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Neuron-derived D-Serine Release Provides a Novel Means to Activate *N*-Methyl-D-aspartate Receptors*

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D-Serine is a coagonist of *N*-methyl-D-aspartate (NMDA) receptors that occurs at high levels in the brain. Biosynthesis of D-serine is carried out by serine racemase, which converts L- to D-serine. D-Serine has been demonstrated to occur in glial cells, leading to the proposal that astrocytes are the only source of D-serine. We now report significant amounts of serine racemase and D-serine in primary neuronal cultures and neurons *in vivo*. Several neuronal culture types expressed serine racemase, and D-serine synthesis was comparable with that in glial cultures. Immunohistochemical staining of brain sections with new antibodies revealed the presence of serine racemase and D-serine in neurons. Cortical neurons expressing serine racemase also expressed the NR2a subunit *in situ*. Neuron-derived D-serine contributes to NMDA receptor activation in cortical neuronal cultures. Degradation of endogenous D-serine by addition of the recombinant enzyme D-serine deaminase diminished NMDA-elicited excitotoxicity. Release of neuronal D-serine was mediated by ionotropic glutamate receptor agonists such as NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, and kainate. Removal of either external Ca^{2+} or Na^{+} blocked D-serine release. Release of D-serine was mostly through a cytosolic route because it was insensitive to bafilomycin A_1, a potent inhibitor of vesicular neurotransmitter uptake. D-Serine was also not transported into purified synaptic vesicles under conditions optimal for the uptake of known transmitters. Our results suggest that neurons are a major source of D-serine. Glutamate-induced neuronal D-serine release provides a novel mechanism for activating NMDA receptors by an autocrine or paracrine way.

The *N*-methyl-D-aspartate (NMDA)[2] type of glutamate receptor plays prominent roles in excitatory neurotransmission (1). In addition to glutamate, the NMDA receptor requires the obligatory binding of a coagonist of the NR1 subunit to mediate ion influx (2). Although glycine was originally suggested as the NMDA receptor coagonist, recent data indicate that endogenous D-serine is a physiologically relevant NMDA receptor ligand at the coagonist site. D-Serine is present at high levels in the brain, with little levels in peripheral tissues (3–5). Destruction of endogenous D-serine by D-amino-acid oxidase in hippocampal cultures promotes a decrease in NMDA receptor responses (6). Likewise, NMDA receptor-mediated responses in the retina and induction of long-term hippocampal potentiation are also diminished by removing D-serine (7, 8). Very recently, D-serine was shown to be required for granule cell migration in the developing cerebellum (9). It has been proposed that Bergman glial cells release D-serine and enhance cell migration through activation of NMDA receptors in migrating granule cells (9). In hippocampal organotypic slice cultures, endogenous D-serine was shown to be the dominant coagonist for NMDA receptor-elicited neurotoxicity, mediating virtually all cell death elicited by NMDA (10).

D-Serine is synthesized from L-serine by serine racemase, a brain enriched enzyme (11–13). Serine racemase activity is stimulated by ATP, and the enzyme also catalyzes deamination of serine into pyruvate and ammonia (14, 15). Both D-serine and serine racemase were shown previously to be enriched in astrocytes (13, 16). Purified astrocytic cultures release D-serine following (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptor activation (16–18) and through an amino acid exchange mechanism by the neutral amino acid transporter ASCT (17). It has been proposed that D-serine released from astrocytes that ensheath the synapse will stimulate nearby neuronal NMDA receptors (16).

The evidence that D-serine possesses a target receptor as well as a biosynthetic and degradative apparatus implies that D-serine is an important transmitter/neuromodulator in the brain (5, 19). On the other hand, the glial localization of D-serine is not compatible with the classical definition of neurotransmitter, which should be present in neurons. We have explored a possible neuronal localization of D-serine and serine racemase utilizing biochemical methods in cell cultures and new antibodies against D-serine and serine racemase. We demonstrate the unambiguous presence of serine racemase and D-serine in neurons. Neuronal release of D-serine mediates a significant fraction of NMDA-elicited excitotoxicity in cortical cultures. Our data indicate that neuronal D-serine may play an important role in NMDA receptor activation, such as occurs in neurotoxicity.

EXPERIMENTAL PROCEDURES

Materials—L-Serine and D-serine were purchased from Bachem. Acetonitrile, AMPA, *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid, bafilomycin A_1, DNase I, anti-glial fibrillary acidic protein (GFAP) and anti-microtubule-associated protein-2 (MAP2) monoclonal antibodies, L-glutamate, glycine, imidazole, kainate, a lactic dehydrogenase-based *in vitro* toxicology assay kit, NADH, NMDA, propidium iodide, tetrahydrofuran, and tetrodotoxin were obtained from Sigma. 6,7-Dinitroquinoxaline-2,3-dione and dizocilpine (MK-801) were purchased from Tocris. Anti-NeuN (neuron-specific nuclear protein) monoclonal antibody was obtained from Chemicon International, Inc. Anti-actin monoclonal antibody was from MP Bioscience. Basal Eagle's medium, minimal essential medium, fetal bovine serum, glutamine, penicillin/streptomycin, trypsin, and soybean trypsin inhibitor were obtained

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[2] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; AMPA, amino-3-hydroxy-5-methylisoxazole-4-propionic acid; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein-2; P, postnatal day; HBSS, HEPES-buffered salt solution; HPLC, high pressure liquid chromatography; TBS, Tris-buffered saline; HCSS, HEPES control salt solution; MOPS, 4-morpholinepropanesulfonic acid.

from Biological Industries (Kibbutz Beit Haemek, Israel). B-27 supplement and diaminobenzidine were obtained from Invitrogen. D-[G-^{3}H]Serine was obtained from PerkinElmer Life Sciences, and L-[G-^{3}H]glutamate was from Amersham Biosciences. Other reagents were analytical grade.

Cell Cultures—Animals were killed by quick decapitation. Serum-free neuronal cultures from the cerebral cortex and striatum were prepared from day 16–18 embryos, and hippocampal cultures were obtained from postnatal day (P) 1 Sprague-Dawley rats as described (20), with the following modifications. After dissection, cortices from 12 embryos were suspended in 1.5 ml of HEPES-buffered salt solution (HBSS) lacking calcium and magnesium (10 mM HEPES, 1 mM pyruvate, 5.6 mM glucose, 0.44 mM KH_2PO_4, 5.4 mM KCl, 0.18 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, and 137 mM NaCl) and digested for 7 min at 37 °C with trypsin at a final concentration of 0.12%. Then, soybean trypsin inhibitor and 400 units of DNase I were added, and the tissue was mechanically dissociated with a fire-polished Pasteur pipette. After adding HBSS containing Ca^{2+} and Mg^{2+} (10 mM HEPES, 1 mM pyruvate, 5.6 mM glucose, 0.44 mM KH_2PO_4, 5.4 mM KCl, 0.18 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 137 mM NaCl, 0.9 mM $MgCl_2$, and 1.1 mM $CaCl_2$) to the suspension and decanting tissue clumps, the cells were plated in poly-D-lysine-coated 12-well plates (Nunc) at a density of 2.2×10^6 cells/well. For immunocytochemistry, the cells were seeded on acid-washed glass coverslips coated with poly-D-lysine (0.2–1 mg/ml). The culture medium consisted of basal Eagle's medium supplemented with B-27, 0.4 mM glutamine, and penicillin/streptomycin. For D-serine synthesis experiments, the cultures were used 4–10 days after plating. The culture medium employed did not contain any L-serine, D-serine, or glycine. To obtain mixed neuronal/glial cultures, 2–5% fetal bovine serum was added to the medium described above, and the cultures were used 10 days after plating. Nearly pure glial cultures were prepared by supplementing the medium with 10% fetal bovine serum and vigorously washing the cells with HBSS four to five times every 2 days to dislodge neuronal cells and microglia. Glial cultures were used 10–14 days after plating. Granule cell cultures, mixed neuronal/glial cultures, and glial cultures of the cerebellum were prepared as described above, except that they were obtained from P2–4 Sprague-Dawley pups.

Determination of Endogenous D-Serine—An aliquot (0.1 ml) of the culture medium was removed, and the samples were processed for HPLC as described (15). Briefly, samples were first deproteinized by addition of trichloroacetic acid to a 5% final concentration. The suspension was centrifuged at 20,000 × *g* for 5 min, and the supernatant was analyzed by HPLC after removal of trichloroacetic acid by four extractions with water-saturated diethyl ether. Because the culture medium contained peaks that ran close to D-serine and sometimes obscured it, all samples were routinely treated with the D-serine deaminase DsdA (EC 4.3.1.18) to confirm D-serine levels and positions in the HPLC chromatogram. L-Serine employed in D-serine synthesis experiments was treated with D-amino-acid oxidase to remove contaminant D-serine as described previously (15).

Antibody Production and Western Blotting—Full-length mouse serine racemase was prepared as a glutathione *S*-transferase fusion protein in mammalian cells as described previously (21) and used to immunize rabbits. The antibody designated Ab2 was purified on a cyanogen bromide column (22) coupled to 1 mg of recombinant full-length mouse serine racemase that was bacterially expressed and purified as described previously (14). The antibody against serine racemase designated Ab1 was generated and characterized in a previous study (13). Except when indicated otherwise, all blots were carried out with antibody Ab2. For Western blotting, the purified antibodies were used at 0.15–0.5 μg/ml, and the blots were revealed with SuperSignal West Dura extended duration substrate (Pierce).

Immunocytochemistry and Cell Counts—Cultured cells were fixed with 4% paraformaldehyde and incubated with 0.5 μg/ml affinity-purified anti-serine racemase antibody Ab2. Controls were carried out by pre-absorption of the antibody with 10 μg of purified antigen. Anti-rabbit Cy3 and anti-mouse Cy2 secondary antibodies (Jackson ImmunoResearch Laboratories) were used. Co-localization with neuronal and glial markers (MAP2 (1:100 dilution) and GFAP (1:1000 dilution), respectively) was analyzed by confocal laser microscopy. For determination of the percentage of glial cell in the different cultures, GFAP-positive cells were counted in several random fields. Total cell number in each field was determined by counting cell nuclei stained with 4′,6-diamidino-2-phenylindole.

Immunohistochemistry for Serine Racemase—For serine racemase staining, Sprague-Dawley rats at P7 or 30 were deeply anesthetized with pentobarbital (100 mg/kg, intraperitoneally) and perfused with phosphate-buffered saline to remove the blood. Then, the perfusion solution was switched to 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4), and brains were cryoprotected with 30% sucrose in 0.1 M sodium phosphate (pH 7.4) plus 5 mM sodium azide. Brain sagittal sections were cut on a cryostat (10 μm) and affixed to slides. Blocking medium contained 10% normal goat serum and 0.05% Triton X-100 in Tris-buffered saline (TBS; 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl). Purified anti-serine racemase antibody Ab2 was used at 0.5–1 μg/ml; and in some experiments, immune serum was used at 1:600 dilution in fresh blocking medium. After overnight incubation at 4 °C, the slides were washed with 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 0.05% Triton X-100. Staining was developed using biotinylated anti-rabbit antibody (1:200 dilution) with diaminobenzidine as the peroxidase substrate (Elite ABC kit, Vector Laboratories).

For double-labeling immunofluorescence, the sections were processed and blocked as described above, and the primary antibodies used were anti-NeuN (1:200 dilution) and anti-NR2a (50 μg/ml; Zymed Laboratories Inc.) monoclonal antibodies and anti-serine racemase immune serum (1:600 dilution, Ab2). NeuN was revealed by applying anti-mouse Cy3 antibody at 1 μg/ml, and serine racemase was revealed by applying biotinylated anti-rabbit antibody (1:600 dilution), followed by fluorescein linked to avidin D (1 μg/ml; Vector Laboratories). The slides were observed under a fluorescence inverted microscope.

Immunohistochemistry for D-Serine—For D-serine staining, the original protocol (16) was modified as follows. After perfusion with phosphate-buffered saline, Sprague-Dawley rats at P9 were perfused with 5% glutaraldehyde, 0.5% paraformaldehyde, and 0.2% sodium metabisulfite in 0.1 M sodium phosphate (pH 7.4) and cryoprotected with 30% sucrose in 0.1 M sodium phosphate (pH 7.4) plus 5 mM sodium azide. Brain sagittal sections were cut on a cryostat or freezing microtome. We used sections of 10 μm instead of 40 μm because they gave lower background staining and higher specific immunoreactivity. Free-floating sections were reduced with TBS containing 0.5% $NaBH_4$ and 0.2% sodium metabisulfite for 10 min. After washing, the sections were affixed to slides. Sections were blocked and permeabilized with 10% normal goat serum and 0.05% Triton X-100 in TBS for 45 min. Then, the sections were incubated for 24–48 h with rabbit anti-D-serine antibody (1:200–600 dilution; MoBiTec) or the anti-D-serine antibody employed previously by Schell *et al.* (16) at 1:200 dilution. Incubation was carried out at 4 °C in TBS containing 10% normal goat serum, 0.05% Triton X-100, and 0.1 mM L-serine-glutaraldehyde conjugate to block any cross-reactivity against L-serine. We avoided using a higher L-serine-glutaraldehyde conjugate concentration because commercial L-serine contains con-

taminant D-serine that might decrease the specific staining as well. Biotinylated anti-rabbit secondary antibody was used at 1:200 dilution in 2% normal goat serum and 0.05% Triton X-100 in TBS. Staining was revealed using the peroxidase Elite ABC kit with diaminobenzidine as the substrate. Controls were carried out by pre-absorbing the primary antibody with 0.5 mM D-serine-glutaraldehyde conjugate. In some experiments, free-floating staining for D-serine was carried out in thick sections (40 μm) using the same technique and antibody employed in previous studies (16, 23).

Double-labeling experiments for D-serine and NeuN were carried out in rats perfused with 3% glutaraldehyde (instead of 5%) to preserve the immunoreactivity against NeuN and to decrease the background fluorescence. This lower glutaraldehyde concentration was highly effective for D-serine fixation, as trichloroacetic acid extraction of the free amino acids from 3% glutaraldehyde-fixed brains released <5% of the total D-serine compared with unfixed tissue. Sagittal sections (10 μm) were cut using a cryostat. Schiff's reagent was employed to quench the background glutaraldehyde fluorescence as described previously (24), with the following modifications. Schiff's reagent (25) was prepared by dissolving 1 g of pararosaniline hydrochloride in 200 ml of boiling water. After cooling to 50 °C, the solution was filtered and acidified by addition of HCl to 0.1 M. After the solution was allowed to cool to 25 °C, 1 g of sodium metabisulfite was added, and the solution was left in the dark for 24 h. Then, the solution was filtered after addition of 2 g of activated charcoal and stored in darkness for no more than 5 days at 4 °C. Free-floating sections were immersed in Schiff's reagent and incubated at room temperature under darkness for 20 min. Then, excess reagent was removed by washing with 0.1 M HCl containing 0.5% sodium metabisulfite. The sections were reduced with TBS containing 0.5% $NaBH_4$ and 0.2% sodium metabisulfite for 10 min and affixed to slides after washing with TBS. Blocking was carried out as described above, and anti-NeuN (1:200 dilution) and anti-D-serine (1:400–1000 dilution; MoBiTec) monoclonal antibodies were incubated for 48 h at 4 °C. NeuN was revealed by applying anti-mouse Cy3 antibody at 1 μg/ml, and serine racemase was revealed by applying biotinylated anti-rabbit antibody (1:600 dilution), followed by fluorescein linked to avidin D at 1 μg/ml.

Subcellular Fractionation—Intact brain nuclei were purified as described previously (26), with some modifications. Adult rat brain homogenates were prepared with a loose homogenizer in 0.32 M sucrose, 1 mM $MgCl_2$, 1 mM potassium P_i (pH 6.4), 0.4 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 1 μg/ml chymostatin. Then, the suspension was filtered through 70-μm mesh nylon. The crude nuclear fraction (P1) was obtained by centrifugation for 10 min at 850 × *g*. Purified nuclei were obtained by applying the washed P1 fraction to a 2 M sucrose cushion as described (26). To obtain cytosolic protein (S3 fraction), the homogenate was centrifuged for 1 h at 120,000 × *g*, and the pellet was discarded.

Recombinant DsdA—DsdA from *E. coli* strain CFT073 containing an N-terminal histidine tag was expressed as described previously (10). Briefly, the protein was induced by addition of 0.8 mM isopropyl β-D-thiogalactopyranoside and affinity-purified with nickel-nitrilotriacetic acid-agarose beads. After extensive washing in buffer containing 500 mM NaCl and 10 mM imidazole, the protein was eluted with 150 mM imidazole and dialyzed overnight against phosphate-buffered saline. The protein was concentrated to 30–40 mg/ml with Centriprep (Amicon) and frozen at −70 °C until used. The purity of each batch of DsdA was virtually 100% as analyzed by SDS-PAGE. Deamination of D-serine into pyruvate by DsdA was monitored by decreases in NADH at 340 nm as the pyruvate was converted to lactate by added lactate dehydrogenase.

NMDA-elicited Neurotoxicity—After 10–15 days in culture, the medium was removed, and cells were washed twice with HEPES control salt solution (HCSS) lacking magnesium (20 mM HEPES, 1.8 mM $CaCl_2$, 15 mM glucose, 5.4 mM KCl, and 120 mM NaCl) and exposed to 100–500 μM NMDA in the same medium for 20 min. Then, cells were washed twice with HCSS, and the original culture medium was returned. Cell death was monitored after 24 h by the release of lactate dehydrogenase using the lactic dehydrogenase-based *in vitro* toxicology assay kit. To destroy endogenous D-serine, 20–50 μg/ml DsdA was added to the culture medium for 20–60 min and was also present in the HCSS medium in which NMDA stimulation was carried out. To evaluate the percentage of cell death caused by NMDA, total lactate dehydrogenase was measured after cell lysis.

D-Serine Release—Neuronal cultures were labeled with 10 μM D-[^{3}H]serine (100,000 cpm/nmol D-serine) during a 20–40-min incubation in HBSS. The cells were washed twice with ice-cold HBSS. Then, cells were exposed to HCSS lacking magnesium for 5–7 min at room temperature with several drugs in triplicates. The radioactivity released was counted. The values were corrected for total protein in each well. To evaluate the total D-[^{3}H]serine taken up, HBSS containing 0.3% Triton X-100 was added, and the radioactivity released was counted. Typically, no more than 7–10% D-serine was released. No significant metabolism of D-serine was observed throughout the experiment monitored as by HPLC analysis. Statistical analysis was carried out by one-way analysis of variance, followed by Tukey's post hoc test.

Neurotransmitter Uptake into Synaptic Vesicles—Synaptic vesicles were purified from rat cerebral cortex as described previously (27) by omitting chromatography on glass beads (28). The vesicles were stored in liquid nitrogen until used. The reaction medium contained 10 mM MOPS/Tris (pH 7.4), 4 mM KCl, 150 mM sucrose, and 50 μM L-glutamate or D-serine. L-[G-^{3}H]Glutamate and D-[G-^{3}H]serine were added at 4 μCi/ml. Uptake was started by addition of 4 mM MgATP, and the reaction was stopped after 10 min at 37 °C by filtration through 0.45-μm Millipore filters (100 μg of protein/filter). The filters were quickly flushed with 50 ml of cold MOPS/Tris (pH 7.4) solution. Glutamate uptake was corrected for the nonspecific binding measured in the absence of ATP. The specificity of uptake was checked by addition of 1 μM bafilomycin A_1. Radioactivity was counted in a liquid scintillation counter.

RESULTS

D-Serine Synthesis and Serine Racemase in Neurons—To evaluate the possibility that D-serine may also be present at significant levels in neurons, we prepared prenatal neuronal cultures containing minimal amounts of glia using medium lacking serum and monitored D-serine levels by HPLC (Fig. 1*A*). In the absence of L-serine, practically no D-serine accumulated in nearly pure neuronal cultures of the cortex (Fig. 1*A*). Addition of 2 mM L-serine led to the accumulation of micromolar amounts of D-serine in the culture medium, reaching steady state after 6 days (Fig. 1*A*). This is compatible with the role of serine racemase, which produces D-serine from L-serine (13). As demonstrated previously for astrocytic cultures (14), the majority of the total D-serine produced in cortical neuronal cultures accumulated in the culture medium (data not shown).

To examine whether formation of D-serine was due to contamination of the neuronal culture by glia, we prepared different cortical cultures and monitored the number of glial cells by GFAP staining (Fig. 1*B*). Our prenatal cortical neuronal cultures contained <2% GFAP-positive cells, whereas mixed neuronal/glial cultures contained ~30% GFAP-positive cells, and nearly 100% of the cells in the glial culture were GFAP-positive (Fig. 1*B*). In the presence of L-serine, comparable amounts of D-serine

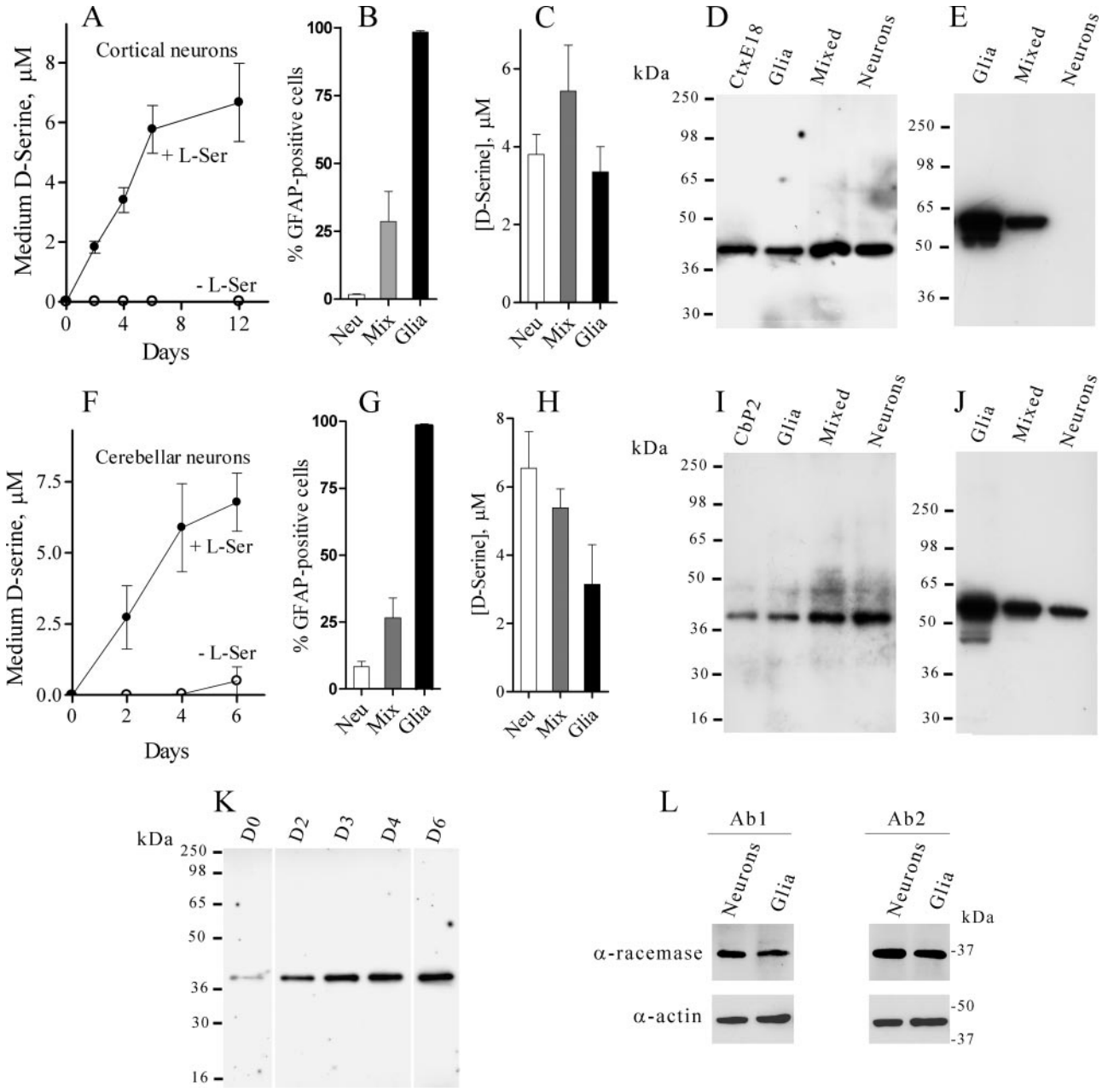

FIGURE 1. **Neuronal synthesis of D-serine and serine racemase expression.** *A*, synthesis of D-serine in purified embryonic cortical neuronal cultures. Addition of 2 mM L-serine 24 h after plating elicited D-serine synthesis. *B*, percentage of GFAP-positive cells in cortical neuronal cultures (*Neu*), mixed neuronal/glial cultures (*Mix*), and purified glial cultures (*Glia*). *C*, comparison of D-serine synthesis by the different cortical culture types. Medium D-serine was monitored 4 days after addition of L-serine. *D*, Western blot analysis of serine racemase expression in dissected cerebral cortex, with the different cortical culture types obtained from animals of the same age. *CtxE18*, embryonic day 18 cerebral cortex. *E*, Western blot analysis of GFAP expression in different culture types from the cerebral cortex. *F*, D-serine synthesis in cerebellar granule cells cultured in serum-free medium. Addition of 2 mM L-serine elicited D-serine synthesis. *G*, percentage of GFAP-positive cells in granule cell cultures (*Neu*), mixed neuronal/glial cultures (*Mix*), and purified glial cultures (*Glia*) from postnatal cerebellum. *H*, comparison of D-serine synthesis by the different cerebellar culture types. *I*, Western blot analysis of serine racemase expression in dissected cerebellum, with the different cerebellar culture types obtained from animals of the same age. *CbP2*, cerebellar postnatal day 2. *J*, Western blot analysis of GFAP expression in the different culture types from postnatal cerebellum. *K*, temporal increase in serine racemase expression in granule cell cultures at culture days (*D*) 0 to 6. *L*, Western blot analysis of serine racemase expression in prenatal neuronal and glial cultures from the cerebral cortex. The blot was revealed with either antibody Ab1 (13) or antibody Ab2 (characterized in this study). The *lower panels* depict actin expression in the cultures using anti-actin monoclonal antibody at 1:10,000 dilution. The data of D-serine synthesis represent the mean ± S.E. with different culture preparations ($n = 3–6$).

were synthesized in neuronal, mixed neuronal/glial, and glial cultures obtained from embryonic cortical cultures (Fig. 1*C*). After 4 days, pure glial cultures synthesized D-serine, which reached ~0.4 μM in the culture medium in the absence of added L-serine (data not shown). This is consistent with their ability to synthesize L-serine, which reached ~100 μM in the glial cell-conditioned medium. However, the levels of D-serine synthesis were at least 10 times lower than those observed after addition of exogenous L-serine.

Serine racemase expression was monitored by Western blot analysis using a new polyclonal antibody we generated (termed Ab2). Comparable levels of serine racemase expression were observed in primary neuronal, mixed, and glial cultures from cerebral cortices of embryos (Fig. 1*D*). The amount of serine racemase protein in the neuronal culture was similar to that found in the cerebral cortices of day 18 embryos (Fig. 1*D*). Note that the levels of D-serine in different cortical culture types correlated well with the levels of serine racemase expression (Fig. 1, compare

C and *D*). On the other hand, serine racemase expression did not reflect the relative amount of glial cells in the cultures as revealed by the percentage of GFAP-positive cells (Fig. 1*B*) or by Western blotting with GFAP (Fig. 1*E*). Robust GFAP expression was observed in glial cultures, with much less in mixed neuronal/glial cultures (Fig. 1*E*). Almost no GFAP expression was observed in purified embryonic neuronal cultures of the cerebral cortex (Fig. 1*E*). Only long exposure of the blot to the film revealed some GFAP expression in our neuronal culture (data not shown), confirming the purity of the preparation.

Postnatal cerebellar granule cells cultured in the absence of serum also synthesized large amounts of D-serine (Fig. 1*F*). The amount of contaminating glia in cerebellar granule cell cultures prepared in the absence of serum was higher than that in embryonic cortical neurons (10% compared with <2%) (Fig. 1*G*). As in cortical neuronal cultures, similar levels of D-serine were detected in different types of cerebellar cultures containing various degrees of glial cells (Fig. 1*H*). Comparable serine racemase expression was observed in the different cerebellar culture types (Fig. 1*I*). As in cortical cultures, serine racemase expression and D-serine synthesis were not correlated with the amount of glial cells as determined by GFAP-positive cell numbers (Fig. 1*G*) or Western blotting with GFAP (Fig. 1*J*). Serine racemase expression in granule cell cultures increased with culture age, reaching steady state after 6 days *in vitro* (Fig. 1*K*). This pattern is similar to that reported previously for intact tissue (29).

To evaluate the specificity of the Western blots, we performed immunoblotting with a different anti-serine racemase antibody (Ab1) shown previously to recognize the enzyme in postnatal glial cultures (13). Using an extended duration chemiluminescent substrate, we found that antibody Ab1 equally recognized prenatal neuronal and glial serine racemases, similar to our antibody Ab2 (Fig. 1*L*).

Immunocytochemical Localization of Serine Racemase—Neuronal expression of serine racemase was confirmed by immunocytochemical experiments. In embryonic neuronal cultures from the cerebral cortex, virtually all neurons were immunoreactive for serine racemase, with perfect co-localization with the neuronal marker MAP2 (Fig. 2, *A–C*). Postnatal hippocampal cultures also displayed clear neuronal serine racemase staining (Fig. 2, *G–I*). We also stained purified neuronal cultures from the cerebral cortex and hippocampus for GFAP (Fig. 2, *D–I*). This revealed astrocytes, which were present in low quantity and in selected fields only. Surprisingly, the few glial cells in the neuronal cultures contained little serine racemase. In some glial cells, no clear racemase staining was detectable (Fig. 2*F*, *arrowhead*), whereas modest staining was observed in others (Fig. 2*I*, *arrowheads*). Neurons (GFAP-negative cells) were strongly positive for serine racemase in these cultures (Fig. 2, *D* and *G*). This clearly indicates that contaminant glial cells were not the source for D-serine synthesis observed in nearly pure neuronal cultures.

The very low levels of serine racemase expression in the few contaminant glial cells present in neuronal cultures may be due to the use of serum-free medium, which lacks growth factors required for optimal survival and proliferation of glia (20). Thus, we checked serine racemase expression in purified glial cultures produced with serum-containing medium. We found strong serine racemase expression in these cultures (Fig. 2*J*) and co-localization with GFAP staining (Fig. 2, *K* and *L*). A large majority of neurons in primary cultures from other brain regions, such as in granule cell cultures of the primary cerebellum and striatal cultures, were also immunoreactive for serine racemase (Fig. 2, *M* and *N*).

Serine Racemase Localization in Forebrain Areas—Because the pattern of serine racemase expression (neurons *versus* glia) may vary depending on the culture conditions, it may not reflect the *in vivo* situation. Previous immunohistochemical analysis of serine racemase expression in rat brain did not detect its presence in neurons (13). However, the previously employed antibody was suitable only for fresh frozen sections fixed with methanol, and immunoreactivity for serine racemase was reported to be absent in conventional aldehyde-fixed tissue (13). In addition to poor morphological preservation in brain sections fixed with methanol, part of the immunoreactivity may have been obscured. Thus, we now carried out immunohistochemistry in P7 and adult rats with our new antibody that recognizes paraformaldehyde-fixed in addition to methanol-fixed cells. In accordance with the biochemical and immunocytochemical methods in neuronal cultures (Figs. 1 and 2), we observed significant neuronal as well as astrocytic staining for serine racemase (Fig. 3).

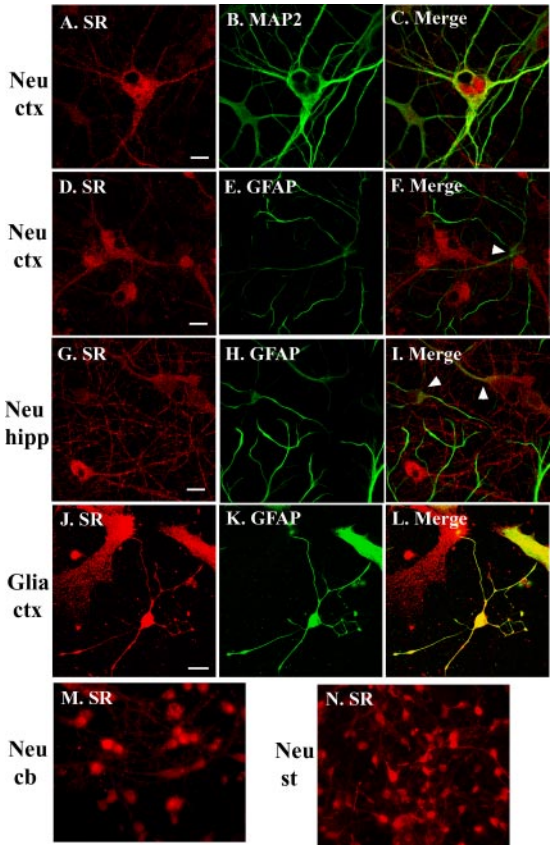

FIGURE 2. **Confocal laser microscopy analysis of serine racemase expression in primary cultures.** *A* and *B*, serine racemase (*SR*; *A*) and MAP2-positive neurons, respectively, in cortical neuronal cultures (*Neu ctx*). *C*, co-localization of serine racemase and MAP2. *D* and *E*, serine racemase and GFAP expression, respectively, in cortical neuronal cultures. *F*, presence of a GFAP-positive cell (*arrowhead*) expressing nearly undetectable serine racemase. Neurons that were not stained for GFAP exhibited strong immunoreactivity against serine racemase. *G* and *H*, serine racemase and GFAP expression, respectively, in postnatal hippocampal neuronal cultures (*Neu hipp*). *I*, presence of GFAP-positive cells expressing serine racemase (*arrowheads*), with other glial processes appearing negative. *J* and *K*, serine racemase and GFAP expression, respectively, in nearly pure prenatal cortical glial cultures (*Glia ctx*). *L*, co-localization of serine racemase and GFAP. *M*, serine racemase staining in purified cerebellar neuronal cultures (*Neu cb*). *N*, serine racemase staining in purified striatal neuronal cultures (*Neu st*). *Scale bars* = 11 μm.

Neuronal serine racemase staining was observed in all layers of the cerebral cortices from P7 rats. Pyramidal cell neurons of the cerebral cortex exhibited particularly strong serine racemase staining (Fig. 3, *A* and *B*). In the hippocampus, serine racemase immunoreactivity was observed in the pyramidal layers of the CA1–CA3 fields, the subiculum region, and granule cells of the dentate gyrus (Fig. 3*C*). Pre-absorption with excess antigen abolished immunostaining (Fig. 3*D*). At high magnification, clear neuronal staining could be observed in the neurons of

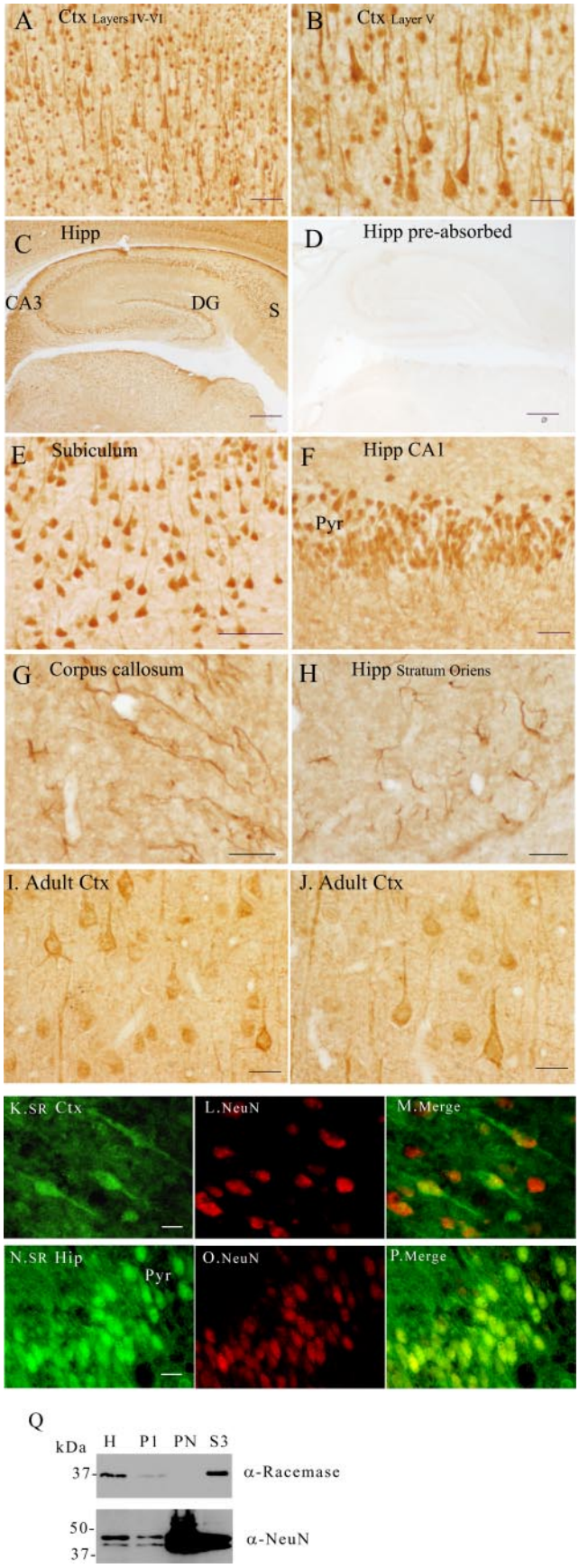

FIGURE 3. **Localizations of serine racemase in the forebrain.** *A*, staining for serine racemase in the cerebral cortex (*Ctx*) of a P7 rat (layers IV–VI). *B*, staining for serine racemase in layer V of the cerebral cortex of a P7 rat. *C*, staining for serine racemase in the hippocampal formation (*Hipp*). *DG*, dentate gyrus; *S*, subiculum. *D*, pre-absorbed control of hippocampal staining. *E*, high magnification image of the subiculum region of the hippocampus of a P7 rat. *F*, staining of neurons in the stratum pyramidale (*Pyr*) of the CA1 region of the hippocampus of a P7 rat. *G*, glial staining in the corpus callosum of a P7 rat. *H*, glial staining in the stratum oriens of the hippocampal formation of a P7 rat. *I*, serine racemase staining in neurons of layers II and III of the cerebral cortex of an adult rat. *J*, serine racemase staining in neurons of layers IV and V of the cerebral cortex of an adult rat. *K* and *L*, immunofluorescence for serine racemase (*SR*) and NeuN, respectively, in the cerebral cortex of a P7 rat. *M*, coexpression of serine racemase and NeuN. *N* and *O*, immunofluorescence for serine racemase and NeuN, respectively, in the CA1 region of the hippocampus of a P7 rat. *P*, coexpression of serine racemase and NeuN in pyramidal cells. *Q*, Western blot analysis of subcellular protein fractions of adult rat brain for serine racemase (*upper panel*) and the NeuN marker (*lower panel*). Each lane contained 15 μg of protein. *H*, homogenate; *PN*, purified nuclei. P1 and S3 are the crude nuclear and cytosolic fractions, respectively. *Scale bars* = 20 μm (*K–P*), 50 μm (*B* and *F–J*), 100 μm (*A* and *E*), and 500 μm (*C* and *D*).

the subiculum region (Fig. 3*E*) and in the pyramidal cells of the CA1 region (Fig. 3*F*).

In agreement with a previous study (13), we also observed that glial cells were immunoreactive for serine racemase (Fig. 3, *G* and *H*). Prominent glial staining was observed in places reported to exhibit strong D-serine immunoreactivity, such as the corpus callosum (Fig. 3*G*) and the stratum oriens of the hippocampus (Fig. 3*H*).

In adult rats, we also detected neuronal staining for serine racemase in several layers of the cerebral cortex (Fig. 3, *I* and *J*). This indicates that the neuronal staining for serine racemase was not due to transient expression in young rats.

In double-labeling experiments in P7 rats, serine racemase was present in cells positive for NeuN (30). Neurons coexpressing serine racemase and NeuN were observed both in the cerebral cortex (Fig. 3, *K–M*) and in the pyramidal cell layer of the CA1 field of the hippocampus (Fig. 3, *N–P*). The immunofluorescence was specific because omission of the primary antibody abolished the signal (data not shown).

Because NeuN strongly labels neuronal nuclei, with lighter staining in the cytosol, we carried out subcellular fractionation of serine racemase to verify that the enzyme can be found in the nucleus as well (Fig. 3*Q*). The highest levels of serine racemase were observed in the S3 cytosolic fraction, whereas lower levels were observed in the P1 crude nuclear fraction. Highly purified brain nuclei contained little or no serine racemase, but displayed clear enrichment in the NeuN marker (Fig. 3*Q*, *PN*).

D-Serine Localizations in the Forebrain—Previous studies have demonstrated that D-serine is found in astrocytes (23). The presence of serine racemase in neurons prompted us to reinvestigate D-serine localizations. We employed a different antibody raised against D-serine-glutaraldehyde conjugate and shown previously to recognize D-serine in serine racemase-transfected cells (14). This antibody recognizes D-serine (but not L-serine)-glutaraldehyde conjugate as determined by dot blotting (Fig. 4*H*). Other amino acid-glutaraldehyde conjugates such as glycine, L-glutamate, L-threonine, L-aspartate, and L-alanine were not immunoreactive with this antibody to D-serine (data not shown).

The use of a prolonged incubation with the new antibody to D-serine in sections thinner than those employed previously revealed clear staining for D-serine in neuronal cell bodies and processes in all layers of the cerebral cortices of P9 rats (Fig. 4, *A–C*). Astrocytic staining was also prominent, especially in the corpus callosum (Fig. 4*D*). The staining for D-serine was specific because it completely disappeared when the antibody was pre-absorbed with D-serine-glutaraldehyde conjugate (Fig. 5) (data not shown). To further verify the specificity of the antibody, we checked the distribution pattern of D-serine in the adult rat brain (Fig. 4*I*). We detected little staining for D-serine in the adult cerebellum (Fig. 4*I*), which is in agreement with the dramatic developmental decrease in D-serine levels measured by HPLC in this region (23).

To confirm the presence of D-serine in neurons, we carried out double-labeling immunofluorescence studies in the cerebral cortex. The

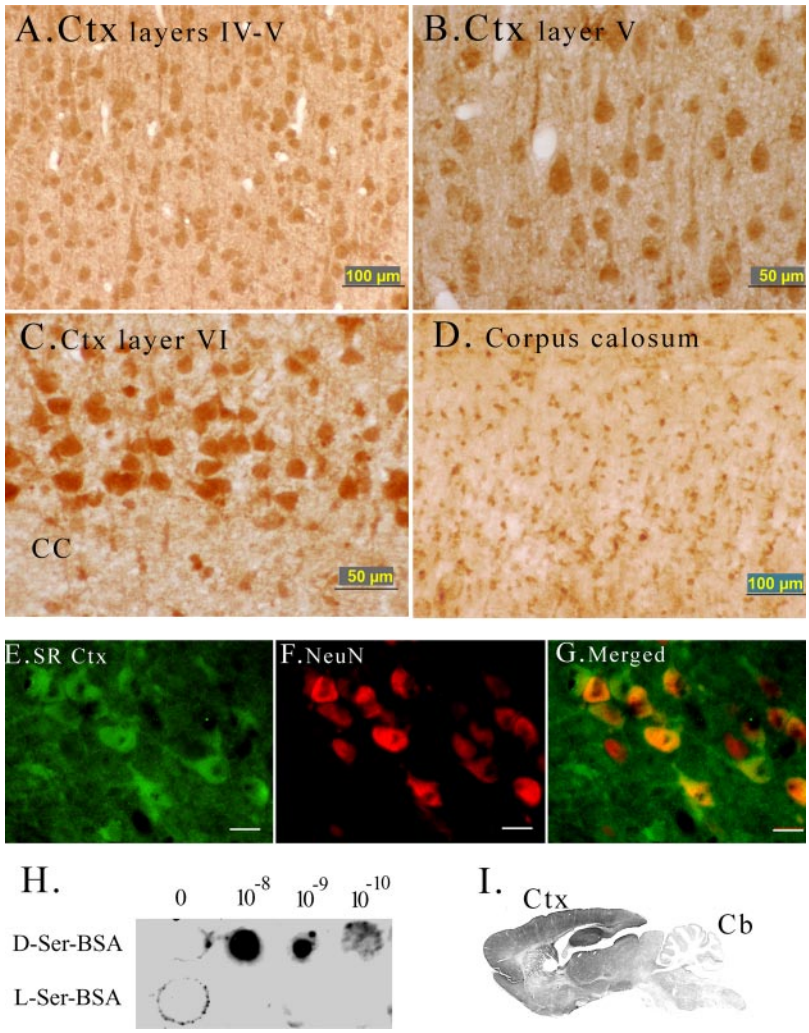

FIGURE 4. **D-Serine localizes to neurons and astrocytes in the brain.** *A–C*, staining for D-serine in neurons of several layers of the cerebral cortex (*Ctx*) of a P9 rat. *CC*, corpus callosum. *D*, glial staining for D-serine in the corpus callosum. *E* and *G*, double-labeling immunofluorescence for serine racemase (*SR*) and NeuN, respectively, in layer VI of the cerebral cortex of a P9 rat. *G*, coexpression of D-serine and NeuN. *H*, dot blot containing increasing amounts of D-serine- and L-serine-glutaraldehyde-bovine serum albumin (*BSA*) conjugates. Primary antibody incubation (1:200 dilution; MoBiTec) was carried out in the presence of 100 μM L-serine-glutaraldehyde liquid conjugate to block any cross-reactivity with L-serine. *I*, immunohistochemistry of adult rat brain for D-serine showing less D-serine staining in the cerebellum (*Cb*). *Scale bars* = 20 μm (*E–G*), 50 μm (*B* and *C*), and 100 μm (*A* and *D*).

glutaraldehyde fixation normally used for amino acid immunohistochemistry precluded any immunofluorescence study due to the strong autofluorescence of glutaraldehyde conjugates. We overcame this technical problem using the method developed by Brusco and co-workers (24) to quench glutaraldehyde autofluorescence, which employs incubation with Schiff's reagent, followed by reduction with sodium borohydride. Using this methodology, we detected coexpression of D-serine with NeuN in neurons of the cerebral cortex, confirming the presence of D-serine in cortical neurons (Fig. 4, *E–G*). In other parts of the brain, neuronal cell bodies were faint, and astrocytic staining seemed to predominate (data not shown).

Previous D-serine localizations were carried out in thick sections using a free-floating technique (16, 23). We wondered if the apparent discrepancies in D-serine staining between the present and previous studies was due to different staining techniques rather than antibody specificity. We compared the staining patterns for D-serine obtained with the antibody generated by Schell *et al.* (16) and the new anti-D-serine antibody using thin sections and longer incubation times (Fig. 5). In thin sections, we observed clear staining of neuronal somata (Fig. 5*A*, *large arrows*) and processes (*small arrows*) in the cerebral cortex using the antibody of Schell *et al*. This antibody also stained glia, and the intensity of glial staining was comparable with that of neuronal staining of layer VI of the cerebral cortex (Fig. 5*B*). Pre-absorption of the antibody with D-serine-glutaraldehyde conjugate abolished the staining (Fig. 5*C*). Furthermore, under identical experimental conditions, we observed a similar staining pattern for D-serine with the new antibody to D-serine (Fig. 5, *D–F*). Primary antibody incubation for <16 h decreased neuronal and glial staining to a similar extent in thin sections (data not shown). The use of the free-floating technique with thick sections (Fig. 5, *G–I*) (16) resulted in higher background staining, which was not optimal for high power observation of neurons. In thick sections, almost only astrocytes were observed, especially in low background areas such as the corpus callosum (Fig. 5*G*). In the cerebral cortex, however, astrocytes appeared less clear due to higher background staining (Fig. 5, *H* and *I*). In this region, we observed a few cell bodies resembling neuronal somata (Fig. 5, *H* and *I*, *arrows*).

Neuronal D-Serine and NMDA Receptor Neurotoxicity—To investigate a possible functional link between NMDA receptors and D-serine, we first carried out double-labeling immunofluorescence studies in brain sections (Fig. 6, *A–C*). We found that cortical neurons expressing serine racemase also expressed the NR2a subunit *in situ* (Fig. 6, *A–C*).

On the basis of the immunohistochemical data, we wondered if neuronal D-serine could regulate NMDA receptors. We investigated whether neuronal D-serine is required for NMDA receptor activation in a cellular model of NMDA-elicited neurotoxicity (31). We employed a strategy to selectively remove D-serine from neuronal cultures using recombinant DsdA. DsdA is a bacterial enzyme (not present in mammals) that catalyzes the deamination of D-serine into pyruvate and ammonia (32). Recombinant DsdA exhibits a very high affinity for

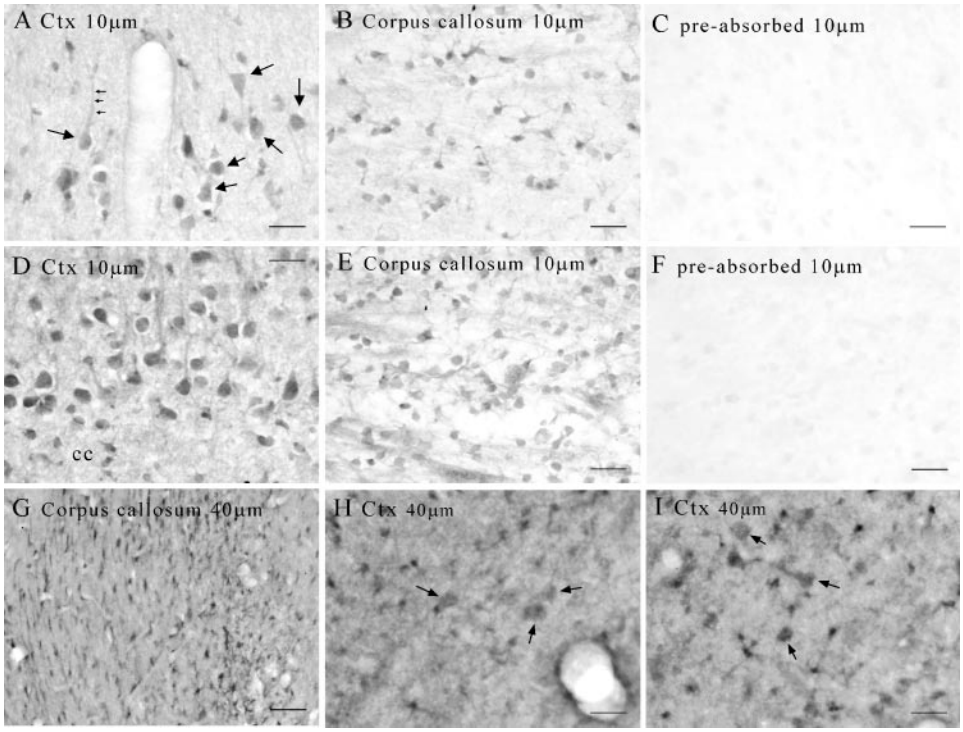

FIGURE 5. **Comparison of different antibodies to D-serine and staining techniques.** *A* and *B*, staining for D-serine in layer VI of the cerebral cortex (*Ctx*) and corpus callosum, respectively, of a P8 rat. Sections (10 μm) were cut and affixed to slides, and immunostaining was carried out with the antibody to D-serine generated by Snyder and co-workers (16). *Large arrows* depict neuronal somata, and *small arrows* depict neuronal processes. *C*, pre-absorption control with D-serine-glutaraldehyde conjugate. *D* and *E*, staining for D-serine in layer VI of the cerebral cortex and corpus callosum (*cc*), respectively. Sections (10 μm) were cut and affixed to slides, and immunostaining was carried out with the new antibody to D-serine (MoBiTec) as described in the legend to Fig. 4. *F*, pre-absorption control with D-serine-glutaraldehyde conjugate. *G–I*, staining for D-serine in the corpus callosum and in layer VI of the cerebral cortex as indicated. Sections (40 μm) were cut, and immunostaining was carried out using anti-D-serine antibody and the free-floating technique as described (16). *Arrows* depict apparent neuronal somata, which are significantly larger that glial cells. *Scale bars* = 50 μm (*A–F*, *H*, and *I*) and 100 μm (*G*).

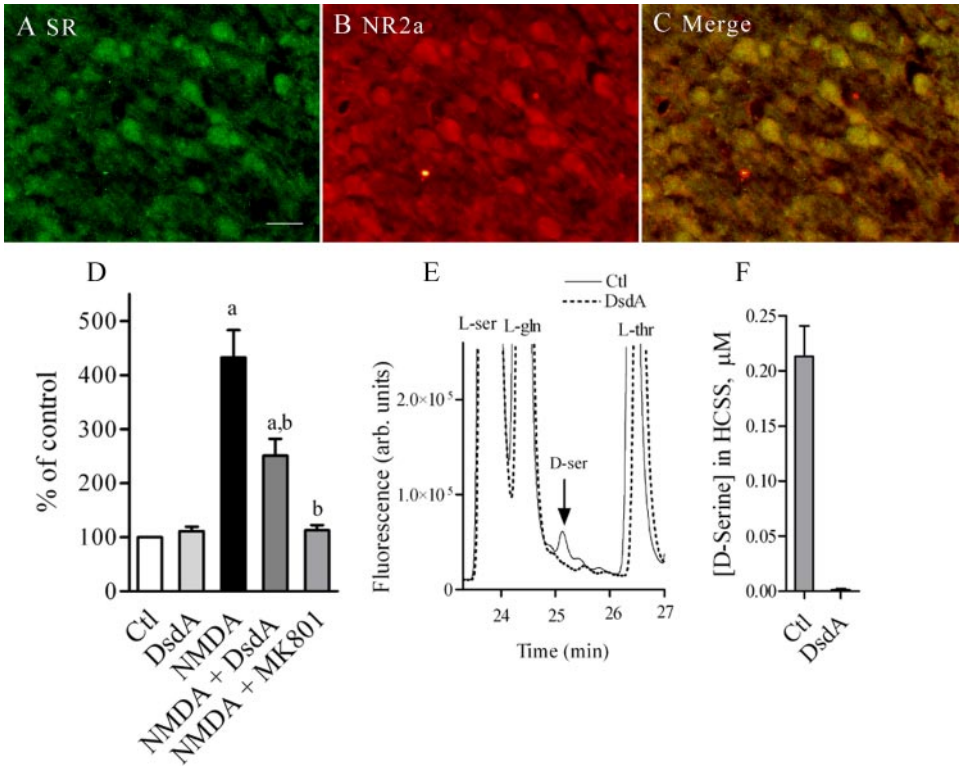

FIGURE 6. **Coexpression of the NR2a subunit and serine racemase in the brain and neuroprotection by depletion of neuron-derived D-serine in cultures.** *A* and *B*, expression of serine racemase (*SR*) and the NR2a subunit, respectively, in the cerebral cortex of a P8 rat. *C*, coexpression of serine racemase and the NR2a subunit. *Scale bar* = 100 μm. *D*, removal of D-serine by DsdA (20 μg/ml) significantly decreases NMDA-elicited cell death in pure cortical neuronal cultures. Cultures were treated with 500 μM NMDA in HBSS without Mg^{2+} for 20 min; the original culture medium was returned; and cell death was assayed 24 h later by release of lactate dehydrogenase. *a*, different from the control (*Ctl*) at $p < 0.001$; *b*, different from NMDA treatment at $p < 0.01$. *E*, HPLC analysis of culture medium reveals a discrete D-serine peak (*solid line*) that was completely destroyed by DsdA treatment (*dashed line*). *arb.*, arbitrary. *F*, quantification of D-serine released into HCSS after the 20-min incubation. No D-serine was observed in cultures treated with DsdA. The data represent the mean ± S.E. of 12 (*D*) and 5 (*F*) experiments. The data in *E* are representative of three experiments.

D-serine and quickly degrades D-serine, without affecting the levels of other amino acids (10).

To check NMDA receptor-elicited neurotoxicity, we monitored cell death by the standard assay of lactate dehydrogenase release into the culture medium (33). Treatment of the culture medium from purified cortical neuronal cultures with DsdA decreased NMDA receptor-elicited cell death by ~50%, whereas the specific antagonist MK-801 abolished it (Fig. 6*D*). In the absence of NMDA, DsdA had no effect (Fig. 6*D*). Controls with heat-inactivated DsdA did not show any inhibition of NMDA-elicited cell death (data not shown). The DsdA effect was specific to its ability to destroy D-serine because addition of glycine at a high concentration (200 μM) to maximally stimulate NMDA receptors completely prevented the DsdA effect (data not shown).

DsdA efficiently destroyed D-serine in the cell culture medium as detected by HPLC analysis (Fig. 6*E*). In the absence of DsdA, significant levels of endogenous D-serine (~0.2 μM) were released to the HCSS medium in which the NMDA insult was carried out (Fig. 6*F*). DsdA completely destroyed the D-serine released into HCSS (Fig. 6*F*). These data

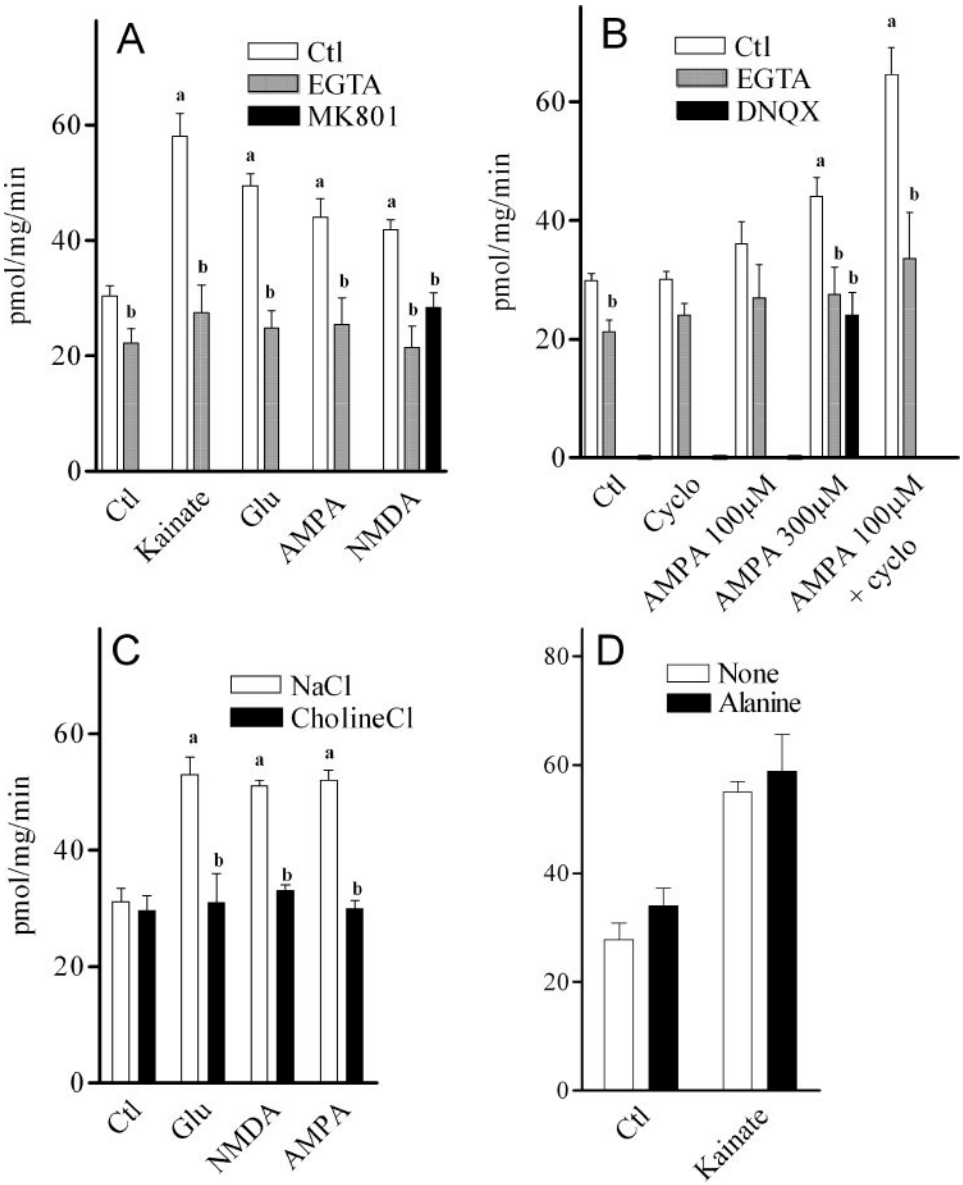

FIGURE 7. **Release of D-serine from neuronal cultures by glutamate receptor agonists.** *A*, cells were exposed to 100 μM kainate, 0. 5 mM L-glutamate, 300 μM AMPA, 0.5 mM NMDA, or 0.5 mM NMDA plus 30 μM MK-801. Release was blocked by omitting external calcium and adding 0.5 mM EGTA. *B*, the AMPA effect was potentiated by 70 μM cyclothiazide (*Cyclo*) and blocked by 20 μM 6,7-dinitroquinoxaline-2,3-dione (*DNQX*). *C*, the substitution of choline chloride for NaCl blocked glutamate, NMDA, and AMPA effects. [a], statistically different from the control (*Ctl*) at $p < 0.01$; [b], statistically different from the efflux observed in the presence of the agonist at $p < 0.01$. *D*, L-alanine (100 μM) did not affect D-serine release. The results are the mean ± S.E. of four to eight measurements done in triplicate.

indicate that neurons are a major source of the D-serine that contributes to NMDA receptor activation in this cellular model.

D-Serine Release from Neurons—The presence of D-serine in HCSS in neurotoxicity experiments led us to investigate whether D-serine can be released by neurons through a regulated mechanism. To verify this possibility, nearly pure neuronal cultures were first loaded with D-[^{3}H]serine, and release was induced with several pharmacological agents. Similar to results reported previously for glial cultures (16, 18), activation of glutamate receptors induced a significant increase in neuronal D-serine release, indicating that transported D-serine probably labels endogenous stores. Kainic acid was the most potent agonist, followed by glutamate and AMPA (Fig. 7*A*). To investigate a possible role of NMDA receptors, magnesium was omitted from the medium to relieve the voltage-dependent blockage of NMDA receptors. Interestingly, NMDA elicited a significant increase in D-serine release that was blocked by MK-801 (Fig. 7*A*).

Omission of Ca^{2+} from the release medium and addition of EGTA abolished the effects of all ionotropic glutamate receptor agonists (Fig. 7*A*), indicating that D-serine release is dependent on extracellular Ca^{2+}. Cyclothiazide (an inhibitor of AMPA receptor desensitization) greatly potentiated AMPA-elicited D-serine release (Fig. 7*B*). The combined effects of cyclothiazide and AMPA elicited a 2-fold increase in the rate of D-serine release (Fig. 7*B*). This effect was abolished by chelating extracellular Ca^{2+} or by 6,7-dinitroquinoxaline-2,3-dione, an AMPA/kainate receptor antagonist (Fig. 7*B*). Agonist-induced D-serine release was blocked when choline was substituted for Na^+ (Fig. 7*C*), indicating that external Na^+ is required in addition to Ca^{2+}.

The metabotropic glutamate receptor agonist *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (known to induce increases in intracellular calcium by activating the inositol phosphate cascade) induced only a minimal release of D-serine (~10%) (data not shown). In addition, depletion of internal calcium stores by addition of thapsigargin (1 μM) had no effect on D-serine release promoted by any of the ionotropic glutamate agonists tested (data not shown).

D-Serine release was action potential-independent. A 20-min preincubation with 1 μM tetrodotoxin (which blocks synaptic activity) did not affect D-serine release mediated by any of the glutamate agonists tested (data not shown).

In astrocytic cultures, amino acid exchange catalyzed by the neutral amino acid transporter ASCT causes release of D-serine (17). Thus,

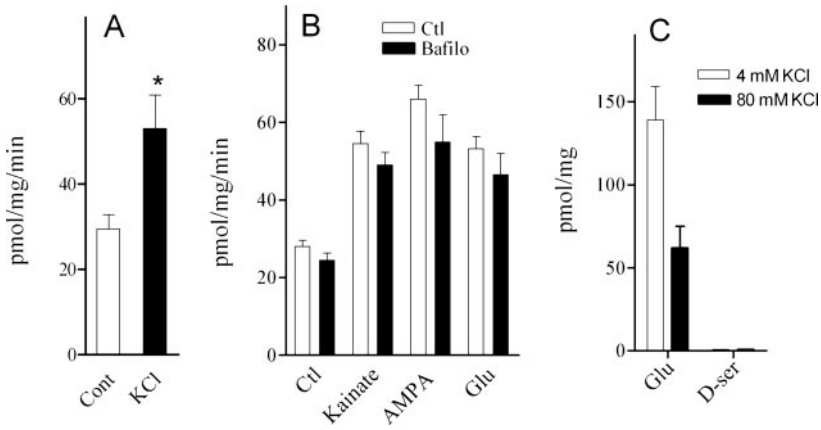

FIGURE 8. **Release of D-serine by membrane depolarization and lack of D-serine transport into synaptic vesicles.** *A*, KCl (40 mM) depolarization for 1 min increased D-serine release. *, statistically different from the control (*Ctl*) at $p < 0.05$. *B*, preincubation with bafilomycin A_1 (*Bafilo*; 1 μM) for 1 h did not inhibit D-serine release. *C*, L-glutamate (but not D-serine) was taken up into a purified synaptic vesicle preparation from rat brain. The results are the mean ± S.E. of three (*A*) and four (*C*) measurements done in triplicate. *B* is representative of three experiments done with a different culture preparation.

addition of micromolar concentrations of L-alanine or L-serine induces robust release of D-serine from astrocytes (17). By contrast, practically no D-serine was released by L-alanine in neuronal cultures (Fig. 7*D*). This suggests that, in contrast to astrocytes, D-serine release from neurons is not mediated by amino acid heteroexchange.

Depolarization with KCl almost doubled the amount of D-serine release (Fig. 8*A*). HPLC analysis of the release medium revealed concomitant release of endogenous glutamate and aspartate by KCl, which was ~10-fold higher than the basal control levels (data not shown).

Conceivably, the calcium dependence and the KCl-induced D-serine release are due to vesicular storage and exocytotic release. However, prolonged preincubation with bafilomycin A_1 (1 μM; which blocks the vesicular uptake of transmitters by inhibiting the vacuolar H^+-ATPase) had no significant effect on agonist- or KCl-induced D-serine release (Fig. 8*B*) (data not shown). The possibility that D-serine might be stored in synaptic vesicles was also directly investigated. Purified synaptic vesicles from the cerebral cortex did not take up D-serine under conditions in which glutamate uptake is optimal (Fig. 8*C*). Addition of bafilomycin A_1 to our synaptic vesicle preparation completely blocked glutamate uptake (data not shown). We also tested a high chloride medium (80 mM), which is known to increase the proton gradient across the vesicles and to facilitate the uptake of other transmitters such as acetylcholine and biogenic amines (34, 35). D-Serine uptake into synaptic vesicles was also undetectable at a high chloride concentration (Fig. 8*C*). Moreover, D-serine (up to 10 mM) did not affect the rate of glutamate uptake into synaptic vesicles (data not shown). Thus, D-serine is not a substrate for the vesicular glutamate transporters present in synaptic vesicles.

DISCUSSION

A role for neurons in the manufacture and release of D-serine has not been explored previously. In this study, we provide evidence that D-serine is also synthesized in neurons both *in vitro* and *in vivo*. Synthesis of D-serine by cultured neurons was not due to an artifact of the culture procedure. Serine racemase was present in different culture types, including postnatal neurons. Most important, neuronal staining for D-serine and serine racemase was confirmed *in vivo* by immunohistochemistry using different antibodies. We found that neuron-derived D-serine accounts for a significant fraction of the NMDA-elicited excitotoxicity in neuronal cultures. We also identified a route for D-serine release from neurons. Our results suggest that neurons are an important source of D-serine, with implications for the regulation of NMDA receptor transmission.

Synthesis of Neuronal D-Serine—Our experiments with neuronal cultures clearly demonstrate that neurons synthesize D-serine (Figs. 1 and 2). Embryonic neuronal cultures expressed serine racemase at levels at least as high as pure glial cultures obtained from embryos of the same age. This was shown with two different antibodies to serine racemase. Serine racemase expression was shown previously to be enriched in postnatal type 2 astrocytic cultures, which exhibited higher racemase expression compared with embryonic neuronal cultures (13). These apparent differences in serine racemase expression may be related to the different developmental stages and culture conditions of the cells used in the previous study (purified postnatal astrocytes *versus* prenatal neurons).

In pure neuronal cultures, practically no D-serine synthesis was observed in the absence of added L-serine (Fig. 1). In agreement, neurons express low levels of enzymes of the "phosphorylated pathway" for L-serine biosynthesis and thus possess limited ability to synthesize their own L-serine from glucose (36). Our results indicate that synthesis of D-serine in neurons requires the uptake of extracellular L-serine, which may be supplied by glial cells under normal conditions (37). Failure to add L-serine to the culture medium may explain the reported absence of D-serine synthesis and release from pure neuronal cultures (18). However, the more limited ability of neurons to synthesize L-serine from glucose does not necessarily indicate that they lack enough L-serine to make D-serine *in situ*. L-Serine is one of the most abundant amino acids in brain extracellular fluid (38). Of 21 amino acids, L-serine is present at the third highest level in cerebrospinal fluid (39). Neurons also express serine hydroxymethyltransferase, which catalyzes the conversion of glycine to L-serine (40). Acutely isolated and highly purified synaptosomal preparations contain high levels of free L-serine, consistent with the occurrence of L-serine in neurons *in situ* (41).

Despite evidence suggesting the presence of L-serine in neurons, the threshold concentration of L-serine is anticipated to be higher for D-serine synthesis compared with some other neuronal activities. Serine racemase displays a relatively high K_m for L-serine, ranging from 4 to 10 mM (12, 42). A similar low affinity for L-serine (K_m = 4 mM) is also displayed by the cystathionine β-synthase, an enzyme that is present in neurons and that produces cystathionine from serine and homocysteine (43, 44). The intracellular L-serine concentration will not likely reach such high values, and these enzymes will probably work under suboptimal conditions. By contrast, sphingolipid metabolism by serine palmitoyltransferase displays a 10-fold higher affinity for L-serine (45), whereas protein synthesis requires only micromolar amounts of L-serine due to the high affinity of the seryl-tRNA synthetase (46). Additional studies will be needed to define the thresholds of L-serine required for the different serine-dependent processes in neurons.

Previous work failed to detect serine racemase and D-serine in neurons (13, 16, 23). The differences between our work and previous studies might be related to the different sensitivities of the antibodies and techniques employed. The anti-serine racemase antibody used in a previous

study was reported to be devoid of immunoreactivity against paraformaldehyde-fixed tissue and was suitable only for methanol-fixed sections (13). Methanol fixation causes the precipitation of proteins and may generate artifacts due to cytoplasmic shrinkage and poor preservation of cell morphology (47). Although this technique was useful for revealing serine racemase in glia, it might have negatively affected the antigenicity of serine racemase and precluded the observation of neuronal staining. Using a new antibody suitable for paraformaldehyde fixation, we detected serine racemase in neurons at levels comparable or higher than those found in glia (Fig. 3).

D-Serine was also detected in neurons using two different antibodies (Figs. 4 and 5). This is in agreement with the expression of serine racemase in these cells both in culture and *in vivo*. This was made possible by employing a prolonged incubation of the tissue and utilizing sections thinner than those employed previously (13, 16). This combination resulted in improved immunoreactivity with lower background staining, which was crucial for the observation of neuronal staining (Fig. 5). One possible reason for the quality improvement in thinner sections may be related to improvement of antibody penetration into the section.

Our results clarify a number of observations that were hard to explain based on the previously reported localization of serine racemase and D-serine in glia: 1) the presence of D-serine in purified synaptosomes reported by Wood *et al.* (41), which is probably not attributable to glial contamination in the preparation; 2) the slight immunoreactivity to D-serine detected in some cortical neurons in a brief study that used an amplification system (48), which may be attributable to the neuronal synthesis of D-serine by serine racemase that we have described in this study; and 3) the specific neuronal expression of the Asc-1 neutral amino acid transporter (49–51), which displays much higher affinity for D-serine compared with the glial neutral amino acid transporters (17). The presence of serine racemase and D-serine in neurons implies that Asc-1 plays a role in D-serine transport.

While this manuscript was under review, Pow and co-workers (52) reported a new antibody against D-serine using paraformaldehyde fixation. They observed staining in glia as well as in a subset of hindbrain neurons. Paraformaldehyde fixation shows a very low retention ability of amino acids that are rapidly released from the cells during fixation (53). This might explain why patches of the cortex were totally devoid of immunolabeling using their antibody and the lack of cortical neuronal staining in their study (52).

Role and Release of Neuronal D-Serine—Cortical neurons expressing serine racemase also contained NMDA receptors *in situ* (Fig. 6), and NMDA receptor activation promoted an increase in neuronal D-serine release (Fig. 7). This provides a functional link between NMDA receptors and the neuronal pool of D-serine. We showed that removal of endogenous D-serine by DsdA significantly decreased cell death elicited by NMDA in pure neuronal cultures (Fig. 6). This result highlights the importance of neuron-derived D-serine in mediating the NMDA receptor activation that occurs in excitotoxicity. However, because *in vivo* neurons do not occur in the absence of glia, it is not possible to conclude from neuronal culture studies that *in vivo* neurotoxicity acts predominantly via neuronal and not glial D-serine. Our results also do not exclude a role for glia in modulating NMDA receptor neurotoxicity by producing growth factors or by regulating extracellular glycine levels. For instance, the action of endogenous glycine in mediating NMDA receptor neurotoxicity is prevented mostly by the action of the powerful glycine transporter GlyT1 (10), which is expressed mainly in glial cells (54, 55). In support of this hypothesis, complete removal of D-serine from our neuronal cultures was only partially protective against NMDA-elicited neurotoxicity (Fig. 6*D*). This is probably attributable to the activation of NMDA receptors by glycine.

The regulated Ca^{2+}-dependent release of D-serine, such as that promoted by ionotropic glutamate receptor agonists (Fig. 7), supports an important role for D-serine. D-Serine release was insensitive to prolonged incubation with bafilomycin A_1, a potent and selective blocker of the vesicular H^+-ATPase (Fig. 8). Bafilomycin A_1 blocks the generation of the electrochemical proton gradient across the vesicles, which leads to quick release of previously accumulated transmitters (56) and exocytosis of empty vesicles (57). In addition, D-serine uptake in purified brain synaptic vesicles was not detectable under conditions that are optimal for the uptake of known neurotransmitters (Fig. 8*C*). This is compatible with the very high specificity of the vesicular transporters. Thus, D-serine release under our experimental conditions is most probably from a cytosolic non-vesicular pool.

Interestingly, similar to D-serine, other amino acids can display a Ca^{2+}-dependent release from either neuronal or glial cells that is of non-vesicular origin. This has been very recently demonstrated for L-aspartate (58) as well as for L-glutamate (59). However, our results cannot exclude the possibility that D-serine release also occurs through a subset of vesicles exhibiting a "non-classical" D-serine uptake system (*i.e.* that does not depend on the electrochemical proton gradient to operate and that cannot be detected by conventional transport studies).

Our demonstration that NMDA can induce neuronal D-serine release sheds light on a recent puzzling observation that NMDA promoted D-serine release in the striatum by *in vivo* microdialysis, despite the scarce presence of NMDA receptors in astrocytes (60). NMDA is known to elicit a predominant Ca^{2+} influx into the cells, and its effect on D-serine release was blocked by removal of external Ca^{2+} (Fig. 7). AMPA/kainate receptor activation also increases intracellular Ca^{2+} either through opening Ca^{2+}-permeable receptors (61, 62) or by favoring the reverse mode of the Ca^{2+}/Na^+ exchanger (63). In this context, it is possible that Ca^{2+} influx is required for D-serine release through a still unidentified amino acid transporter or channel. Additional studies will be required to unravel the pathway(s) for D-serine release.

Glial Versus Neuronal D-Serine—Our study raises major questions regarding the relative roles of the neuronal *versus* glial pool of D-serine. Because neurons expressing serine racemase also contain NMDA receptors, it is conceivable that neuronal D-serine exerts an autocrine or paracrine activation of NMDA receptors. The presence of neuronal D-serine does not imply that glia play a less important role. Our micrographs typically highlight neurons because they are much larger than glia, but D-serine and serine racemase are present in most, if not all, glia. We detected clear neuronal D-serine only in the cerebral cortex, whereas in other brain areas, glial staining was predominant. In these other areas, glial D-serine will play a more prominent role. Moreover, because glial cells outnumber neurons in the brains of higher mammals, the glial pool of D-serine is most likely larger than the neuronal pool, even in the cerebral cortex. As proposed previously by Snyder and co-workers (16, 64), glial D-serine should be important in modulating NMDA receptor activity by neuronal/glial cross-talk. Because neuronal/glial communication plays a key role in neurotransmission (65), glial D-serine would be anticipated to be important in most, if not all, NMDA-dependent processes. Bidirectional neurotransmitter fluxes between neurons and glia have been demonstrated for the neurotransmitter glutamate (65). Because glutamate induces release of D-serine from both astrocytes and neurons, it is possible that the direction of glutamate fluxes between these cells will determine the direction of D-serine fluxes as well.

By adopting a more liberal conceptualization of a neurotransmitter, Snyder and Ferris (19) first suggested a transmitter role for glial D-serine. Very recently, this proposal was strengthened by data suggesting that D-serine may be released through exocytosis upon AMPA/kainate receptor activation in cultured glial cells (18). Although we confirmed the presence of serine racemase in glia, our results indicate that D-serine is a neuronal/glial rather than a specific glial coagonist. Nevertheless, our results do not support a role for D-serine as a conventional neurotransmitter. Whether or not D-serine satisfies all the criteria for a neurotransmitter, its coagonist action on the NMDA receptors indicates an important physiological role (64). Similar to D-serine, several physiologically relevant neural modulators (*e.g.* NO and CO) are not accumulated in synaptic vesicles, but play prominent roles in signaling in the nervous system (64).

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