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# D-Serine, the Shape-Shifting NMDA Receptor Co-agonist

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## Abstract

Shape-shifting, a phenomenon wide-spread in folklore, refers to the ability to physically change from one identity to another, typically from an innocuous entity to a destructive one. The amino acid D-serine over the last 25 years has "shape-shifted" into several identities: a purported glial transmitter activating *N*-methyl-D-aspartate receptors (NMDARs), a co-transmitter concentrated in excitatory glutamatergic neurons, an autocrine that is released at dendritic spines to prime their post-synaptic NMDARs for an instantaneous response to glutamate and an excitotoxic moiety released from inflammatory (A1) astrocytes. This article will review evidence in support of these scenarios and the artifacts that misled investigators of the true identity of D-serine.

### Keywords

Astrocytes; D-Serine; Excitotoxicity; Glutamic acid; γ-Amino-butyric acid (GABA); Serine racemase

## Introduction

Shape-shifting refers to the ability to physically change from one identity to another, often from an innocuous one to an evil one. Shape-shifting is a common theme in folklore as manifest by vampires and werewolves. The amino acid D-serine presents a molecular example of shape-shifting as its perceived role and cellular localization has changed dramatically over the last two decades. In part, this confusion over the role of D-serine resulted from artifacts and misinterpretation of experimental results and in part from faulty assumptions. This article will review the changes in perceived roles and cellular localizations of D-serine over the last 30 years.

One of the fundamental precepts in biology is that with fewer minor exceptions, eukaryotes do not synthesize D-amino acids whereas prokaryotes such as bacteria make them in

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This review is dedicated to Michael B Robinson, PhD, Professor of Pharmacology and Pediatrics at Perelman School of Medicine at the University of Pennsylvania, who spent the years of 1985-1989 as a post-doctoral fellow in the Coyle laboratory in the Department of Neuroscience at Johns Hopkins School of Medicine and played a seminal role in the laboratory's 45-year-long research program on the role of glutamatergic neurotransmission in health and disease.

abundance [1]. In this regard, D-cycloserine was developed as an effective antibiotic because it mimics bacterial D-alanine and disrupts bacterial peptidoglycan synthesis by inhibiting alanine racemase, resulting in faulty bacterial cell wall synthesis and cell lysis [2]. Nishikawa and his colleagues were the first to report that D-serine was present in the adult rat brain in substantial quantities (0.25μmole/g) and represented over a quarter of total brain serine [3]. D-serine was found to have an uneven regional distribution in brain with high concentrations in cerebral cortex and low levels in the cerebellum and brainstem [4].

The discovery of D-serine seemed to be anticipated by two previous seemingly incongruous findings. First, D-amino acid oxidase (DAAO) was found to be present in the mammalian brain with highest activity in the cerebellum [5]. This finding preceded by a quarter of a century the discovery of its primary substrate, D-serine, where regional brain levels were roughly the inverse of DAAO expression [4]. Secondly, Johnson and Ascher [6] discovered that the *N*-methyl-D-aspartate receptor (NMDAR) expressed on cultured neurons required the binding of glycine for glutamate to open the cation channel. Subsequently, Klecker and Dingledine [7] using Xenopus eggs found that D-serine as potent as glycine at this site on the NMDAR, a finding that finally made sense with the discovery of endogenous D-serine in brain.

The discovery of brain D-serine raised important questions: how was it synthesized, where was it located, how was it released, how did it interact with NMDARs? This article will address each of these issues.

## D-serine as a Glial Transmitter

The laboratory of Solomon Snyder had a long-term interest in atypical neurotransmitters such as nitric oxide and carbon monoxide [8, 9], Thus, the report of a D-amino acid in brain with potent modulatory effects on the NMDAR attracted his attention [3]. Using antibodies with a high degree of specificity for D-serine (100-fold specificity for D-serine over L-serine in a dot blot assay), Schell et al. [10] reported that D-serine occurs in high concentrations in forebrain (cortex, striatum and anterior olfactory bulb), low in midbrain and virtually absent from the cerebellum. They found that D-serine was localized exclusively in astrocytes. This regional distribution of D-serine correlated inversely with DAAO expression measured by immunocytochemistry and as previously reported in neurochemical assays [4], Williams et al. [11], using a different immunocytochemical method to stain for D-serine, also reported strong labeling of a subset of astrocyte-like cells in the grey matter of the cortex, as well as astrocytes in the white matter. Double labeling with anti-glial fibrillary acidic protein (GFAP) further confirmed the apparent astrocytic localization of D-serine in the cerebellum.

The study by Williams et al. [11] described the imrnunostaining in a punctate pattern with light microscopy and gold labeling associated with glial intracellular vesicles with electron microscopy. To further characterize the glial localization of D-serine, primary cortical glial cultures were studied. Consistent with immunocytochemistry results in rat brain sections, D-serine appeared to be concentrated in astrocytes cultures [10]. Furthermore, stimulation of the azole-4-propionic acid (AMPAR) and kainic acid (KAR) subtypes of glutamate receptors in cultured astrocytes resulted in release of D-serine in a $Ca^{2+}$-sensitive and SNARE protein dependent fashion [12].

Since there was considerable regional variation in the concentration of D-serine in brain, it seemed likely that that a biosynthetic pathway existed. Using standard biochemical methods, Wolosker et al. [13] were able to identify and purify to homogeneity serine racemase (SR) from rat brain. The protein had a molecular weight of 37 kDa, required pyridoxal 5'-phosphate for activity and exhibited very high substrate specificity for L-serine. Using a partial amino acid sequence from the purified protein, Wolosker et al. [14] cloned the rat SR, demonstrating homology to the bacterial racemase enzymes. Polyclonal antibodies were generated against bacterially expressed rat SR. To obtain optimal immunostaining, paraformaldehyde-fixed brain tissue was subject to partial trypsinization. Under these conditions, SR immunoreactivity was reported to be localized to astrocytes in the rat cerebral cortex, consistent with the prior finding of D-serine concentrated in astrocytes [10]. The role of endogenous D-serine in permitting NMDAR function was established by showing that perfusing cultured neurons and brain slices with purified DAAO abolished NMDAR activation as demonstrated by intracellular recordings and by nitric oxide synthesis. This loss of NMDAR function could be reversed by perfusion with exogenous D-serine [15].

Given the unusual notion of glia actually controlling NMDAR function, which plays a central role in learning, memory and neural plasticity [16], the Snyder laboratory sought to establish the mechanisms of this "cross-talk" between the glutamatergic terminal and the astrocytic processes ensheathing the synapse. Some of the studies relied on primary astrocyte cultures, which consist of predominantly the A1 subtype astrocytes [17]. Kim et al. [18] reported that glutamate stimulation of AMPARs on astrocytes caused $Ca^{2+}$ influx and activation of protein kinase C (PKC). This promoted the dissociation of glutamate receptor interacting protein (GRIP) from the AMPAR, resulting in GRIP binding to and activating SR [19], The newly synthesized D-serine diffuses from the astrocyte to the NMDARs on the post-synaptic spine, permitting them to respond to the glutamate released into the synapse [20].

Early on after the glial localization of D-serine in astrocytes was reported, a number of studies were published that provided evidence in support of astrocytes being the source of synaptic D-serine, which was deemed the "co-agonist" at forebrain NMDARs. Perfusion of cortex or hippocampal slices with purified or recombinant DAAO caused a profound inhibition of NMDAR mediated long-term potentiation (LTP), which could be restored by perfusion with exogenous D-serine [21, 22], Given the apparently convincing evidence that SR was expressed in astrocytes, astrocytes acquired the mystique of orchestrating neural plasticity, learning and memory, prompting Diamond [23] to title a *Cell* review: "Astrocytes put down the broom and take up the baton." Nevertheless, more rigorous confirmation of the source of D-serine in intact brain tissue seemed necessary. Fossat et al. [24] used fluoroacetate, a purported glial specific toxin, to show that poisoning the astrocytes in the prefrontal cortex inhibited LTP, which could be restored by perfusion with D-serine, thus implicating astrocytes as the source of D-serine. In another strategy, Henneberger et al. [25]

electrophysiologically clamped internal $Ca^{2+}$ in the astrocytes at identified Schaffer collateral-CAl synapses and showed that this blocks D-serine release and LTP.

One issue that arose in considering the astrocytic localization of D-serine is the method of its release. Reputed $Ca^{2+}$ dependence of D-serine release [24, 25] pointed to an exocytotic release mechanism. A series of experiments reported that D-serine was concentrated in vesicles with SR bound to the outer membrane [26–29]. However, the interpretation of these findings and the extrapolation to normal brain function was clouded by the fact that the results were obtained primarily from cultured astrocytes, which have the characteristics of A1 inflammatory astrocytes and not the resting astrocytes normally present in the brain [17]. To circumvent this limitation, studies have been carried out using a mouse line with a doxycycline-dependent, reversible expression of a dominant negative SNARE domain of synaptobrevin-2 under the control of an astrocyte-specific GFAP promoter (dnSNARE) to block vesicular D-serine release [30]. With this model, Sardinha et al. [31] found EEG abnormalities and cognitive impairments that were reversed by perfusion with exogenous D-serine. Sultan et al. [32] used the same dnSNARE mouse line to show that adult born hippocampal neurons, but not mature ones had reduced number of dendritic spines (glutamatergic synapses) in dendritic segments intersecting with the transgene expressing astrocytes. However, this dnSNARE construct has been reported to also be expressed in neurons, thereby clouding the interpretation of these studies [33].

In aggregate, the results of these studies attracted considerable interest because they strengthened the case that astrocytes were not simply passive handmaidens to neurons, providing metabolic and structural support, but actively modulated neuronal function. Thus D-serine, which is the gatekeeper for forebrain NMDAR function, joined astrocytic ATP through its purinergic transmission as a “glial transmitter” [34]. However, as reviewed below, because of several artifacts and misinterpretations, this conclusion has been called into question [35].

## D-serine and Serine Racemase are Expressed in Neurons

The Wolosker laboratory developed the first evidence that SR and D-serine were located in neurons, as well as astrocytes using a combination of tissue culture and immunocytochemical techniques [36]. When the culture medium was supplemented with L-serine, relatively pure neuronal cultures, mixed neuron and glial cultures and pure glial cultures all synthesized substantial amounts of D-serine. The expression of SR in both cell types under these culture conditions was confirmed by immunocytochemistry with specific antibodies against SR that they had developed. They also reported SR expression in striatal neurons and cerebellar granule cells in culture. Using these reagents, they observed NeuN positive cells in the mouse cerebral cortex expressing SR, but also astrocyte staining in areas where neuronal staining was “faint”. Kartvelishvily et al. [36] concluded that SR and D-serine are primarily localized to neurons in forebrain. Given that neurons containing D-serine also express the NR1 subunit, they speculated presciently that D-serine may be acting as a paracrine modulator rather than a co-transmitter.

One of the most fundamental rules in immunocytochemistry is that the antibodies must be specific for the protein of interest, typically documented by a single band on Western blot. Currently, most antibodies are purchased from commercial laboratories, which usually generate them from synthetic peptides representing “specific” epitopes in the protein of interest. However, epitopes may be shared among other unrelated proteins, resulting in multiple bands on Western blots. Remarkably, some purveyors recommend antibodies for use “only for immunocytochemistry” but not for Western blots because they exhibit more than one protein band on Western blots. With the availability of transgenic technology, it is now possible to generate mice that do not express the protein of interest. With tissue from the homozygous null mutant as a control, no Western blot bands or immune-staining of the knock-out tissue should be observed. Thus, “knock-out controls” have become the “gold standard” for determining antibody specificity. Notably, several commercially available antibodies against SR do not pass this rigorous test (unpublished observation).

In 2008, Miya et al. [37] pioneered the use of Srr−/− mice as their negative controls to validate the specificity of their antiserum in characterizing SR expression in brain. Using SR antiserum that exhibited no immunocytochemical staining in the Srr−/− mouse, they reported that SR was expressed nearly exclusively in neurons and not in astrocytes. SR was predominantly expressed in pyramidal neurons in the cerebral cortex and in the CA1 region of the hippocampus. Dual labeling studies revealed that SR co-localized to cells expressing neuron-specific nuclear protein, but not to the astrocyte markers GFAP and 3-phosphoglycerate dehydrogenase. In the striatum, they made the counterintuitive discovery that SR is heavily expressed in the striatal intrinsic neurons, presumably the medium spiny GABAergic neurons, as well as weakly expressed in the cerebellar granule cells. The Srr−/− mice exhibited approximately a 90% reduction in D-serine in the frontal cortex, hippocampus and striatum. Ishiwata et al. [38] reported that D-serine released from neurons into the extracellular space in the hippocampus was reduced by over 40%. Notably, the serum levels of D-serine were essentially unchanged in Srr−/− mice as compared to WT, revealing an additional source of D-serine, probably gut bacteria [39]. An incidental finding of the Horio et al. [39] study is that SR may also synthesize D-aspartate.

In 2012, Benneyworth et al. [40] used a genetic approach to establish the cellular localization of SR by conditionally silencing its expression in specific cell types. To achieve cell specificity, mice with the first coding exon of *Srr* flanked with lox-P sites were crossed with mice bearing a Cre-recombinase gene driven by an inducible GFAP promoter for expression in astrocytes or the α-subunit of $Ca^{2+}$/calmodulin-dependent kinase II (CaMK IIα) promoter for expression in cortical pyramidal neurons. The astrocyte expression of Cre-recombinase was activated by treatment with tamoxifen whereas neuronal expression of Cre-recombinase commences at approximately 18 days *post-partum*. Western blots revealed a minimal reduction in SR in the forebrain of astrocyte knock-out whereas D-serine levels were unaffected. In contrast, SR expression was reduced in neuronal knock-outs by nearly 70% in the cortex and hippocampus at 12 weeks post-partum and by 40% in the striatum. The neuronal knock-out exhibited a 70% reduction in LTP at the Schaffer collateral-CA1 synapse induced with a one second stimulus train; however, induction of LTP with three one second stimuli trains restored full LTP, consistent with the recruitment of glycine release from astrocytes. Astrocyte-specific suppression of SR expression did not affect LTP. The results from conditional knock-out of SR comported with the findings in the Miya et al. [37] report on the predominant localization of SR in neurons.

Using an anti-SR antiserum validated with the *Srr−/−* mice [41], Balu et al. [42] established conditions for immunocytochemically visualizing the cellular localization of D-serine as the SR knock-out contained less than 15% of WT levels in brain. To minimize cross-reactivity with cellular L-serine, the antiserum incubation medium included exogenous L-serine. Concentrations of blocking L-serine that were used in prior studies [10, 11] resulted in intense immunostaining of forebrain astrocytes in *Srr−/−* mice, indicating inadequate concentrations of blocking L-serine to quench cross-reactivity. Thus, the conditions used by Schell et al. [10] revealed an artifactual localization of apparent D-serine in astrocytes because of their extremely high concentrations of endogenous cross-reacting L-serine. Increasing the concentration of the blocking L-serine to 10 mM in the incubation medium eliminated astrocyte staining to reveal D-serine immunoreactivity restricted to neurons. Forebrain sections *Srr−/−* exhibited no immune-staining under these conditions, thus confirming the specificity of the neuronal staining.

The Balu et al. [42] studies revealed that nearly all the D-serine in neocortex and hippocampus was found in neurons with virtually no D-serine co-localizing with two astrocyte markers: GFAP or S100β. Notably, only a subset of D-serine containing neurons expressed SR, suggesting that D-serine can be taken up and concentrated in neurons that do not synthesize the ligand. More than half of the D-serine positive neurons were GABAergic interneurons with the majority of them containing parvalbumin or somatostatin, the subtype of cortical GABAergic neuron vulnerable in schizophrenia [43]. Only 25–40% of the inter-neurons expressed SR in the neocortex and the hippocampus. Using the conditions established in the mouse, Balu et al. [42] further demonstrated in human post-mortem cerebral cortex that SR is expressed in pyramidal neurons and parvalbumin-expressing GABAergic interneurons. Ehmsen et al. [44] also used *srr−/−* mice as negative controls to optimize immune-staining for D-serine. They observed that over 80% of D-serine was localized to neurons in the cortex and hippocampus with only much smaller amounts associated with glia. D-serine staining occurred in the pyramidal neurons in all layers of the cortex and all sectors of the hippocampus.

Recent studies have been probing the remarkably high concentrations of L-serine in the astrocytes and retinal Muller cells that caused the unsuspected cross-reactivity with the D-serine antibodies in initial immunocytochemical studies [10, 11, 45]. Yamasaki et al. [46] demonstrated that 3-phosphoglycerate dehydrogenase (Phgdh), which is the first committed step in the synthesis pathway for L-serine, is expressed exclusively in astrocytes in brain. In mice in which the expression of Phgdh has been genetically silenced in astrocytes, the levels of cortical L-serine were reduced by approximately 60% and the levels of D-serine by more than 80%, thereby demonstrating that astrocytes are primary source of L-serine for neuronal D-serine synthesis [47]. Notably, the Phgdh−/− mice exhibited microcephaly, indicating that astrocytic Phgdh was also important for L-serine availability during brain development. Ehmsen et al. [44] studied the impact of silencing the expression of Phgdh in astrocytes using immunocytochemical staining of both L-serine and D-serine. L-serine immunoreactivity was markedly reduced in astrocytes and D-serine was reduced substantially in neurons, consistent with the neurochemical results of Yang et al. [47].

These findings on astrocytic Phdgh prompted Wolosker and Radzishevsky [48] to propose the “glia-neuron serine shuttle” as critical to D-serine disposition in the brain. Thus, L-serine is synthesized in astrocytes, whereupon it is released by facilitated transport to be taken up by neurons expressing SR and converted to D-serine. This mechanism explains a number of the artifacts that sustained the belief that D-serine was a glial transmitter for over twenty years. First, the intense staining of astrocytes with antibodies “specific” for D-serine [10, 11] undoubtedly resulted from unappreciated cross-reactivity with the very high concentrations of L-serine in astrocytes [35, 42, 44]. The use of flouroacetate, a purportedly ‘specific” glial toxin, to prevent the release of D-serine from astrocytes in electrophysiologic studies [23, 25, 49, 50] in fact, disables the source of L-serine for neuronal synthesis of D-serine, thus reducing extracellular levels. This hypothesis is supported by the finding that perfusion of hippocampal slices poisoned with fluoroacetate with L-serine restored LTP [50].

An inducible dnSNARE mutant mouse line was designed to reduce D-serine release from astrocytes because the particular SNARE protein was thought to be restricted to astrocytes [30]. However, this dnSNARE construct has been reported to also be expressed in neurons [33]. While the transgene does “appear” to reduce D-serine release, it is unclear whether in fact it is impairing L-serine release from astrocytes or D-serine release from neurons. Given the compelling evidence that SR and D-serine are predominantly localized to neurons and virtually absent from resting astrocytes [35], it is not surprising that artifacts supporting the predominant astrocyte localization are now understandable. What is surprising is that nine years after the immunocytochemical studies of Miya et al. [37], 5 years after the conditional knock-out studies of SR of Benneyworth et al. [40] and four years after the immunocytochemical confirmation of the neuronal localization of SR and D-serine [42], investigators continue to ignore these findings and rely on flawed methods to argue that D-serine is released from resting astrocytes as a purported gliotransmitter [51, 52] (Table 1)

## Is D-serine an Autocrine?

The term “co-transmitter” implies that two or more signaling molecules, one of which is typically a neuropeptide, are released from the same neuron at its terminals by exocytosis [59, 60]. The demonstration that SR and D-serine were localized to glutamatergic neurons led to a reasonable conclusion that D-serine was a co-transmitter with glutamate [37, 40]. But, such an inference is inconsistent with the evidence that D-serine is not released by exocytosis but by facilitated transport [61–63] and that it is co-localized to GABAergic neurons [37, 42]. GABA receptors are not known to contain a glycine modulatory site.

Ma et al. [64] first demonstrated the interaction of SR with post-synaptic proteins localized to the dendritic spine. In co-precipitation experiments, they showed that SR, PSD95 and stargazin form a tertiary complex. Given the interaction between stargazin and AMPARs, they proposed a model whereby AMPAR activation causes a dissociation of SR from the complex, which drives D-serine synthesis. Using cortical cultures that are amenable to rapid fixation and high-resolution confocal fluorescent microscopy, Lin et al. [65] also demonstrated that SR and D-serine co-localize with PSD-95 and NMDARs in the post-synaptic spine, but not with the pre-synaptic vesicular glutamate transporter 1 (vGLUT1). They demonstrated the association of SR and D-serine with PSD-95 and the NR1 subunit early in glutamatergic synaptic development on both glutamatergic and GABAergic neurons. Addition of D-serine to the culture medium increased the number of vGLUT1 and PSD-95 synapses. Furthermore, they found that adding exogenous D-serine, but not glycine to the culture medium enhances this association of SR with PSD95 and NR1 in co-precipitation experiments using either SR, PSD95 or NR1 as the target antigen. This effect was blocked by the NMDAR antagonist, 2-amino-5-phosphonopentanoicacid and 7-chlorokynurenic acid, a specific antagonist at the glycine modulatory site on the NMDAR.

Neurophysiologic evidence in support of D-serine's role as an autocrine substance is still scarce, but the results of a study by Li et al. [66] is consistent with such a function. Recording from neurons in the lateral nucleus of the amygdala, which receive glutamatergic inputs from the thalamus, they monitored NMDAR activity under different conditions of presynaptic activity. The NMDAR function during spontaneous (low) frequency input associated with mini-excitatory post-synaptic potentials (mEPSCs) was reduced by perfusion with DAAO but not with glycine oxidase, which degrades glycine, the other endogenous glycine site agonist on NMDARs. This finding indicates a marked dependence on "ambient" D-serine released at the post-synaptic spine but not by glycine. In contrast, stimulation of inputs in the internal capsule at 0.1 Hz, NMDAR function was drastically reduced with glycine oxidase treatment but not with DAAO treatment, suggesting that glycine released from astrocytes subsumes the role of the glycine modulatory agonist during periods of high presynaptic activity.

## Inflammatory Astrocytes

Since the first studies of the cellular localization of SR and D-serine, primary cultures of astrocytes have been exploited to understand the dynamics of D-serine disposition. Thus, Schell et al. [10] in the first publication on the localization of SR and D-serine utilized primary cultures of astrocytes, which the authors identified as "A2 type astrocytes" which express SR, synthesize D-serine and release it by stimulation with the glutamate receptor subtype agonist, kainic acid. However, as reviewed above [34, 38, 40, 42], astrocytes (quiescent) in the healthy brain do not express SR nor contain D-serine. So, the relevance of these findings to normal brain function is unclear. Nevertheless, reactive astrocytes appear in brain in a number of neurodegenerative disorders.

Barres and Liddelow [17] recently described how reactive astrocytes, which respond to CNS injury, differ markedly from "resting" astrocytes and how reactive astrocytes can be further subdivide into toxic (A1) or trophic (A2) astrocytes, each with their unique gene expression profiles. The major distinctions between quiescent and reactive astrocytes did not deter many investigators from utilizing astrocyte cultures to study D-serine disposition as a model for what was occurring in quiescent astrocytes of the healthy brain [67]. Shao et al. [68] used primary cultures of cortical neurons and astrocytes to study expression of the transporters for D-serine, ASCT-1 and −2. Using primary astrocyte cultures, Vargas-Lopez et al. [69] reported that the activation of protein kinase C (PKC) in cultured astrocytes resulted in phosphorylation of serine residues on SR, inhibiting the production of D-serine. While a similar phenomenon was observed in the hippocampus after a learning task, these changes in SR undoubtedly occurred in neurons. Ma et al. [70] showed that the putative schizophrenia risk gene, DISCI, when expressed in cultured astrocytes, blocks WT DISC1 binding to SR, thereby increasing its degradation and reducing D-serine synthesis. They argue that this mechanism accounts for behavioral phenotype of mice with DISC1 conditionally expressed in astrocytes, whereas DISC1 more likely disrupted the glia-neuron D-serine shuttle. In contrast, using cortical neuronal cultures, Jacobi et al. [71] demonstrates that DISC1 and SR form complexes in glutamatergic and GABAergic neurons. These complexes are concentrated in the nuclei and dendrites, a process that is stimulated by exogenous D-serine.

Liebl's laboratory has been interested in the role of NMDARs in mediating neuronal damage after traumatic brain injury (TBI) Perez et al. [72]. Using a controlled cortical impact (CCI) model, Perez et al. [73] examined the effects on SR as prior results pointed to a decrease in D-serine levels in the week after the CCI in the hippocampus. During the week after CCI, western blots revealed little change in the levels of SR. However, immunocytochemical studies told a different story. SR immunostaining was fading in hippocampal pyramidal neurons over the 7 days postinjury but appeared in reactive astrocytes that proliferated at the injury site. Furthermore, while the levels of D-serine in the hippocampus under the injury fell nearly 50% 3 days after injury, the levels of D-serine in isolated astrocytes from the lesion sit rose dramatically. The CCI is associated with substantial synaptic dysfunction and memory impairments in a fear-conditioning paradigm. However, CCI-associated LTP and memory deficits were prevented in mice in which SR expression was conditionally silenced only in astrocytes prior to the injury. Conditionally silencing astrocytic SR expression in control mice had no effects on either LTP or memory. Furthermore, administering D-serine after CCI to mice with conditionally silenced astrocytic SR expression reversed the enhanced synaptic potentiation, validating their conclusion that the CCI injury-induced synaptic dysfunction is aggravated by increased D-serine levels.

These findings provide compelling evidence that D-serine released by inflammatory astrocytes that proliferate after CCI are responsible for the neuronal damage in the hippocampus. Since release of D-serine from astrocytes would be into the extra-cellular space and not at the synapse, the D-serine would preferentially activate extra-synaptic receptors. While extra-synaptic NMDARs have been linked to excitotoxic neuronal damage, synaptic NMDARs are responsible for functional and structural neuroplasticity [74, 75]. This conclusion is entirely consistent with the findings from primary astrocyte cultures, which not only express SR and synthesize and release D-serine into the medium but also express lipocalin-2 and complement C3 (C3), markers for the A1 reactive or neurotoxic phenotype [76].

Given the failures of all clinical trials with drugs and monoclonal antibodies directed at increasing the clearance or decreasing the production of amyloid Ap peptide [77], recent *post-mortem* and genetic findings have shifted attention to the role of inflammation in the pathophysiology of AD [78, 79]. Such a mechanism might explain why these treatments fail since inflammation may progress in an autonomous manner after the amyloid pathology is well established. Such a scenario seems feasible since it is now apparent that AD pathology commences and progresses for twenty years before the onset of cognitive symptoms [80]. Based on the findings in the TBI study on the proliferation of A1 toxic astrocytes expressing SR and D-serine causing excitotoxic neuronal damage, neurophysiologic abnormalities and cognitive impairments [73], Balu et al. [81] examined the neuropathology of AD with eye towards a role for excitotoxic damage caused by inflammation.

Balu et al. [81] found that SR was robustly expressed in A1 neurotoxic astrocytes in the hippocampus and the entorhinal cortex in AD and in a transgenic rat model of AD with an APPsw/PS1ΔE9 transgene [82]. In the AD entorhinal cortex, the increase in GFAP + and SR + astrocytes went from negligible in the age-matched controls to ~ 20,000 cells per $mm^3$ in Layers I-III and to 10,000 per $mm^3$ in Layers IV-VI. These astrocytes highly expressed C3, a marker for A1 neurotoxic astrocytes. In the subfields of AD hippocampus, the dentate gyrus exhibited the greatest increase in GFAP + SR + astrocytes (~ 10,000 per $mm^3$), followed by CA4, CA2 and CA1 (~ 7,000 per $mm^3$) with the least in the CA3 region (~ 4,000 per $mm^3$). The AD rat exhibited an age-related increase in C3 + and SR + GFAP-expressing astrocytes in the hippocampus with the most robust response in the CA1 sector (~ 40,000 per $mm^3$). Phosphorylation of GluN2B at Ser1303 by death-associated protein kinase (DAPK) is linked to cell death pathways via extra-synaptic NMDAR activation [83]. Consistent with the hypothesis that the extra-synaptic release of D-serine by A1 neurotoxic astrocytes preferentially extra-junctional NMDARs, the AD rat hippocampus exhibited a significant increase in DAPK as well as phospho-GluN2B.

In summary, these results suggest that D-serine is an important mediator of neurotoxicity of the A1 inflammatory astrocytes that proliferate in neurodegenerative disorders. In essence, the designation “reactive” is a misnomer since the TBI studies indicate that D-serine released from the A1 astrocytes is actually responsible for the neuronal damage because silencing SR expression only in astrocytes, which has no effect in controls, provides robust neuronal protection in the traumatized region of the hippocampus. These findings have major therapeutic implications since inhibiting D-serine synthesis and/or release from A1 astrocytes should provide neuroprotection in neurodegenerative disorders associated with the proliferation of A1 astrocytes.

## Conclusion

D-serine appears to be unique among signaling molecules in the brain. While there are trace amounts of other D-amino acids in brain, such as D-aspartate, D-serine stands alone as the dominant endogenous modulator of a neurotransmitter receptor. Contrary to long the held belief that D-serine is a “glial transmitter” released from quiescent astrocytes [51, 52], SR and its product, D-serine, are expressed in neurons, both glutamatergic and GABAergic neurons [37, 40, 42]. In the forebrain, D-serine appears to be the primary agonist at the glycine modulatory site on the GluNl subunit of the NMDAR that permits the neurotransmitter, glutamate, to open the cation channel [16, 41, 66]. However, in spite of its primary neuronal expression, D-serine is not a co-transmitter, as it is not released by vesicular exocytosis but rather by facilitated transport by Asc-1. [61–63].

Evidence points to D-serine acting as an autocrine receptor modulator. In other words, it is released by the post-synaptic neurons to bind to their own NMDARs so as to “prime” the receptors to respond immediately to synaptic glutamate. D-serine and SR are concentrated in the dendritic spines [61, 62]. Asc-1, which drives D-serine facilitated transport, has been shown to be expressed primarily on the dendrites and somata of neurons [84], which is consistent with the D-serine being an autocrine signaling molecule. Biochemical studies demonstrate the physical association with PSD-95 [61] as do confocal microscopy of cultured cortical neurons [62]. Another signaling molecule involved in synaptic plasticity, brain derived neurotrophic factor (BDNF), has also been shown to be an autocrine signaling molecule, released at the post-synaptic spine by activation of NMDARs [85]. Most evidence supports the notion that the glycine modulatory sites on NMDARs are only partially occupied by D-serine [66, 86]. However, the availability of D-serine is actively regulated by changes in the expression of SR based upon changes in NMDAR activity as shown by studies in the basolateral nucleus of the amygdala [87].

The best example of the “shape shifting” nature of D-serine is its dual role of driving neuronal plasticity or neurodegeneration. These dueling effects of NMDAR activation appears to be determined by the neuronal localization of the activated NMDARs, whether at the synaptic NMDARs, prompting trophic effects or extra-synaptic NMDARs on the dendrite or soma, driving excitotoxicity [74, 75]. The D-serine synthesized by SR in the spine is obviously situated to preferentially bind to synaptic NMDARs, thereby facilitating glutamatergic neurotransmission. However, the proliferation of inflammatory A1 astrocytes as a consequence of brain trauma, infarction, infection or endogenous toxic molecules creates a new source of D-serine that is released into the extracellular space to permit extra-synaptic NMDARs to respond to ambient glutamate [73, 81]. It is important to emphasize that the extra-synaptic NMDARs are generally silent unless the glycine modulatory site on GluN1 is occupied by D-serine (or glycine) although a small amplitude tonic current mediated by extra-synaptic receptors has been described in CA1 pyramidal neurons [88]. Thus, the D-serine released from A1 astrocytes may be the proximate cause of excitotoxicity in neurodegenerative disorders where A1 astrocytes proliferate.

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## Table 1

Purported glial source of d-serine in NMDA receptor mediated processes

| Authors | Journal | Evidence | Mechanism |
|---|---|---|---|
| Beltrán-Castillo et al. [49] | *NatCommun* | Flouroacetate | Regulates breathing |
| Wu et al. [53] | *Addict Biol* | Inhibition of astrocyte AMPARs | Morphine effects |
| Meunier et al. [54] | *J Neurosci* | Astroglial Cx43 Hemichannels | Cortex NMDARs |
| Papouin et al. [55] | *Neuron* | “Astrocyte-specific” dnSNARE mice | Sleep |
| Terrillion et al. [56] | *Neuropsychopharm* | Effects reversed by D-serine | DISC1 in astrocytes |
| Sherwood et al. [57] | *Glia* | Heparin blockade of $IP_3Rs$ | LTP |
| Sardinha et al. [31] | *Glia* | “Astrocyte-specific” dnSNARE mice | Theta EEG |
| Robin et al. [58] | *Neuron* | dnSNARE mice: “Source …is still under debate” | CB1 receptors and memory |

The above articles that reported D-serine was released from astrocytes were published 9 years after Miya et al. [37] using SR−/− validated immunocytochemistry and 5 years after Benneyworth et al. [40] using conditional knock-outs of SR demonstrated that SR was expressed in neurons and not astrocytes