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D-Serine and Serine Racemase are Localized to Neurons in the Adult Mouse and Human Forebrain

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Abstract

D-Serine, a co-agonist at the NMDA receptor (NMDAR), is synthesized from L-serine by the enzyme serine racemase (SR), which is heavily expressed in the forebrain. Although SR was originally reported to be localized exclusively to astrocytes, recent conditional knock out results demonstrate that little SR is expressed in forebrain astrocytes. As a consequence, the cellular location of its product, D-serine, in the brain is also uncertain. Immunocytochemistry now indicates that SR is expressed primarily in forebrain glutamatergic neurons with the remainder in GABAergic interneurons. We utilized SR deficient (SR^{-/-}) mice, which have <15 % of normal D-

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Conflict of interest JTC has served as a consultant for EnVivo, and Abbvie in the last 2 years. A patent owned by Massachusetts General Hospital for the use of D-serine as a treatment for serious mental illness could yield royalties for Dr. Coyle. The remaining authors have no conflict of interest.

serine levels, to validate and optimize a D-serine immunohistochemical method. Nearly all of the D-serine in neocortex and hippocampus (HP) is found in neurons, with virtually no D-serine co-localizing with two astrocyte markers. Interestingly, only a subset of the D-serine positive neurons contained SR in the neocortex and HP. Greater than half of the D-serine positive neurons were GABAergic interneurons, with a majority of these neurons containing parvalbumin and/or somatostatin. Only ~25–40 % of interneurons expressed SR in the neocortex and HP. Finally, we demonstrate in human *post-mortem* neocortex that SR is found in both excitatory and inhibitory neurons, but not in S100 β -containing astrocytes. In sum, these findings conclusively demonstrate that the majority of D-serine is both synthesized and stored in neurons. It will be important to determine the functional significance for the separation of synthesis and storage of D-serine in neurons, as well as the presence of this NMDAR co-agonist in GABAergic interneurons.

Keywords

NMDA receptor; Parvalbumin; Somatostatin; CAMKII α ; Hippocampus; Cortex

Introduction

D-Amino acids are now well established as mediators and modulators of neuronal activity in mammals (Boehning and Snyder 2003; Wolosker et al. 2008). Activation of the N-methyl-D-aspartate receptor (NMDAR) requires the binding of either glycine or D-serine at the glycine modulatory site (GMS) on the GluN1 subunit (Kishi and Macklis 2004). D-Serine is enriched in corticolimbic regions of the brain, where its localization closely parallels that of NMDARs (Schell et al. 1995). Therefore, D-serine is thought to be the primary NMDAR co-agonist in the forebrain because of its high concentration in this region and because elimination of synaptic D-serine reduces NMDAR-mediated currents (Mothet et al. 2000; Schell et al. 1995). Recent evidence also suggests that D-serine is the primary co-agonist for synaptic, but not extra-synaptic NMDARs in the hippocampus (HP) (Papouin et al. 2012), via non-vesicular release through alanine-serine-cysteine-1 (Asc-1) transporters (Rosenberg et al. 2013).

The cloning and characterization of serine racemase (SR) demonstrated that D-serine is synthesized endogenously in the mammalian brain through the conversion of L- to D-serine (Wolosker et al. 1999). Deletion of the SR gene reduces cortical D-serine levels by ~85 % (Basu et al. 2009). Initial in vitro studies and immunohistochemical studies suggested that SR was present mainly in astrocytes, which, therefore, were the major source of D-serine in the brain (Mothet et al. 2005; Schell et al. 1995; Wolosker et al. 1999). However, recent immunohistochemical studies have suggested a more prominent neuronal expression of SR (Ding et al. 2011; Miya et al. 2008; Ehmsen et al. 2013). Furthermore, using mice with conditional SR deletions either in excitatory forebrain neurons (nSR $^{-/-}$) or astrocytes (aSR $^{-/-}$), we have demonstrated that the majority (~65 %) of SR is expressed in forebrain excitatory neurons, particularly in the neocortex and HP, and that 15 % or less is expressed in astrocytes (Benneyworth et al. 2012). Notably, the cellular localization of SR has not been demonstrated in human brain.

The cellular location of D-serine itself also is not well characterized. Some immunohistochemical studies, both from brain tissue and cell culture, suggest a primary localization in astrocytes (Fossat et al. 2012; Schell et al. 1995; Martineau et al. 2013), while other studies in rats found that D-serine was located primarily in neurons (Kartvelishvily et al. 2006; Curcio et al. 2013). However, the specificity of D-serine immunostaining in brain tissue has yet to be established. Thus, we utilized SR^{-/-} mice as a negative control to optimize D-serine staining and used this new protocol to accurately quantify the cellular localization of D-serine, as well as SR, in the rodent brain. Finally, we demonstrate the cellular location of SR in the human brain, which comports with the findings in mice.

Materials and Methods

Animals

Constitutive SR^{-/-} mice (Basu et al. 2009) were generated as previously described. SR[±] sires and dams were bred to produce wild-type (WT), as well as SR^{-/-} offspring. Adult male mice (3–5 months old) were used for all experiments. Animals were group housed in polycarbonate cages and maintained on a 12:12 h light/dark cycle in a temperature (22 °C) and humidity-controlled vivarium. Animals were given access to food and water ad libitum. All animal procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee.

Drugs

SR^{-/-} mice received a single subcutaneous (s.c.) injection of D-serine (300 mg/kg; Sigma-Aldrich, St. Louis, MO) at a volume of 5 ml/kg dissolved in 0.9 % sodium chloride. Mice were perfused 2 h following the injection.

Antibody Characterization

See Table 1 for a complete list of antibodies and working concentrations. The rabbit polyclonal antibody to D-serine (cat # ab6472; Abcam) was characterized in this manuscript by immunohistochemistry using SR^{-/-} mice.

Amino Acid–BSA conjugation

L- or D-Serine (100 mM) was dissolved in 1 ml of a 1.5 M sodium acetate solution (pH 8). Then, 500 µl of a 0.5 M glutaraldehyde (25 % = 2.5 M stock) solution was added for 5 min, with stirring. The mixture containing activated L-serine was mixed with 1 ml of a 1.5 M acetate solution (pH 8) containing 15 mg of BSA and was left to react for 10 min with stirring. 500 µl of a 0.1 M sodium borohydride solution was then added for 5 min with stirring. The conjugate was subject to overnight dialysis (Spectrum Laboratories; 12–14 kDa MWCO; Before use, the dialysis membrane was boiled several minutes in a large excess of 10 mM sodium bicarbonate, followed by several minutes in boiling 10 mM sodium EDTA. This was repeated and the membrane was washed several times in distilled water) at 4 °C in 0.1 M sodium phosphate buffer (pH 7.6). Insoluble material was then removed by centrifugation at 10,000×g for 15 min.

D-Serine Immunohistochemistry

Tissue for immunohistochemical studies was obtained from transcardially perfused mice. Animals were deeply anesthetized with sodium pentobarbital (180 mg/kg, i.p.) and perfused with 0.1 M phosphate-buffered saline followed by a fixative of 3 % glutaraldehyde (25 % stock; Fisher Scientific), 1 % paraformaldehyde (16 % stock; Electron Microscopy Sciences), 0.2 % sodium metabisulfite (Sigma Aldrich), and 10 U/ml Heparin salt (Sigma Aldrich). Brains were post-fixed for 24 h in fixative and cryoprotected in 30 % sucrose. Immunohistochemistry was performed on 20 µm free-floating sagittal or coronal sections. Sections were treated with freshly made 0.2 % sodium metabisulfite (Sigma Aldrich) and 0.5 % sodium borohydride (Sigma Aldrich) for 10 min (to reduce the free aldehydes). Sections were treated for 60 min with blocking solution (0.02 M Tris-buffered saline (TBS) containing 10 % normal goat serum and 0.1 % Triton X-100).

D-Serine was localized using a primary antibody of rabbit origin (1:60K) diluted in blocking solution including L-serine–glutaraldehyde–BSA conjugate (10–200-fold dilution of 100 mM dialyzed stock). Incubation in primary antibody was done for ~40 h at 4 °C. Sections were incubated with biotinylated secondary antibody (1:1,000) for 90 min and Elite ABC reagent (1:100 dilution of each reagent in TBS w/0.1 % Triton X-100, ABC Elite kit, Vector Laboratories) for 60 min. Colorimetric detection was performed with 3,3-diaminobenzidine (0.02 %) enhanced with nickel (II) sulfate (0.08 %) in 0.1 M phosphate buffer containing 0.01 % hydrogen peroxide. In between each incubation step, sections were washed 3 times for 10 min each in 0.02 M TBS [except prior to the metabisulfite/borohydride incubation and colorimetric detection when only 0.1 M phosphate buffer (PB) was used]. Experimental and corresponding control samples were processed in parallel. Immunostaining was visualized on a Zeiss Axioskop microscope using StereoInvestigator software (MBF Bioscience; Welliston, VT) to capture the digital images under constant conditions for subjects of each comparison. For the 20× brain region images, multiple overlapping images were brought into register using Photoshop CS5 (Adobe Systems Inc., San Jose, CA) to create the resulting collage images.

D-Serine Immunofluorescence

Brain sections were treated with Schiff's Reagent (Sigma Aldrich) for 20 min at room temperature with shaking, followed by washing with 0.1 M hydrochloric acid containing 0.5 % sodium metabisulfite for 10 min at room temperature. The sections were then incubated 4× with freshly made 0.2 % sodium metabisulfite and 1.0 % sodium borohydride for 15 min each wash, in order to decolorize the sections. Sections were washed 3× in TBS and then incubated for 60 min with blocking solution (10 % normal goat serum and 0.1 % Triton X-100). D-Serine was localized using a primary antibody of rabbit origin diluted in blocking solution containing a 10-fold dilution of 100 mM L-serine–glutaraldehyde–BSA conjugate. For neuronal co-localization, sections were also incubated with mouse anti-NeuN. For astrocyte co-localization, sections were incubated with mouse anti-GFAP or mouse anti-S100β. Mouse anti-GAD67, mouse anti-parvalbumin, and rat antisomatostatin were used to label GABAergic neurons. Incubation in primary antibodies was done overnight with agitation at room temperature. Sections were washed 3× in TBS and then incubated with biotinylated secondary antibody for 90 min at room temperature. After washing 3× in TBS,

sections were incubated in the dark with streptavidin Alexa Fluor-488 and species appropriate secondary Alexa Fluor-555 IgG (H+L) (1:500 or 1:3,000 for GFAP) or Alexa Fluor-647 IgG (H+L) for 90 min at room temperature. After washing, sections were mounted on slides, allowed to dry for at least 10 min, and coverslipped using ProLong Gold Antifade Reagent (Life Technologies). Immunofluorescence was visualized on a Zeiss Axio ImagerM2 equipped with an AxioCam MRm camera and Apotome 2.0. Immunofluorescent images appearing in figures were obtained using a Zeiss Axio ImagerM2 at 40 $\times$. Pseudocoloring was performed in StereoInvestigator. In PowerPoint 2011, the images were adjusted for contrast and brightness and were cropped to improve the display of the regions of interest.

Quantification of D-Serine Localization

D-Serine positive cells were counted in the prefrontal cortex (PFC), primary somatosensory barrel field cortex (S1), and HP. From each mouse ($n = 3$), cells were counted from 4 to 5 sections per region that spanned across the rostral-caudal axis according to Paxinos and Franklin, 2001 (PFC 2.34 to 1.54, Cg1, PrL, IL; S1 0.38 to -1.94; HP -1.34 to -2.5), and were counterbalanced for hemisphere. For each section, all the D-serine positive cells were counted in the defined region at 20 $\times$ and it was then determined whether these same cells co-localized with the other cellular marker. The determination for whether a cell was positive or not for each marker was made in the same focal plane. Due to the almost complete lack of D-serine staining in astrocytes, only one section per brain region per mouse ($n = 3$) was used to quantify D-serine co-localization with S100 β and GFAP. Furthermore, due to the restricted expression of GFAP in the cortex, D-serine co-localization was only assessed in the areas of defined cortex that contained GFAP positive cells. Regions of interest and cell counts were performed using StereoInvestigator (MBF Bioscience; Williston, VT).

Quantification of SR Localization and Density

Due to the high density of SR positive cells, all of the PV and SOM cells were first counted in the HP [not including *stratum pyramidale* and the granule cell layer (GCL)], PFC, and S1 ($n = 3$ mice; 4–5 sections per region). It was then determined whether these PV and/or SOM cells co-localized with SR. The density of SR cells in the analyzed PFC and S1 sections (4–5 sections per region) was stereologically estimated at 20 $\times$ using the Optical Fractionator module of StereoInvestigator (MBF Bioscience, Williston, VT). The chosen grid size (PFC 250 $\times$ 250 μ m; S1 400 $\times$ 400 μ m) and counting frame (100 $\times$ 100 μ m) estimated densities with high precision (coefficient of error <0.07). The density of PV and SOM cells was calculated by dividing the total number of counted cells by the area of the analyzed region.

Human Post-Mortem Immunofluorescence

Formalin-fixed primary motor cortex (BA4) brain tissue from 3 male control cases (AN18870, AN10180, AN16218) was provided by the Harvard Brain Tissue Resource Center that is supported in part by PHS Grant number R24-MH068855. Case AN18870 was 58-years old, died of cancer with a post-mortem interval (PMI) of 20.5 h, and the brain tissue was in formalin for 1.3 years. Case AN10180 was 73-years old with a PMI of 24 h,

and the brain tissue was in formalin for 1.3 years. Case AN16218 was 72-years old with a PMI of 27.7 h, and the brain tissue was in formalin for 3.3 years. Tissue blocks were cryoprotected (0.1 M PB, 33 % glycerol, 33 % ethylene glycol) for 14 d at 4 °C and then transferred to −20 °C. Brain tissue was cut at a thickness of 40 µm on a freezing microtome and sections were stored in cryoprotectant at −20 °C. All solutions were made in 0.1 M PBS with 0.5 % TritonX-100 (washing solution) except where noted. The tissue sections were washed 3× for 5 min between all steps. Tissue sections were washed and then incubated in a citric buffer (0.1 M citric acid, 0.2 M Na₂HPO₄ made in water) antigen unmasking solution. The sections were placed into citric buffer that was heated to 80 °C and then allowed to cool for 20 min without agitation. Sections were washed, incubated in a water solution containing 0.3 % H₂O₂ and 10 % methanol, and washed again. The tissue was blocked at room temperature in 2 % BSA and then placed in blocking solution containing the primary antibodies for two nights at 4 °C. Sections were washed and placed in a 1 % BSA solution containing biotinylated secondary antibody for 2 h at room temperature. Following washing, the sections were placed in a 1 % BSA solution containing a streptavidin–fluorophore conjugated antibody and a species appropriate fluorophore conjugated secondary antibody for 4 h at room temperature. Lipofuchsin autofluorescence was quenched by placing the tissue in a cupric sulfate (0.5 M sodium acetate, 0.001 M cupric III sulfate in water) solution for 10 min at room temperature. After washing in 0.1 M PB, sections were mounted on slides, allowed to dry for at least 10 min, and cover slipped using ProLong Gold Antifade Reagent (Life Technologies).

Results

Optimized Immunostaining Protocol for D-Serine in Brain Tissue

We first wanted to validate the specificity of the D-serine antibody for immunohistochemistry using brain tissue from our constitutive SR knockout mouse (SR^{−/−}), the cortex of which has <15 % of the D-serine levels of wild-type (WT) mice. Our initial validation was based on a protocol used to demonstrate that D-serine was found in neurons (Kartvelishvily et al. 2006). This protocol used glutaraldehyde as the main fixative, as the antibody was created using a D-serine–BSA glutaraldehyde conjugate (D-ser–BSA), and included a 0.1 mM L-ser–glutaraldehyde conjugate to block the antibody from non-specifically binding L-serine in the tissue sections. However, when 200 (Fig. 1a, b) or 20-fold (Fig. 1c, d) dilutions of a dialyzed 100 mM L-serine–BSA conjugate were used, there was intense cellular staining in SR^{−/−} tissue, although less intense than staining in WT tissue. Therefore, we increased the concentration of the conjugate in the primary antibody incubation to determine if the staining in SR^{−/−} mice was due to non-specific L-serine binding. A 10-fold dilution of 100 mM L-serine–BSA allowed for detection of D-serine in WT tissue (Fig. 1e) and prevented staining in SR^{−/−} tissue (Fig. 1f). Furthermore, fixation conditions, which did not include glutaraldehyde (4 % paraformaldehyde) fixation and L-serine–BSA blocking during the primary antibody incubation, resulted in identical staining across WT and SR^{−/−} subjects (data not shown).

Distribution of D-Serine in the Adult Brain

With this new validated immunostaining protocol, we next wanted to assess the distribution of D-serine across brain regions. In the several cortical brain regions that we investigated (Fig. 2a–d), primary somatosensory barrel field cortex (S1), primary motor cortex (M1), medial prefrontal cortex (mPFC), and primary visual cortex (V1), D-serine was evenly distributed across cortical laminae. In the HP (Fig. 2e), there was dark staining in the ventral molecular layer (Mol) of the dentate gyrus (DG) and cellular staining in the hilus (Hi), *stratum radiatum* (s.r.), *stratum oriens* (s.o.), and *lacunosum moleculare* (l.m.). Interestingly, there was very limited cellular staining in the *stratum pyramidale* (s.p.) or GCL, which is consistent with what was observed in prior studies even when L-serine–glutaraldehyde conjugate was excluded from the primary antibody incubation (Schell et al. 1997; Schell et al. 1995; Wolosker et al. 1999).

D-Serine is Primarily Expressed in Neurons

Due to the uncertainty of whether D-serine is stored in astrocytes or neurons, immunofluorescence was used to determine the cellular location of D-serine. First, we validated our D-serine immunofluorescence protocol and demonstrated the absence of D-serine staining in: WT tissue lacking primary antibody (Fig. 3a), SR^{−/−} mouse brain (Fig. 3b, c), and in WT cerebellum (Fig. 3d–f). Streptavidin-conjugated fluorophores must be used as avidin conjugated antibodies produce “cellular-like” non-specific staining in glutaraldehyde fixed tissue in the absence of primary antibody (data not shown). We found that in S1 and HP (Fig. 3g–i) subfields that nearly all (Table 2; 83–96 %) of the D-serine positive cells co-localized with the neuronal marker, NeuN. In the mPFC and S1, D-serine cells are evenly distributed across laminae, although in S1 there appears to be fewer cells in layer I and enrichment in layer IV. In the dorsal and ventral HP, D-serine cells were concentrated in the s.o and s.r. of CA1/CA3 and in the Hi and Mol of the DG, as well as subiculum. There were, however, sporadic D-serine positive cells located in s.p. and GCL.

In contrast to what was found with NeuN, there was negligible co-localization (Table 2; 0–6 %) of D-serine in all of the brain regions examined with two astrocyte markers, S100β (Fig. 4a–c) and GFAP (Fig. 4d–f). S100β labeled a greater proportion of astrocytes and was more evenly distributed throughout the brain than GFAP (data not shown). The lack of GFAP staining in intermediate cortical layers was due neither to glutaraldehyde fixation nor primary antibody (data not shown).

We next wanted to demonstrate that our protocol does not bias against astrocytic staining. Therefore, we peripherally injected SR^{−/−} mice with 300 mg/kg D-serine, a dose that restores cortical D-serine levels (Balu et al. 2013), and then stained for D-serine, since nearly all of the amino acid would have to pass through astrocytes from the bloodstream into the brain. In SR^{−/−} mice perfused 2 h after injection, there was D-serine staining in both astrocytes, primarily perivascular (Fig. 5a–c), and in neurons (Fig. 5d–f).

D-Serine is Found in Some, But Not All SR-Expressing Neurons

To date, no studies have examined the co-expression of SR and D-serine in brain tissue. The SR antibody used here was previously validated in SR^{−/−} mice (Miya et al. 2008). Under the

current fixation conditions, the antibody recognized SR and produced a pattern of expression consistent with the literature, in that there was high forebrain expression with little to no hindbrain expression and no labeling in SR^{-/-} mice (data not shown). As shown in Table 2, only ~50 % of D-serine positive cells expressed SR in the PFC and S1 (Fig. 6a–c). The amount of co-localization was even lower in the HP, ranging from 23 to 41 %. The DS/SR cells in the HP were located outside of the s.p. and GCL.

D-Serine is Abundantly Found in GABAergic Neurons

The location and morphology of many of the D-serine-containing neurons, particularly in the HP, suggested that they were gamma amino butyric acid (GABA) interneurons. We first used an antibody against the 67 kDa isoform of the enzyme glutamic acid decarboxylase (GAD67), which is responsible for producing the majority of cortical GABA.

Approximately 50–60 % of D-serine positive neurons co-localized with GAD67 in the PFC and S1 (Fig. 7a–c), while there was approximately 75 % co-localization in the HP (Fig. 7d–f). GAD67/DS neurons were found in the deeper cortical layers (IV–VI) in S1. In the hippocampal formation, a majority of the GAD67/DS cells were found in s.o., s.r., and Mol, with fewer cells located in l.m. and Hi. There were also GAD67/DS neurons in s.p. and at the base of the GCL.

There are many types of GABAergic neurons that are classified according to various characteristics, including morphology, electrophysiology, calcium-binding protein expression, and co-localized neuropeptides (DeFelipe et al. 2013). We used parvalbumin (calcium-binding protein; PV) and somatostatin (neuropeptide; SOM) to identify in which population(s) of GABA neurons D-serine is localized (Fig. 8). Triple labeling for D-serine, PV, and SOM revealed that in the PFC and S1, respectively, 30 and 90 % of D-serine positive neurons expressed PV and/or SOM (Fig. 9a). Consistent with previous reports, there was virtually no overlap between PV and SOM in the cortex (Rudy et al. 2011; Xu et al. 2010). The lower percentage of D-serine cells containing PV or SOM in the PFC was because there are fewer PV and SOM GABAergic neurons located in the PFC. In addition, the higher overlap of D-serine with PV or SOM in S1 cortex as compared to GAD67 reflects difficulty in determining co-localization due to intense GAD67 neuropil staining in the intermediate cortical layers. When PV and SOM, respectively, were used as the primary markers in the PFC, 30 and 10 % of these neurons contained D-serine, while in S1, 50 % of PV and 17 % of SOM neurons had D-serine (Fig. 9b).

Due to the discrete localization of GABAergic neuron populations to different layers in the HP (Klausberger 2009; Houser 2007), we assessed the co-expression of D-serine within the different layers of each subfield. First, we found that a majority of D-serine cells were located in the Hi and Mol of the DG, as well as in the s.o. and s.r. of CA3 and CA1 (Fig. 9c, e, g). In the Hi, GCL, and Mol 35, 47, and 7 %, respectively, of D-serine cells contained PV and/or SOM (Fig. 9d). In s.o. and s.p. of CA3 and CA1, 75 % of D-serine cells expressed PV and/or SOM, while only 30 and 11 % were localized with these GABAergic markers in s.r. of CA3 and CA1, respectively (Fig. 9f, h). The low percentages of D-serine overlap with PV/SOM in the s.r. and l.m. were due to the lack of PV/SOM-expressing neurons in these laminae, which is in agreement with the literature (Klausberger 2009).

Inhibitory Neurons Express SR

Given the abundant expression of D-serine in cortical and hippocampal GABAergic neurons, we examined whether these same cells also express SR. Although we found that 25–30 % of PV and SOM neurons contain SR (Fig. 10a), they represent only a small subpopulation of the total number of SR-containing neurons. The estimated density of all SR neurons in the PFC and S1 is 900–1,200 cells/mm², while the total number of PV and SOM cells is only 75–200 (Fig. 10b). These numbers are in agreement with the estimate that inhibitory neurons constitute ~10–20 % of all neurons in the cortex (Rudy et al. 2011). In the HP, SR expression was very high in excitatory neurons of the GCL and in the s.p. of CA1 and CA3 (data not shown), as previously reported. We counted PV and SOM neurons first and then determined what percentage of these cells also expressed SR. Most of the PV and/or SOM neurons were located in the Hi and GCL of the DG, and in s.o. and s.p. of CA3 and CA1 (Fig. 10c, e, g). The percentage of SR-expressing PV and SOM cells varied across subfield and lamina. In the DG, co-localization (25 %) was mainly restricted to SOM neurons in the Hi and Mol (Fig. 10d). In CA3 and CA1, 30 % of PV and/or SOM neurons expressed SR in s.o. and s.p. (Fig. 10f, h). There was also some co-localization in s.r and l.m., but the number of PV and SOM neurons in these layers was much lower than in s.o. and s.p.

Excitatory and Inhibitory Neurons Express SR in Human Cortex

Finally, we wanted to determine the cellular localization of SR in the human brain. We tested three different SR antibodies in paraformaldehyde-fixed human primary motor cortical (BA4) tissue and found that only the antibody directed against human SR was effective for immunohistochemistry (Fig. 11a). As immunostaining with this antibody has never been published, we validated its specificity for SR in WT and SR^{-/-} tissue (Fig. 11b, c). There was intense SR staining in pyramidal neurons, as well as in other neuronal cell types. Therefore, we used immunofluorescence to determine whether SR is expressed in neurons (calmodulin kinase II alpha (CAMKII α), excitatory neurons; PV, inhibitory neurons) or astrocytes (S100 β). We found that SR was expressed in both excitatory (Fig. 12a–c) and inhibitory (Fig. 12d–f) neurons, but not in S100 β -containing astrocytes (Fig. 12g–i).

Discussion

Although SR was initially thought to be an astrocytic enzyme, it is now known that it is located almost exclusively in neurons in the forebrain (Benneyworth et al. 2012). However, the cellular location of D-serine in the brain still remains unresolved. Thus, we utilized SR^{-/-} mice to validate a D-serine immunohistochemistry protocol and determined that D-serine is almost exclusively stored in neurons, particularly GABAergic neurons in the neocortex and HP. We also showed that SR and D-serine co-localized within the same cell, although there was a discrepancy between the sites of D-serine synthesis and storage. Finally, we demonstrate for the first time that SR is found in both excitatory and inhibitory neurons, but not astrocytes, in human *post-mortem* neocortex.

We utilized brain tissue from SR^{-/-} mice, which have <15 % of normal D-serine levels, as a negative control to determine the specificity of our immunohistochemical protocol. We

established that the brains need to be fixed with glutaraldehyde and a high concentration of L-serine–BSA conjugate must be included during the primary antibody incubation to block binding of the D-serine antibody to tissue L-serine. The first studies (Schell et al. 1997; Schell et al. 1995; Wolosker et al. 1999) showing astrocytic D-serine localization used a D-serine antibody generated by Schell et al. (1995) and did not use the L-serine blocking conjugate, while more recent studies (Fossat et al. 2012; Martineau et al. 2013) used a GemacBio antibody and paraformaldehyde as the primary fixative, and did not include the L-serine–BSA blocking conjugate. The different staining patterns obtained for D-serine between our study and previous studies are likely due to differences in fixation and the inclusion of high concentrations of blocking L-serine–BSA. The D-serine antibody is derived from immunizing rabbits with glutaraldehyde conjugated D-serine–BSA. Thus, glutaraldehyde needs to be the fixative as it is part of the epitope recognized by the D-serine antibody. In addition, paraformaldehyde is not very effective in retaining amino acids inside cells, which are rapidly released during fixation (Aprison 1990). The source of the antibody does not likely contribute to the staining differences, as neuronal D-serine localization was found using the Schell et al. 1995 and MoBitec (same antibody sold by GemacBio and Abcam) antibodies (Kartvelishvily et al. 2006; Ehmsen et al. 2013) along with 0.1–0.2 mM L-serine glutaraldehyde conjugates. It is also possible that low amounts of D-serine in small astrocytic processes or other cell populations might be below the sensitivity of our immunohistochemistry protocol.

Interestingly, we found that a majority of cortical D-serine-containing neurons were in fact GABAergic, as determined by co-localization with the enzyme GAD67. Because there are numerous subtypes of GABAergic interneurons, we further characterized which type of inhibitory neurons contained D-serine based on the presence of the calcium-binding protein PV and neuropeptide SOM. In the mouse S1 cortex, PV and SOM: label distinct cell populations, are found almost exclusively in layers II–VI, and account for approximately 60–70 % of all GABA neurons (Rudy et al. 2011; Xu et al. 2010). We found that in S1 cortex, 87 % of the D-serine positive cells contained PV and 6 % contained SOM. Although we could not discriminate between the classes of PV neurons with our staining, we hypothesize that basket cells would be the predominant D-serine-containing population, since their axons target proximal dendrites of pyramidal neurons, where NMDARs would be present. Basket cells are likely the dominant inhibitory system in the cortex and have been implicated in maintaining fast (gamma frequency) cortical rhythms (Cardin et al. 2009) and controlling excitation/inhibition balance. Therefore, if basket cells could also release D-serine, this would be another mechanism by which an important population of GABAergic neurons could regulate network activity by enhancing their NMDAR responsiveness to pyramidal neuron afferents. SOM expression in the neocortex has been most associated with Martinotti cells which have axons that innervate dendrites of pyramidal cells. Future studies will be needed to determine whether inhibitory neurons can in fact release D-serine, a process that has been shown to occur in the HP via neuronal Asc-1, a high affinity transporter of D-serine (Rosenberg et al. 2013).

Similar to the cortex, D-serine positive cells in the HP were also predominantly GABAergic. Immunostaining with PV and SOM highlighted the subfield and laminar-specific differences across interneuron populations. In the DG, a majority of D-serine positive cells that

expressed PV were located in the GCL and to a lesser extent the Hi, while SOM-containing D-serine neurons were found almost exclusively in the Hi. PV cells in the DG are predominantly basket and chandelier cells that target excitatory granule cells. SOM neurons are all virtually located in the Hi and synapse primarily with granule cell dendrites in the outer molecular layer where perforant path (pp) fibers terminate, ideally positioning these neurons to modulate the influence of the pp on dentate granule cell activity (Houser 2007). In s.o. of CA3 and CA1, the majority of D-serine neurons expressed PV and/or SOM, while in s.p. almost all were only PV positive. Pyramidal cells in CA1 receive 92 % of their GABAergic input onto dendrites (Megias et al. 2001). The inhibitory PV and/or SOM-containing neurons in s.o. project to the dendrites of CA1 pyramidal cells (Klausberger 2009). These D-serine-containing inhibitory neurons are in a prime location to regulate hippocampal activity in all components of the circuit. The ability of these inhibitory neurons to also release D-serine in an activity-dependent manner would add another layer of regulation. Future studies will be needed to understand the functional consequences of D-serine-containing GABA neurons in the HP.

SR is the enzyme that catalyzes the racemization of L-serine to D-serine. However, we found that only 50 % of D-serine positive cells expressed SR in the cortex. In the HP, the co-expression varied depending on the subfield and lamina, but was generally around 25–40 %. In addition to racemization, SR also catalyzes the α - β elimination of water from both L- and D-serine to generate pyruvate and ammonia, with the latter reaction prevailing over the former (Foltyn et al. 2005). Under physiological conditions, SR would consume both enantiomers by α - β elimination and prevent the accumulation of D-serine (Wolosker 2011). Thus, it has been proposed that SR and D-serine need to be compartmentalized, with neuronal SR producing the majority of D-serine that is then transported to astrocytes for activity-dependent release (Wolosker 2011). Our results support this hypothesis with the important exception that D-serine is not being shuttled between neurons and astrocytes, but rather D-serine is being shuttled between neurons, consistent with recent electrophysiological findings (Rosenberg et al. 2013). In S1 cortex and HP, nearly all of the D-serine were found in PV and/or SOM neurons, while only 25 % of these inhibitory neurons expressed SR. This percentage of inhibitory neurons represents a small percentage (2 % in cortex) of the total SR neuron population, which is comprised mostly of excitatory neurons. Presumably this D-serine transport would occur via Asc-1, which is expressed exclusively on neurons and is localized to cell bodies and dendritic fields (Helboe et al. 2003).

Finally, we determined whether what we observed in mice with regard to SR localization in the brain translated to humans. We found that similar to mice, SR was primarily expressed in excitatory neurons and to a lesser extent in PV+inhibitory neurons, but not in S100 β -containing astrocytes from human *post-mortem* BA4 cortex. To our knowledge, only one other study has performed SR immunohistochemistry in human *post-mortem* tissue (Verrall et al. 2007). This study found hippocampal and cortical SR expression to be greater in white matter than in gray matter, which is inconsistent with what is observed in rodents and what we found in human cortex. Our studies differed in the SR antibody used, tissue storage, as well as in the sectioning, antigen unmasking, and staining of tissue. Future work examining

other brain regions and using additional cell-specific markers will more fully establish the location of SR throughout the human brain.

In summary, our findings demonstrate that D-serine and SR are located almost exclusively in neurons in the neocortex and HP of the adult mouse. In addition, the co-expression of SR with excitatory and inhibitory neurons in the human cortex suggests that SR is a predominantly neuronal enzyme in the mammalian brain. Most of the D-serine is found in GABAergic neurons, particularly those containing PV and SOM. This finding could have major implications on how GABAergic neurons regulate forebrain activity, especially given the important role PV neurons play in controlling gamma oscillations (Cardin et al. 2009). Furthermore, our findings could provide a possible link between reduced NMDAR activation and GABAergic dysfunction, which are thought to underlie such disorders as schizophrenia (Balu and Coyle 2011).

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Fig. 1.

Optimized conditions for D -serine immunohistochemistry in adult rodent brain. Sagittal brain sections ($\times 10$) from a wild-type (WT; **a, c, e**) and $SR^{-/-}$ **b, d, f** mouse incubated with D -serine antibody and increasing concentrations of L -ser-BSA. Low concentrations of **a, b** 200-fold and **c, d** 20-fold dilutions of 100 mM L -ser-BSA produce intense cellular staining in WT and $SR^{-/-}$ tissue. **e, f** A 10-fold dilution of 100 mM L -ser-BSA is required to block non-specific binding of the D -serine primary antibody to L -serine in the glutaraldehyde fixed mouse brain tissue, as demonstrated by the lack of cellular D -serine staining in $SR^{-/-}$ tissue. Scale bars represent 50 μ m. L -ser-BSA L -serine-bovine serum albumin glutaraldehyde conjugate

Fig. 2.

Regional distribution of D -serine in the adult brain. Sagittal brain sections from wild-type (WT) mice demonstrating the distribution of D -serine positive cells in the **a** S1, **b** M1, **c** mPFC, **d** V1, and **e** hippocampus. There is an even distribution of D -serine positive cells across layers I through VI of the cortical laminae. In the hippocampus, cellular D -serine staining occurs in the Hi, s.o., s.r., and Mol layers. However, there was almost no cellular staining in the GCL or in the s.p. *Hi* hilus, *GCL* granule cell layer, *M1* primary motor cortex, *mPFC* medial prefrontal cortex, *Mol* molecular layer, *S1* primary somatosensory cortex, s.o., *stratum oriens*, s.p. *stratum pyramidale*, s.r. *stratum radiatum*, *V1* primary visual cortex. Each panel represents tiled $\times 20$ images

Fig. 3.

D-serine is found mainly in neurons of the adult brain. Representative coronal brain sections ($\times 20$) from adult WT and SR^{-/-} mice. There is no immunofluorescent D-serine signal when **a** primary antibody, but not L-serine-BSA conjugate, is omitted. There is also no immunofluorescent D-serine signal in SR^{-/-}. **b** cortex and **c** hippocampus using the D-serine primary antibody. **d-i** shows double immunofluorescent labeling of D-serine **d, g** with the neuronal marker, neuronal nuclei (NeuN; **e, h**) in Cb, and hippocampus (CA1). Merged images are shown in *panels (f, Cb; i, CA1; D-serine, green; NeuN, magenta)*. There are no D-serine positive cells in Cb, while in S1 and CA1, D-serine positive cells almost exclusively co-localize with NeuN. *Arrows* indicate D-serine positive cells. *Asterisks* indicate DS/NeuN overlap. *CA1* cornu ammonis, *Cb* cerebellum, *Ctx* cortex. *Scale bars* represent 50 μ m in **a-f** and 25 μ m in **g-i** (Color figure online)

Fig. 4.

D-Serine is not found in S100β or GFAP-containing astrocytes of the adult brain.

Representative coronal brain sections from adult WT mice. D-Serine positive cells (*arrows*) in **a** S1 and **(d)** CA1 hippocampus do not overlap with the astrocyte markers **b** S100β or **e** GFAP. Merged images are shown in *panels (c, S1; f, CA1; D-serine, green; S100β, and GFAP, magenta)*. CA1 cornu ammonis, S1 primary somatosensory cortex. Scale bars represent 25 μm (Color figure online)

Fig. 5.

D -Serine can be visualized in astrocytes and neurons of $SR^{-/-}$ mice injected with D -serine (300 mg/kg). Representative coronal brain sections ($\times 40$) from adult $SR^{-/-}$ mice perfused 2 h after D -serine injection. When perfused 2 h after injection, clear D -serine staining was found in both astrocytes and neurons in $SR^{-/-}$ mice. D -serine was found both in astrocytes (**a**; S1; arrows), with some of them unsheathing blood vessels, as well as in neurons (**d**; CA1; arrows). Merged images are shown in (**c**, S1; **f**, CA1; D -serine, green; NeuN, magenta). Asterisks indicate DS/SR overlap. CA1 cornu ammonis, S1 primary somatosensory cortex. Scale bars represent 25 μ m (Color figure online)

Fig. 6. D-Serine partially co-localizes with serine racemase in the adult brain. Representative coronal brain sections ($\times 40$) from adult WT mice. A portion of D-serine positive cells (arrows) in **a** S1 overlap with the SR (**b**; arrows). Merged images are shown in *panels* (**c**; D-serine, green; SR, magenta). Asterisks indicate DS/SR overlap. *S1* primary somatosensory cortex. *Scale bars* represent 25 μm (Color figure online)

Fig. 7.

D-Serine co-localizes with GAD67 in the adult brain. Representative coronal brain sections ($\times 40$) from adult WT and SR $^{-/-}$ mice. A portion of D-serine positive cells (arrows) in **a** S1 and **d**; CA1 hippocampus overlap with GAD67 (**b**, **e**; arrows). Merged images are shown in panels **c**, S1; **f**, CA1; D-serine, green; GAD67, magenta). Asterisks indicate DS/GAD67 overlap. CA1 cornu ammonis, GAD67 glutamic acid decarboxylase of 67 kDa, S1 primary somatosensory cortex. Scale bars represent 25 μ m (Color figure online)

Fig. 8. D-Serine co-localizes to parvalbumin and somatostatin positive inhibitory neurons. Representative coronal brain sections from adult WT mice. A portion of D-serine positive neurons (arrows) in **a** S1 and **(e)** CA1 hippocampus overlap with PV (**b**, **f**; arrows) and/or SOM (**c**, **g**; arrow). Merged images are shown in panels **(d)**, S1; **(h)**, CA1; D-serine, green; PV, blue; SOM, magenta). Asterisks indicate DS/PV overlap and (^) indicates DS/PV/SOM overlap. CA1 cornu ammonis, PV parvalbumin, SOM somatostatin, S1 primary somatosensory cortex. Scale bars represent 25 μ m (Color figure online)

Fig. 9.

Quantification of D-serine-containing parvalbumin and somatostatin neurons in the neocortex and hippocampus. **a** Percentage of D-serine positive cells that express PV (open bars), SOM (striped), or PV+SOM (gray bars) in the PFC and S1. Total (black bars) represents the total percentage of D-serine positive cells that express PV, SOM, and PV+SOM. **b** Percentage of PV (open bars) or SOM (striped bars) positive neurons that contain D-serine in the PFC and S1. Distribution of D-serine positive cells in each subfield of the hippocampus (**c**, DG; **e**, CA3; and **g**, CA1). The numbers in each bar represent the total number of counted cells from three animals. Percentage of D-serine positive cells that express PV (open bars), SOM (striped), or PV+SOM (gray bars) in each subfield of the hippocampus (**d**, DG; **f**, CA3; and **h**, CA1). Total (black bars) represents the total percentage of D-serine positive cells that express PV, SOM, and PV+SOM. CA1 cornu ammonis, DG dentate gyrus, Hi hilus, GCL granule cell layer, Mol molecular layer, PV parvalbumin, PFC prefrontal cortex, SOM somatostatin, s.o. stratum oriens, s.p. stratum pyramidale; s.r. stratum radiatum; S1 primary somatosensory cortex. All values represent the mean $\pm$ SEM (Color figure online)

Fig. 10.

Quantification of serine racemase expressing parvalbumin and somatostatin neurons in the neocortex and hippocampus. **a** Percentage of parvalbumin PV (open bars) and somatostatin SOM (striped bars) positive neurons that express SR in the PFC and S1. The numbers above each bar represent the total number of counted cells from three animals. **b** The estimated density of all SR (black bars), as well as PV or SOM (PV, SOM; gray bars) neurons in the PFC and S1. Distribution of PV (open bars), SOM (striped), and PV+SOM (gray bars) neurons in each subfield of the hippocampus (**c**, DG; **e**, CA3; and **g**, CA1). The numbers above each bar represents the total number of counted cells from three animals. Percentage of PV (open bars), SOM (striped), and PV+SOM (gray bars) positive neurons that express SR in each subfield of the hippocampus (**d**, DG; **f**, CA3; and **h**, CA1). CA1 cornu ammonis, DG dentate gyrus, Hi hilus, GCL granule cell layer, Mol molecular layer, PV parvalbumin, PFC prefrontal cortex, SOM somatostatin; s.o. stratum oriens, s.p. stratum pyramidale, s.r. stratum radiatum, S1 primary somatosensory cortex, SR serine racemase. All values represent the mean $\pm$ SEM

Fig. 11.

Validation of serine racemase antibody used for human *postmortem* immunofluorescence.

(a, BA4) Human primary motor cortex incubated with a rabbit anti-(SR) antibody showed intense staining of pyramidal neurons ($\times 20$). This antibody was then validated using **b** WT and **c** SR $^{-/-}$ brain tissue. Sagittal brain sections from SR $^{-/-}$ mice ($\times 10$) did not show any SR staining. *SR* serine racemase. *Scale bars* represent 50 μ m

Fig. 12. Serine racemase is expressed in neurons in human *postmortem* cortex. Representative coronal brain sections of human primary motor cortex (BA4; $\times 40$). SR (**a, d, g**; arrows) were expressed mainly by excitatory neurons (**b**; CAMKII α ; arrows) and a subset of GABAergic neurons (**e**; PV; arrows), but not S100 β -containing astrocytes **h**. Merged images are shown in panels (**c, f, i**; SR, green; CAMKII α , PV, and S100 β , magenta). CAMKII α calcium-calmodulin kinase II alpha, PV parvalbumin, SR serine racemase. Scale bars represent 25 μ m

Table 1
List of antibodies used for immunohistochemistry and immunofluorescence

Antibody	Antigen/immunogen	Species/dilution	Source/cat no.
Mouse			
Primary			
D-Serine (colorimetric)	Synthetic D-Serine conjugated to bovine serum albumin (BSA)	Rabbit polyclonal 1:60K	Abcam ab6472
D-Serine (immunofluorescence)	Synthetic D-serine conjugated to bovine serum albumin (BSA)	Rabbit polyclonal 1:15K	Abcam ab6472
GAD67 (clone 1G10.2)	Recombinant GAD67 protein	Mouse 1:500	Millipore MAB5406
GFAP	Preparation of full-length human recombinant GFAP expressed in bacteria and highly purified	Rabbit polyclonal 1:500	Abcam ab7260
GFAP (clone GA5)	Purified GFAP from porcine spinal cord	Mouse 1:10K	Millipore MAB360
NeuN	Purified cell nuclei from mouse brain	Mouse 1:1,000	Millipore MAB377
Parvalbumin (clone PARV-19)	Frog muscle parvalbumin	Mouse 1:10K	Sigma P3088
S100 β (clone SH-B1)	Bovine brain S100 β	Mouse 1:5K	Sigma S2532
Serine racemase (clone A-17; immunofluorescence)	Raised against N-terminal amino acids 1–50 of mouse serine racemase	Goat polyclonal 1:50	SCBT sc-5751
Serine racemase (colorimetric)	C-terminal amino acids 191–340 of human serine racemase	Rabbit polyclonal 1:1,000	SCBT sc-48741
Somatostatin (clone YC7)	Synthetic peptide corresponding to amino acids 1–14 of cyclic somatostatin conjugated to bovine thyroglobulin using carbodiimide	Rat monoclonal 1:200	Millipore MAB354
Secondary			
Biotinylated goat anti-rabbit		1:1,000	Vector BA-1000
Goat anti-mouse AlexaFluor 555 IgG (H+L)		1:500, 1:3K	Life Technologies A-21422
Goat anti-mouse AlexaFluor 647 IgG (H+L)		1:1,000	Life Technologies A-21235
Goat anti-rat AlexaFluor 555 IgG (H+L)		1:500	Life Technologies A-21434
Rabbit anti-goat AlexaFluor 555 IgG (H+L)		1:500	Life Technologies A-21428
Streptavidin AlexaFluor-488		1:1,000	Life Technologies S-32354
Human			
Primary			
CAMKIIa (6G9)	Partially purified full length native rat protein	Mouse 1:300	Novus Biologicals NB100-1983
Parvalbumin (clone PARV-19)	Frog muscle parvalbumin	Mouse 1:5K	Sigma Aldrich, P3088
S100 β (clone SH-B1)	Bovine brain S100 β	Mouse 1:2,500	Sigma Aldrich S2532
Serine racemase (immunofluorescence)	C-terminal amino acids 191–340 of human serine racemase	Rabbit polyclonal 1:50	SCBT sc-48741
Secondary			

Antibody	Antigen/immunogen	Species/dilution	Source/cat no.
Biotinylated goat anti-rabbit		1:250	Vector Laboratories BA-1000
Goat anti-mouse Alexa Fluor 647 IgG (H+L)		1:250	Life Technologies A-21235
Streptavidin Alexa Fluor-488		1:2,500	Life Technologies S-32354

Table 2
D-serine is almost exclusively localized to neurons in the hippocampus and cortex

	PFC	S1	DG	CA3	CA1
NeuN (<i>n</i> = 3 mice)					
% Co-localization with D-serine	88 ± 1.4	84 ± 6.1	83 ± 2.3	83 ± 3.3	96 ± 0.9
Average # D-serine cells counted/mouse	537	691	116	297	381
S100β (<i>n</i> = 3 mice)					
% Co-localization with D-serine	4 ± 1.1	1 ± 0.6	6 ± 1.1	5 ± 3.8	4 ± 2.3
Average # D-serine cells counted/mouse	50	149	27	44	35
GFAP (<i>n</i> = 3 mice)					
% Co-localization with D-serine	0	0	0	3 ± 2.1	1.4 ± 0.7
Average # D-serine cells counted/mouse	43	152	7	31	46
SR (<i>n</i> = 3 mice)					
% Co-localization with D-serine	56 ± 4.2	52 ± 4.7	23 ± 7.1	41 ± 6.3	37 ± 0.9
Average # D-serine cells counted/mouse	47	389	43	50	120

The percent co-localization of D-serine positive cells with NeuN (neurons), S100β (astrocyte), GFAP (astrocyte), and SR was quantified from 3 WT mice in the PFC, S1, and hippocampus (DG; CA3; CA1). The percent co-localization is represented as the average ± SEM

CA1/3 cornu ammonis, DG dentate gyrus, GFAP glial fibrillary acidic protein, PFC prefrontal cortex, NeuN neuronal nuclei, SR serine racemase