession numbers AA170919 and AA032965, respectively), which corresponded to the 5′ and 3′ ends of the gene: forward primer, 5′-ATG TGT GCT CAG TAC TGC ATC TCC-3′; reverse primer, 5′-TTA AAC AGA AAC CGT CTG GTA AGG-3′. Several other mouse ESTs also covered parts of the sequence of serine racemase (GenBank accession numbers AI322578, AI173393, AA833469, and AA197364). The ORF and stop codon were confirmed by an independent 3′ rapid amplification of cDNA ends reaction using a primer against 5′ untranslated region (5′-AAA CAC AGG AGC TGT CAG C-3′). The mouse serine racemase sequence was deposited in GenBank (accession number AF148321).

Cell Culture and Transfection. HEK293 cells were cultured in DMEM/penicillin-streptomycin/10% FBS media. Cells were transfected with serine racemase constructs subcloned in pRK5-KS vector with a cytomegalovirus promoter (provided by A. Lanahan and P. Worley, Johns Hopkins University) by using the calcium chloride method. Serine racemase mutant K56G was constructed by PCR. Equal expression of wild-type and mutant enzyme was confirmed by Western blot analysis of transfected cells.

D-Serine Synthesis. In studies of D-serine synthesis, cells were cultured in media supplemented with increasing concentrations of L-serine. Unsupplemented media contained 0.4 mM L-serine. L-serine used in the experiments was rendered free of contaminating D-serine as described (10). D-serine in cells was measured in 25-mm culture dishes 36 hr after transfection. The cells were washed twice with cold PBS, followed by addition of 5% trichloroacetic acid (TCA) to extract free amino acids. After removing TCA with diethyl-ether, D-serine content was analyzed by both HPLC and a luminescence method as described (10). For measurement of D-serine in culture media, an aliquot was deproteinized by adding TCA (5% final concentration) and processed as described above for D-serine in cells. Blanks were performed by analyzing medium alone. For measurement of D-serine synthesis in cell homogenates, transfected cells were disrupted by sonication in 10 mM Tris·HCl (pH 8.0), 10 μM pyridoxal 5′ phosphate, and 0.2 mM PMSF. Reactions were started by adding 10 mM L-serine to cell protein extract. After 2 hr at 37°C, the reaction was stopped by addition of TCA 5%. Blanks were performed with heat-inactivated cell extract.

Immunocytochemistry. HEK293 cell cultures were fixed 36 hr after transfection with 4% glutaraldehyde in 0.1 M sodium phosphate

Abbreviations: NMDA, *N*-methyl-D-aspartate; EST, expressed sequence tag; GFAP, glial fibrillary acidic protein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF148321).

*Present address: Departamento de Bioquimica, ICB/CCS, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941–590, Brazil.

†Present address: Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115.

‡To whom reprint requests should be addressed. E-mail: ssnyder@jhmi.edu.

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buffer (pH 7.4) and 0.1% sodium metabisulfite for 40 min. After reduction of free aldehyde groups by 30-min incubation in 0.5% sodium borohydride, cells were incubated with a purified anti-D-serine antibody raised against a reduced glutaraldehyde conjugate of D-serine (3). The antibody detects as few as 10 pmol of D-serine and is 100-fold less sensitive to L-serine. To avoid any cross reactivity with L-serine present in the cells, incubations were performed in the presence of 0.5 mM L-serine-glutaraldehyde conjugate. Preabsorption of the antibody with 0.5 mM D-serine-glutaraldehyde conjugate abolished immunoreactivity. Staining was developed with an immunoperoxidase ABC Elite kit (Vector Laboratories) by using 3,3′-diaminobenzidine as substrate.

Primary Cultures. Mixed neuronal-glial cell cultures were prepared as described (27). Cortical embryonic neuronal cultures (E18) virtually free of glia were prepared in a serum-free media supplemented with B27 (28). Primary astrocyte cultures were prepared from cerebral cortex as described (29). Astrocytes type 1 are polygonal flat cells that adhere strongly to the tissue culture flasks. Type 2 astrocytes are nonadherent process-bearing cells that can be detached by shaking the cultures, leaving the adherent cell population enriched in type 1 astroglia. Although both types of astrocytes strongly express astrocytic markers, the *in vivo* counterparts of type 1 and type 2 astroglia are still controversial, as astrocytes type 2 were not found in the cerebral cortex *in vivo* (30). Control experiments were conducted to assure that the differences observed in serine racemase expression were not caused by differences in components of media used in each culture. Accordingly, we cultured neurons with serum containing media after diminishing glia proliferation with 5-fluoro-2′-deoxyuridine or cultured astrocytes in DMEM/10% FBS/penicillin-streptomycin media. Changes in the media did not alter the expression pattern of serine racemase.

Antibody Production. Polyclonal antibody against his-tagged and bacterially expressed serine racemase was raised in rabbits and affinity-purified as described (31).

Immunohistochemistry. Sprague–Dawley rats were used at 13 days of age. For serine racemase staining, saggital sections of 10 μm were cut on a cryostat and fixed by immersion in methanol for 5 min at room temperature. With our antibody, serine racemase immunoreactivity was very low or absent when aldehyde fixatives were used. Partial trypsinization of the tissue fixed with 4% paraformaldehyde restored some of the antigenicity, suggesting that aldehyde fixation blocked access of the antibody to the antigenic epitopes. Purified antibody was used at 0.5 to 1 mg/ml. For D-serine staining, rats were perfused with 5% glutaraldehyde and 0.5% paraformaldehyde and processed as described (3). For double-labeling experiments, brain sections fixed in methanol were incubated with affinity-purified antiserine racemase (0.2 mg/ml) and monoclonal anti-glial fibrillary acidic protein (GFAP) antibodies (Sigma, at 1:800 dilution) for 48 hr at 4°C. Secondary antibodies consisted of a Cy3-conjugated anti-rabbit and a FITC-conjugated anti-mouse used at 1 mg/ml (Jackson ImmunoResearch). Images were captured on a Noram (Philadelphia) confocal microscope.

Results

We obtained amino acid sequence for two peptides from purified serine racemase. A search of the GenBank database revealed identity to two ESTs from the mouse genome encoding fragments of a gene of unknown function. Using primers based on EST sequences, we cloned the full-length mouse serine racemase by reverse transcription–PCR. To confirm that we had cloned the complete ORF and stop codon, we also performed 3′ rapid amplification of cDNA ends (RACE) using a primer based on

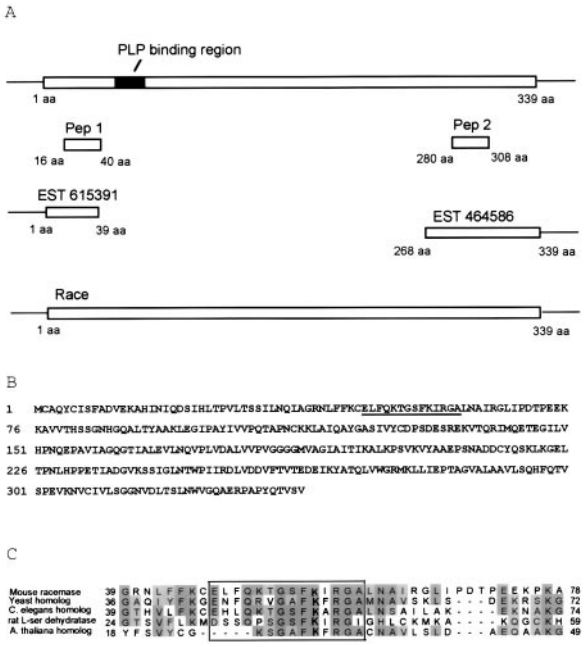


Fig. 1. Cloning and sequence analysis of mouse serine racemase. (*A*) Strategy for serine racemase cloning. Two peptides obtained by internal amino acid sequence of purified enzyme (PEP1, HLNIQDSVHLTPVLTSSILNQIAGR; PEP2, LLIEPTAGVGLAAVLSQHFQTVSPEVK) corresponded to mouse ESTs in GenBank. Full-length serine racemase cloned by reverse transcription–PCR from mouse brain has a pyridoxal-5′ phosphate (PLP) binding region. The ORF and stop codon were confirmed by an independent 3′ rapid amplification of cDNA ends (Race) reaction. (*B*) Amino acid sequence of serine racemase. Underlined sequence corresponds to the amino acid consensus for pyridoxal 5′ phosphate binding (Prosite accession number PS00165). (*C*) Alignment of proteins exhibiting significant homology to serine racemase in the pyridoxal 5′ phosphate binding region (boxed area), which include homologs in yeast, *C. elegans*, *A. thaliana*, and rat L-serine dehydratase (GenBank accession numbers P3600, CAB02298, CAB39935, and DWRTT, respectively). The lysine residue predicted to interact with pyridoxal 5′ phosphate molecule is in bold, and homologous amino acid residues are shadowed.

the 5′ untranslated region of the gene. A single band was observed in the RACE reaction, which contained a full-length sequence of the enzyme (Fig. 1*A*).

Serine racemase comprises 339 aa with a predicted molecular mass of 36.3 kDa (Fig. 1*B*). Close to the N terminus we observed a region that has a consensus sequence for pyridoxal 5′ phosphate. In this region, serine racemase displays homology with enzymes of the serine/threonine dehydratase family, best represented by rat L-serine dehydratase, the closest mammalian sequence, which, like serine racemase, is a pyridoxal phosphate-dependent enzyme (Fig. 1*C*). The overall identity of serine racemase to L-serine dehydratase is only 28%. We observed putative homologs of serine racemase with previously unknown function in yeast *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and the plant *Arabidopsis thaliana*, which display 40%, 37%, and 46% overall amino acid sequence identity, respectively to mouse serine racemase (Fig. 1*C*). The similarity of the sequence between mammals and plants indicates that serine racemase represents an additional family of pyridoxal phosphate enzymes distinct from serine dehydratase, whose mammalian sequence shows less similarity to mammalian serine racemase than the plant gene.

To establish that the cloned protein displays serine racemase activity, we transfected HEK293 cells that do not possess endogenous D-serine. Augmenting D-serine concentration in the medium led to striking increases in D-serine generation in both

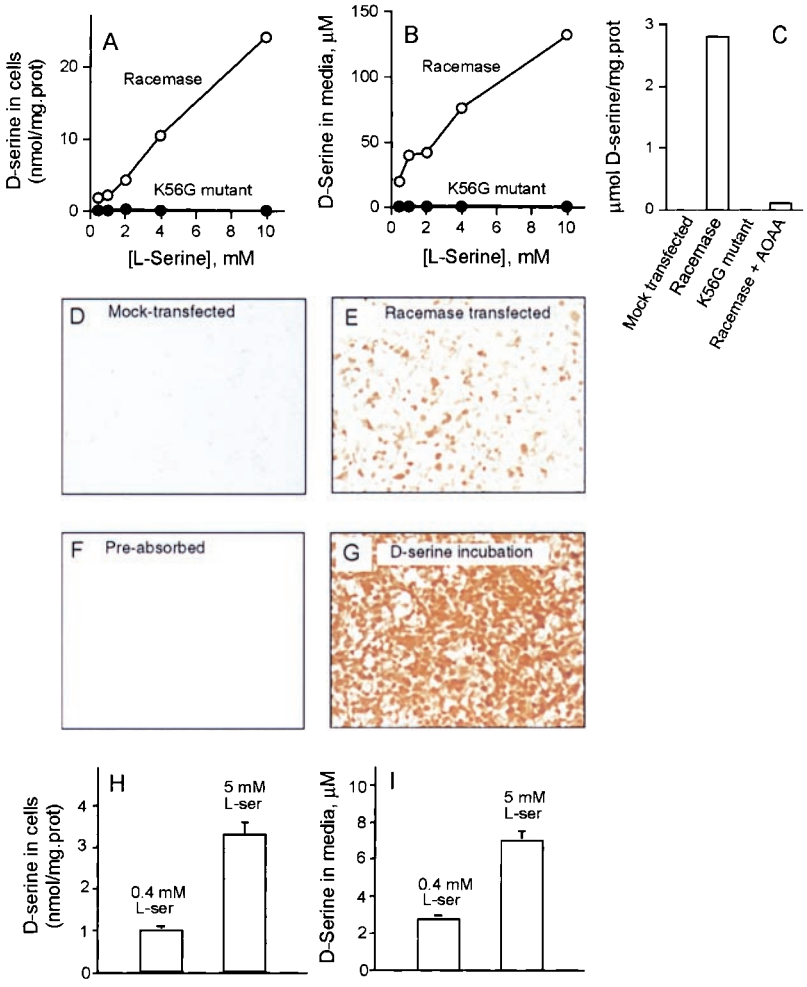


Fig. 2. Serine racemase catalyses the formation of D-serine *in vivo*. (*A*) Analysis of D-serine synthesis in transfected cells. HEK293 cells were transfected either with full-length mouse serine racemase (○) or serine racemase mutant K56G (●). (*B*) Analysis of D-serine synthesis in culture media from transfected cells. (*C*) Synthesis of D-serine in cell homogenates. Mock-transfected cells or K56G mutant exhibited no activity, whereas addition of 0.5 mM amino-oxyacetic acid (AOAA) inhibited most of the activity in serine racemase-transfected cell extracts. (*D–G*) HEK293 cells were transfected with serine racemase and further analyzed for D-serine content by immunocytochemistry. (*D*) Mock-transfected cells incubated in DMEM media supplemented with 10 mM L-serine. (*E*) Serine racemase-transfected cells incubated in media supplemented with 10 mM L-serine. (*F*) Serine racemase-transfected cells incubated in media supplemented with 10 mM L-serine and stained with antibody preabsorbed with 0.5 mM D-serine-glutaraldehyde conjugate. (*G*) Mock-transfected cells first incubated for 12 hr in media containing 5 mM D-serine. Intense immunostaining represents accumulation of D-serine by the cells, which was not significantly metabolized during the course of the experiment. (*H* and *I*) Synthesis of D-serine by mixed neuronal-glia cell culture. A significant increase in D-serine synthesis was observed by supplementing the media with 5 mM L-serine. D-serine produced in cells (*H*) and released to culture media (*I*) was analyzed 48 hr after addition of L-serine. The results are representative of four independent experiments with different culture preparations (*A–G*) and presented as the mean ± SEM of three independent experiments (*H* and *I*).

cells and culture media (Fig. 2 *A* and *B*). Synthesis of D-serine was specific as HPLC analysis failed to reveal formation of any other D- or L-amino acid. To explore the dependence on pyridoxal 5′ phosphate binding, we constructed a mutant in which lysine-56, predicted to bind pyridoxal 5′ phosphate in serine racemase (Fig. 1*C*), was replaced by glycine (K56G mutant). The mutant enzyme did not catalyze the formation of D-serine when transfected into HEK293 cells (Fig. 2 *A* and *B*). We demonstrated enzyme activity in homogenates of the transfected HEK293 cells but not in the K56G mutant (Fig. 2*C*). Amino-oxyacetic acid, a known inhibitor of pyridoxal 5′ phosphate enzymes, abolished enzyme activity (Fig. 2*C*). Cells transfected with serine racemase and fixed with glutaraldehyde stained positively with a stereospecific antibody to D-serine-glutaraldehyde conjugate, whereas mock-transfected cells were unstained (Fig. 2 *D* and *E*). Preabsorption of the antibody with D-serine glutaraldehyde conjugate abolished the staining (Fig. 2*F*). Intense immunoreactivity was observed in cells that accumulated exogenous D-serine added to media several hours before fixation, demonstrating that the antibody readily recognized D-serine (see Fig. 4*G*). Untransfected mixed neuronal-glial primary cultures from rat brain synthesized D-serine with greater synthesis in the presence of higher concentrations of L-serine in culture media, ensuring that the racemizing enzyme activity is responsible for the formation of endogenous D-serine (Fig. 2 *H* and *I*).

Northern blot analysis revealed a single message of 2.6 kb with highest levels for serine racemase RNA in the liver, second highest values in brain, low levels in the kidney, and very faint message in the other tissues (Fig. 3*A*). Western blot analysis revealed a prominent single band in the brain at about 38 kDa (Fig. 3*B*), fitting with the molecular mass of purified serine racemase (10). Serine racemase protein expression was higher in the brain than liver, with very faint or no detectable expression

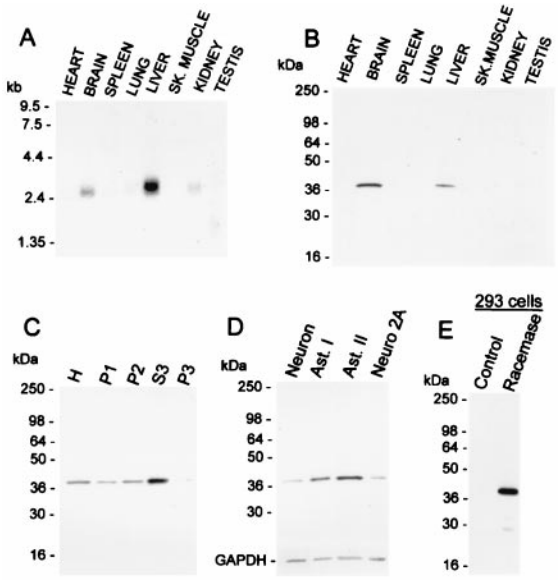


Fig. 3. Distribution of serine racemase. (*A*) Northern blot analysis. A full-length serine racemase probe was used to probe a multiple-tissue rat mRNA membrane (CLONTECH). (*B*) Western blot analysis of serine racemase in rat tissues. Immune serum to serine racemase was used at 1:10,000 dilution. (*C*) Subcellular fractionation of brain extracts showing enrichment of serine racemase in the cytosolic fraction. H, total homogenate; P1, 9,000 × *g* pellet; P2, 30,000 × *g* pellet obtained from P1 supernatant; S3, supernatant of 120,000 × *g* pellet representing soluble cytosolic proteins; P3, 120,000 × *g* pellet. (*D*) Enrichment of serine racemase in glia. Western blot of serine racemase in neuronal culture virtually free of glia, astrocyte type 1 primary culture (Ast. I), astrocyte type 2-enriched culture (Ast. II), and neuroblastoma (Neuro 2A cells). To assure that the same amount of protein was loaded per lane (20 mg homogenate protein), the blot was reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (*E*) Western blot of serine racemase in transfected HEK293 cells. Each lane contains 100 ng of homogenate protein.

in other tissues (Fig. 3*B*). Expression of serine racemase coincides with the high levels of endogenous D-serine in the brain. Rat liver expresses large amounts of D-amino acid oxidase, which completely metabolizes D-serine in most peripheral tissues where D-serine is almost undetectable (11).

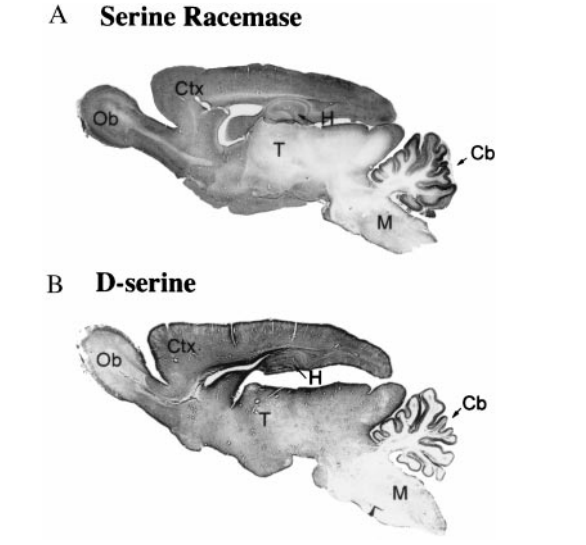
Subcellular fractionation of rat brain revealed the great proportion of serine racemase protein in soluble fractions (Fig. 3*C*). To explore the cellular localization, we prepared neuronal cultures virtually free of glial cells, glial cultures greatly enriched, respectively in type 1 or type 2 astrocytes, and neuroblastoma cell line Neuro-2A. Highest levels of enzyme protein were observed in the astrocyte-enriched cultures with lowest levels in the neuronal cultures and the neuroblastoma cell line (Fig. 3*D*). The molecular mass of serine racemase in the subcellular fractions and the cultures was essentially the same as in HEK293 cells transfected with serine racemase (Fig. 3*E*).

Further evidence that serine racemase accounts for the physiologic biosynthesis of D-serine came from immunohistochemical studies. The distribution of serine racemase was closely similar to that of endogenous D-serine with highest concentrations in the forebrain and negligible levels in the brainstem (Fig. 4 *A* and *B*). Whereas the adult cerebellum possesses very low levels of D-serine(3), the 13-day-old rat brain used in our experiments showed substantial expression of serine racemase and D-serine in the cerebellum. At high magnification we observed both serine racemase and D-serine selectively enriched in astrocytes concentrated in gray matter areas. High densities were observed in astrocytes throughout the cerebral cortex (Fig. 4 *C* and *D*). In the hippocampal formation, serine racemase and D-serine occurred in astrocytes with highest densities in the hilus of the dentate gyrus (Fig. 4 *E* and *F*). The pyramidal cell layers and the granule cell neurons of the dentate gyrus were unstained. In the 13-day-old rat cerebellum, both D-serine and serine racemase were highly enriched in Bergmann glia (Fig. 4 *G* and *H*), where D-serine may play a role in the NMDA transmission that is essential for migration of granule cells in neural development (12). Scattered stellate astrocytes and glial cell processes throughout the granule cell layer also were stained for both serine racemase and D-serine. Staining in cerebellar white matter was much less (data not shown). In the telencephalic white matter, very strong serine racemase and D-serine staining was evident in astrocytes of the corpus callosum (Fig. 4 *I* and *J*), which continuously generates new astrocytes in young rats. Direct colocalization by double labeling of serine racemase and D-serine is not feasible because we found the glutaraldehyde fixation used for D-serine staining incompatible with proper preservation of serine racemase antigenicity. However, like D-serine (3), cells staining for serine racemase also stained for GFAP, a marker for astrocytes in double-labeling experiments (Fig. 5 *A–D*). We did not detect significant neuronal staining above the background for both serine racemase and D-serine, which is consistent with our observation that neurons express very low levels of serine racemase by Western blot analysis of cultures virtually free of GFAP-positive glial cells (Fig. 3*D*). The enrichment of serine racemase in glia indicates that D-serine is formed in the cells that contain endogenous D-serine rather than being synthesized in neurons and transported into glia.

Discussion

Heretofore, demonstrations of D-amino acids in mammalian tissues often had been ascribed to dietary origin or intestinal bacteria (13, 14). Our identification of mammalian serine racemase as the physiologic biosynthetic enzyme for D-serine establishes an endogenous origin. Though several amino acid racemases have been cloned from bacterial sources, no eukaryotic amino acid racemase has been previously cloned. All of the known amino acid racemases have been cloned from archae or eubacteria, including alanine, aspartate, glutamate, serine, and phenylalanine racemases (15–19). None of these display significant amino acid sequence homology to serine racemase. Accordingly, the enzyme we have cloned reflects an additional family of amino acid racemases in eukaryotes. Whether additional mammalian amino acid racemases exist is unclear. The only other D-amino acid consistently observed in significant amounts in mammalian tissues is D-aspartate (20). We localized D-aspartate to selected neuronal populations in the brain and neuroendocrine tissues such as the pineal gland, the posterior pituitary, and the adrenal medulla (21). Biosynthetic mechanisms for D-aspartate have not been elucidated. Failure to detect other D-amino acids in mammalian tissues does not rule out their existence and rapid turnover, which might account for very low or undetectable levels. Our cloning of serine racemase may facilitate the discovery of other mammalian amino acid racemases.

Cloning of serine racemase should greatly enhance characterization of D-serine as a modulator of NMDA neurotransmission. In contrast to glycine, D-serine is selective for NMDA receptors, as it does not activate inhibitory strychnine-sensitive receptors (22). Endogenous levels of D-serine, which are in the micromolar range (9), are adequate to influence the "glycine" site of NMDA receptors for which D-serine is at least as potent as glycine (6–8). Extracellular D-serine concentration is similar to glycine in the forebrain and up to 2.6 times higher than glycine in the striatum (9). D-serine is highly localized to astrocytic foot processes that are in close contacts with neurons in the synaptic cleft (3, 4), whereas the bulk of endogenous glycine generally is distributed in amino acid pools involved in protein synthesis and inhibitory synapses.



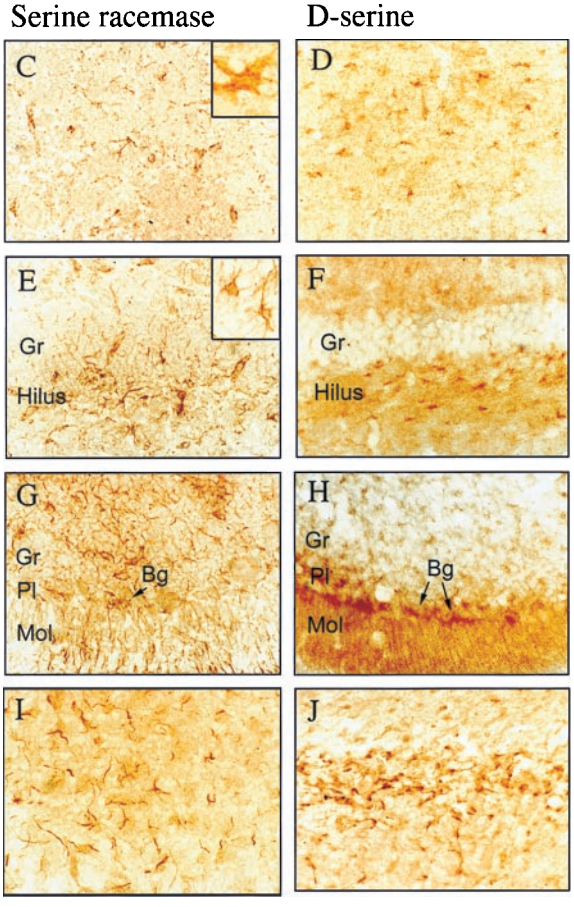


Fig. 4. Colocalization of serine racemase and endogenous D-serine in brain. (*A*) Immunohistochemical staining for serine racemase. (*B*) Immunohistochemical staining for D-serine. In the cerebral cortex (*C* and *D*), both serine racemase and D-serine are evident in glial cells with morphology corresponding to astrocytes. (*C*, *Inset*) A ×1,000 magnification of a glial cell positive for serine racemase in the cerebral cortex. In the hippocampus (*E* and *F*), several glial cells containing serine racemase and D-serine occur in the hilus of the dentate gyrus. Granule cell neurons (Gr) are unstained. (*E*, *Inset*) A ×650 magnification of glial cells positive for serine racemase in the hippocampus. In the cerebellum (*G* and *H*), Bergmann glia cells (Bg) in the Purkinje cell layer (Pl) and scattered stellate astrocytes and glial processes in the granule cell layer (Gr) are positive for both serine racemase and D-serine. Arrows depict Bergmann glia cell bodies that extend processes toward the pia in the molecular layer (Ml) of the cerebellum. In the corpus callosum (*I* and *J*), astrocytes are strongly labeled for both serine racemase and D-serine. Though both serine racemase and D-serine occur in the same types of cells, subtle morphologic differences are noticeable, perhaps reflecting the use of different fixatives and tissue processing for each staining. Except for the insets, magnifications are ×400.

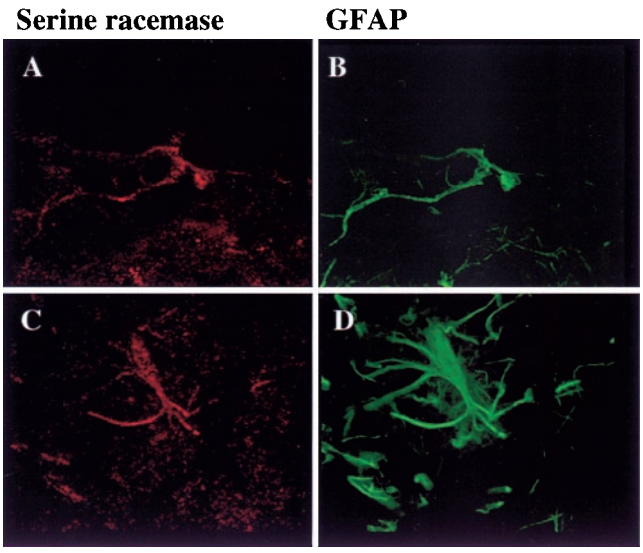


Fig. 5. Colocalization of serine racemase and GFAP in brain. Serine racemase (red) colocalizes with GFAP (green) in double-labeling experiments. Astrocytes of different regions and different shapes are strongly labeled, including accessory olfactory bulb (*A* and *B*) and hindbrain (*C* and *D*). Pictures were taken at ×1,000 magnification.

Recently, we showed that purified D-amino acid oxidase, which is virtually absolutely selective for D-serine as opposed to L-amino acids, depletes endogenous D-serine but no other amino acids from brain slices and cultures. Destruction of endogenous D-serine selectively by application of D-amino acid oxidase greatly reduces NMDA receptor activation (J.P. Mothet, A.T. Parent, H.W., R.O. Brady, Jr., D.J. Linden, C.D. Ferris, M.A. Rogawski, and S.H.S., unpublished observations). Moreover, application of D-serine was found to be 100 times more potent that glycine in enhancing the NMDA component of miniature end-plate synaptic currents recorded in rat hypoglossal motoneurons (23). These findings, taken together with the closely similar regional localizations of D-serine and NMDA receptors (4), strongly imply that D-serine is a major endogenous ligand for the glycine site of NMDA receptors.

The cloned-expressed serine racemase should help clarify biological roles of D-serine. Selective inhibitors of serine racemase should be valuable tools for investigating the regulation of NMDA transmission. Massive release of glutamate to stimulate NMDA receptors after stroke is thought to be responsible for the major portion of neural damage (24). Drugs that block the glycine site of NMDA receptors prevent stroke damage (25, 26). Inhibitors of serine racemase may provide novel therapeutic agents for stroke and other neurodegenerative diseases in which glutamate excitotoxicity plays a pathophysiologic role.

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