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NMDA Receptor Regulation by D-serine: New Findings and Perspectives

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Abstract The *N*-methyl-D-aspartate (NMDA) receptors play key roles in excitatory neurotransmission and are involved in several important processes, including learning, behavior, and synaptic plasticity. The regulation of NMDA receptor neurotransmission has been extensively studied, but many important questions still remain unsolved. One of the most debated aspects of the NMDA receptor regulation relates to the identity, role, and cellular origin of the NMDA coagonist(s). In addition to glutamate, the NMDA receptor activity was believed to be regulated by the coagonist glycine. More recently, D-serine has also been proposed to play a role as a key coagonist for NMDA receptor activity and neurotoxicity. A surprising unique biosynthetic pathway for D-serine has been demonstrated, indicating the conservation of D-amino acid metabolism in mammals. D-Serine was originally shown to be exclusively made in astrocytes, indicating a possible role as a gliotransmitter. Nevertheless, recent data indicate that D-serine has a neuronal origin as well, which raises several new questions on D-serine disposition. In this review, I discuss recent advances in the field and propose a novel model of D-serine signaling that includes a bidirectional flow of D-serine between astrocytes and neurons.

Keyword D-Serine · Serine racemase · NMDA receptor · Glutamate · Coagonist

This review is dedicated to the memory of Dr. Marcos Wolosker.

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The NMDA Receptor Coagonist Site

A fascinating aspect of *N*-methyl-D-aspartate (NMDA) receptor function is the requirement of more than one agonist for the opening of the receptor/channel [1]. In 1987, Johnson and Ascher discovered the existence of a second agonist binding site of NMDA receptors [2]. They observed that the magnitude of the NMDA receptor responses was dependent on the speed of perfusion of the neuronal cultures with the NMDA-containing solution. At slower perfusion rates, the NMDA responses were larger, indicating that the accumulation of a substance tonically released from the cultured cells was able to stimulate the NMDA receptors. Although the substance was not isolated and its identity was not chemically determined, the effects of the conditioned media were reproduced by glycine [2]. Glycine was shown to display high affinity to the NR1 subunit of NMDA receptors, which established the existence of a coagonist site, generally referred to as the strychnine-insensitive "glycine site" [1]. The requirement for a coagonist is a unique property of NMDA receptors, not seen with other neurotransmitter receptors. Binding of the coagonist is an obligatory requirement for NMDA receptor/channel activity, as the channel does not operate without it [2, 3]. Moreover, selective blockers of the coagonist site abolish NMDA receptor activity [4, 5].

The coagonist site seems to be not fully saturated in vivo, suggesting that it exerts a dynamic regulation of the NMDA receptors [6–8]. Accordingly, many studies revealed that the coagonist site exerts major regulatory roles. Binding of glycine to the coagonist site increases the affinity of NMDA receptors for glutamate [9] and decreases the receptor desensitization [10, 11]. Recently, occupation of the coagonist site has been shown to prime the NMDA receptor internalization, even in the absence of glutamate [12].

Whereas the important role of the coagonist site of the NMDA receptors is now widely recognized, the identity of the endogenous coagonist has been a matter of controversy. The original studies of Johnson and Ascher demonstrated the requirement for exogenous glycine to stimulate NMDA receptors, but did not biochemically isolate and confirm the identity of the physiological agonist. Soon after the discovery of the coagonist site, Kleckner and Dingledine demonstrated that D-serine was as effective as glycine in activating NMDA receptors [3]. As D-amino acids were considered "unnatural" isomers, binding of D-serine to the coagonist site was viewed as a nonphysiological event, which did not raise much attention. A number of years later, the demonstration of large amounts of endogenous D-serine in the mammalian brain was to drastically change this view [13]. We now know that D-serine is synthesized and released by neural cells, and its effects on NMDA receptors are in most cases indistinguishable from those of glycine [14–17].

D-Serine: A Novel (Co)Transmitter Candidate

The discovery of brain D-serine came from a serendipitous observation made by Hashimoto, Nishikawa and colleagues when they were testing the effects of *N*-myristoyl-D-serine administration in an animal model of schizophrenia [18]. When they sought to verify if *N*-myristoyl-D-serine was being cleaved to D-serine, they were surprised to observe high levels of endogenous D-serine in the brains of control animals that were not injected with the drug [18]. D-Serine concentration in the brain is about one third that of L-serine, and even higher than some L-amino acids [19]. Microdialysis experiments demonstrated that the extracellular D-serine is comparable to glycine in many brain areas [20, 21], whereas the affinity of NMDA receptors for D-serine is at least as high as for glycine [22]. These data provided indirect evidence indicating that D-serine might be an endogenous agonist of NMDA receptors [23].

The possibility that D-serine is an endogenous coagonist of NMDA receptors was elegantly investigated by Snyder and colleagues, which demonstrated that D-serine is enriched in areas containing the highest levels of NMDA receptors, including the cerebral cortex and the hippocampus [24]. By contrast, glycine is enriched in the brainstem, which has less NMDA receptor densities [25]. At high power examination, D-serine was enriched in astrocytes, indicating that glia is a source for D-serine [24, 25]. Astrocytes are a type of glia that ensheath the synapse, and the densities of D-serine are intimately close to neurons containing NMDA receptors. In addition, cultured astrocytes release [^{3}H]-D-serine when activated by AMPA/kainate type of glutamate receptors [24]. These data led to the proposal that D-serine is released from glia to activate neuronal NMDA receptors [24] (Fig. 1). This proposal suggests a unidirectional flow of D-serine from astrocytes to neurons (termed "unidirectional model", Fig. 1). The innovative aspect of this model was that it conferred to glia the noble task of regulating neuronal activity by means of D-serine release. At that time, the idea that glia affects neuronal activity by releasing chemical transmitters was new and still controversial [26, 27].

At first, the proposal that glia-derived D-serine was a physiological activator of NMDA receptors had many troubling issues. First, there was no direct demonstration that endogenous D-serine acted at the coagonist site of NMDA receptors, whereas the role of glycine as the endogenous coagonist was widely accepted. Second, the origin of D-serine was mysterious, and possible pathways or biosynthetic enzymes to D-serine were unknown at the time. Third, the molecular mechanisms regulating D-serine release had not been characterized in detail. Fourth, the mechanisms leading to termination of D-serine signaling were not clear. For instance, the metabolic enzyme for D-serine, D-amino acid oxidase, is scarcely present in

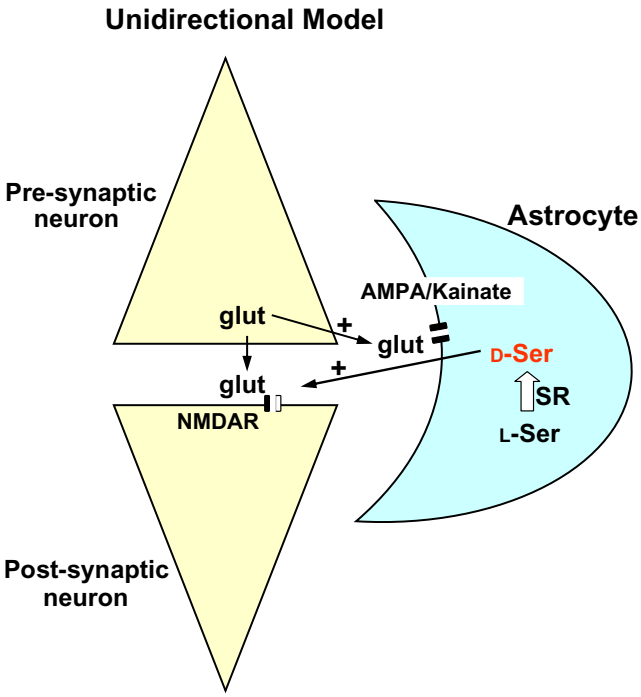

Fig. 1 Unidirectional model of D-serine signaling. The unidirectional model of D-serine flow from astrocytes to neurons was adapted from Schell et al. [24]. Glutamate (*glut*) released from neurons stimulates AMPA/kainate-type of glutamate receptors in astrocytes, leading to D-serine release. D-Serine acts in concert with glutamate to activate postsynaptic NMDA receptors [24]. D-Serine (*D-Ser*) is synthesized from L-serine (*L-Ser*) in astrocytes by the serine racemase (*SR*) enzyme [14]. For simplification, other by-products of SR (pyruvate and ammonia) [56, 58, 60, 104, 105] are not depicted in the model

forebrain areas [24, 28], indicating lack of significant metabolism in these areas [29, 30]. Moreover, high-affinity transporters for D-serine were unknown. Many of these issues were clarified in recent studies, which strongly support the notion that D-serine is an important chemical transmitter [15]. On the other hand, new studies raised major additional questions, especially on the relative roles of glia and neurons in synthesizing D-serine [31]. In the next sections, we discuss the recent advances in each of these issues and point to directions for future investigation.

Glycine or D-serine, Which Coagonist?

Electrophysiological and biochemical studies by several laboratories now support a role of D-serine as an endogenous coagonist of NMDA receptors. [32–36]. Almost all the studies used a similar strategy, which consisted of treating primary cell cultures or slices with purified D-amino acid oxidase enzyme [32]. This enzyme degrades D-serine, generating a keto acid, hydrogen peroxide, and ammonia [37]. Thus, degradation of endogenous D-serine by D-amino acid oxidase enzyme markedly reduces NMDA neurotransmission in hippocampal cultures [32]. Likewise, the induction of hippocampal long-term potentiation are also diminished by removing D-serine with D-amino acid oxidase [34]. In addition, the neuronal cell death caused by overactivation of NMDA receptors in hippocampal organotypic slices is mediated by endogenous D-serine, and not glycine [38]. In the retina, D-serine mediates a significant fraction of NMDA receptor responses, indicating a role as a physiologic coagonist [17, 33]. More recently, D-serine was also shown to mediate the NMDA receptor responses in the supraoptic nucleus of the hypothalamus, indicating that D-serine is a physiologically relevant NMDA receptor coagonist [36].

D-Serine is involved in additional NMDA receptor-related events, including cell migration during development [39]. Snyder and colleagues demonstrated that the removal of endogenous D-serine blocks the NMDA receptor-dependent granule cell migration from the external to the internal granule cell layer [39]. As migrating granule cells do not make conventional synaptic connections, the modulatory action of glial-released D-serine reflects a novel mechanism for neuromodulation [39].

Although it is clear that the data produced so far strongly support a role for D-serine in NMDA receptor neurotransmission, an important point to keep in mind is the experimental problems with the use of D-amino acid oxidase to remove D-serine. The first concern relates to its very low affinity (K_m of about 30 mM), which is more than four orders of magnitude lower than the affinity of NMDA receptors for D-serine [37, 38]. Perhaps because of its intrinsic low affinity, D-amino acid oxidase has not been much effective in some preparations of slices [32]. Moreover, some commercial preparations are not active enough to ensure complete removal of D-serine. Another problem observed in most commercial D-amino acid oxidase preparations relates to the presence of several impurities, including D-aspartate oxidase activity that quickly destroys NMDA itself [38]. This leads to an artifactual decrease in the NMDA receptor responses when employing exogenous NMDA plus D-amino acid oxidase. Therefore, when the enzyme preparation used to remove D-serine has not been thoroughly characterized and D-serine levels are not monitored, it is not possible to assume that the decrease in NMDA effects are specifically related to the removal of endogenous D-serine [40]. Recently, improved enzyme preparations have been employed to overcome these limitations. The use of recombinant D-serine deaminase to remove D-serine provides a high-affinity and pure D-serine degrading enzyme [38]. D-Serine deaminase is a bacterial enzyme that possesses very high affinity and specificity to D-serine and can be purified to homogeneity [41]. Alternatively, the use of a recombinant D-amino acid oxidase preparation diminishes the concern about the purity of the enzyme [42]. These improved enzyme preparations allows a direct comparison of the relative roles of D-serine vs glycine in stimulating NMDA receptors.

We recently utilized an improved enzyme preparation to show that D-serine is the dominant endogenous coagonist for NMDA neurotoxicity (Fig. 2). Massive stimulation of NMDA is a major culprit in the neuronal death that occurs after stroke [43]. In an organotypic slice model, removal of D-serine by the high-affinity recombinant D-serine deaminase practically abolished the NMDA receptor-elicited neurotoxicity (Fig. 2) [38]. By contrast, depletion of D-serine did not affect kainate neurotoxicity. This indicates that endogenous D-serine is the dominant and necessary coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices.

Furukawa and Gouaux proposed that D-serine binds more tightly to the receptor in comparison with glycine because it makes three additional hydrogen bonds and displaces a water molecule [44]. Thus, one possible explanation for the prevailing action of D-serine in NMDA receptor-elicited neurotoxicity in hippocampal slices could be its slightly higher affinity to the coagonist site when compared to glycine [22]. However, the endogenous glycine concentration in the conditioned medium was about tenfold higher than D-serine, which excludes this possibility. Another explanation for the ineffectiveness of endogenous glycine in mediating NMDA-elicited neurotoxicity is the action of the glycine transporter (GlyT1). The GlyT1 is expressed near NMDA receptors and keeps the synaptic glycine concentration below the levels required to saturate

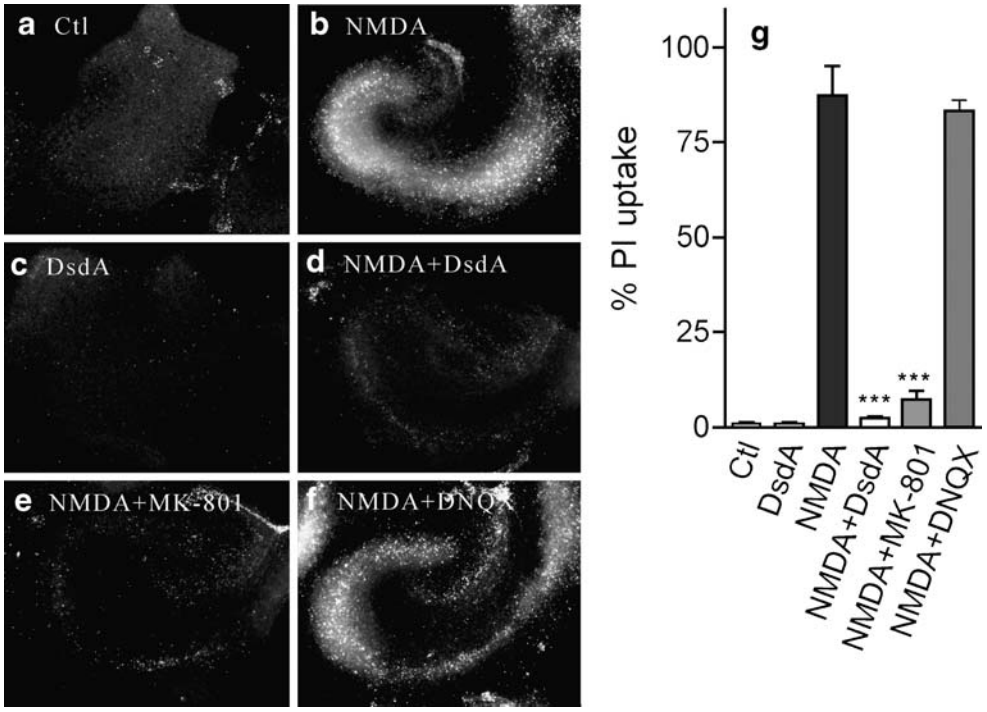

Fig. 2 D-Serine is the dominant coagonist for NMDA receptor elicited neurotoxicity. Removal of endogenous D-serine by D-serine deaminase (*DsdA*) abolishes NMDA receptor elicited neurotoxicity. **a** Control. **b** NMDA (500 μM) elicited robust cell death in all hippocampal areas as measured by propidium iodide (PI) fluorescence (*bright areas*). **c** Control treated with D-serine deaminase (*DsdA*). **d** Destruction of D-serine by DsdA protected against NMDA-elicited cell death. **e** Blockage of NMDA receptor by MK-801 protected against the NMDA insult. **f** Blockage of AMPA/kainate receptors by DNQX did not protect against the NMDA insult. **g** Densitometric analysis revealed that removal of endogenous D-serine by DsdA (*NMDA+DsdA*) practically abolished NMDA receptor-elicited neurotoxicity. Modified from Shleper at al. [38]

NMDA receptors [45–48]. Accordingly, we found that the addition of a specific inhibitor of the GlyT1 transporter elicited neurotoxicity, but only after D-serine was removed by D-serine deaminase treatment [38]. Thus, endogenous glycine was able to mediate NMDA receptor neurotoxicity only after GlyT1 was blocked and all endogenous D-serine had been destroyed [38]. The high efficiency of GlyT1 explains the lack of saturation of the coagonist site, despite the high extracellular glycine concentration. The presence of GlyT1 also explains the findings of Ascher and colleagues who observed that exogenously added D-serine was three orders of magnitude more potent than glycine in mediating NMDA receptor responses in hypoglossal neurons [45].

The dominant role of endogenous D-serine over glycine in regulating NMDA receptors was confirmed by direct electrophysiological experiments in the supraoptic nucleus of the hypothalamus [36]. In this study, Oliet and colleagues determined the NMDA component of neuronal activity and observed that D-serine mediates most of NMDA receptor responses at the synapse. Addition of a more efficient preparation of recombinant D-amino acid oxidase inhibited most of the NMDA receptor responses [36]. On the other hand, removal of endogenous glycine by addition of a specific glycine oxidase enzyme did not affect NMDA transmission. This study concurs with our data on NMDA receptor-elicited neurotoxicity, providing additional evidence that D-serine is the dominant coagonist of NMDA receptors, at least in some brain regions.

Serine Racemase: Biosynthesis of D-serine

The discovery of the biosynthetic enzyme for D-serine paved the way for additional advances in the field [14, 16, 49, 50]. The synthesis of free D-serine in eukaryotes was first described by Alton Meister and colleagues in 1965 [51]. They presented evidence for the occurrence of large amounts of D-serine in silkworms and demonstrated the interconversion of [^{14}C] L- into D-serine in intact animals [51]. Almost four decades after Meister's discovery, Esaki and colleagues directly demonstrated the synthesis of D-serine by a serine racemase activity in partially purified protein extracts of the silkworm *Bombyx mori* [52]. In the brain, the initial studies on the origin of D-serine were contradictory. Patients lacking the ability to degrade glycine by mutations in the glycine cleavage system (GCS) had decreased levels of brain D-serine, suggesting the involvement of GCS in D-serine synthesis [53]. On the other hand,

administration of L-serine to rats increases brain D-serine, also implicating L-serine as a precursor for D-serine synthesis [54, 55].

We found that the racemization activity observed in silkworms was conserved in the mammalian brain and isolated the biosynthetic enzyme for D-serine [49]. D-Serine is synthesized from L-serine by the serine racemase, a vitamin B_6-dependent enzyme [14, 49, 50]. Serine racemase was the first cloned eukaryotic racemase and displays unique properties. The enzyme also catalyzes the conversion of L-serine into pyruvate by β-elimination of water from L-serine [56]. Under normal conditions, about three molecules of pyruvate are synthesized for each D-serine generated. A unique feature of serine racemase is the use of magnesium and ATP as cofactors. Binding of magnesium stimulates the enzyme activity several fold [56–58]. Magnesium also complexes with ATP, and this complex strongly stimulates the enzyme, apparently at a different site [56]. The production of pyruvate by serine racemase has been proposed to play a role in the energy metabolism of glia, but it is still unclear if the amount of pyruvate would be enough to play a significant role. It is conceivable that the β-elimination of serine racemase is reminiscent of its homology to serine/threonine dehydratase class of enzymes from bacteria [50].

A striking feature of serine racemase is its unique ability to transform D-serine itself into pyruvate through its β-elimination activity [59, 60]. This activity allows the modulation of intracellular D-serine levels by limiting the achievable D-serine concentration within the cells. Thus, astrocytes overexpressing serine racemase more rapidly degrade D-serine because of the β-elimination activity [59]. It has been proposed that the β-elimination activity by serine racemase can be a source for metabolism of D-serine in forebrain areas, which lack significant amount of D-amino acid oxidase [59].

Regardless of the exact function of the additional reactions catalyzed by serine racemase, the existence of an enzyme for D-serine synthesis in the brain is perhaps the most compelling evidence that D-serine plays an important physiological role. Moreover, the study of serine racemase provides new tools to understand the role of D-serine. Preliminary biochemical analysis of serine racemase knockout mice revealed a large decrease in brain D-serine, indicating that serine racemase is the main source for brain D-serine [61]. Further analysis of the knockout mice phenotype will be an essential tool to investigate the role of D-serine in vivo.

Dynamic Regulation of D-serine Synthesis

The evidence that D-serine possesses a target receptor as well as biosynthetic and degradative apparatus implies that D-serine is an important neuromodulator/transmitter in the brain [16]. On the other hand, an important question concerning the proposed roles of D-serine as a regulator of NMDA receptors is on whether D-serine levels are dynamically regulated. As it has not been possible to directly monitor synaptic D-serine concentration, one way to approach this issue is to investigate the mechanisms regulating D-serine synthesis by the serine racemase enzyme. Although much remains to be learned about the serine racemase regulation, there is already ample evidence for the modulation of D-serine synthesis by different mechanisms.

Serine racemase activity is regulated by interacting proteins, mainly the glutamate receptor interacting protein (GRIP-1) and Golga3 [39, 62]. GRIP-1 binds to the C terminus of serine racemase through its PSD-95/Disc-large/Zo-1 (PDZ) domain [39]. Serine racemase displays a C-terminal PDZ-binding motif (TVSV), and the interaction is disrupted when the last amino acid Val-339 is replaced by glycine [39]. A significant increase on synthesis/release of D-serine has been observed by overexpression of GRIP-1 in C6 glioma cells. It is interesting to note that GRIP-1 mediates the activation of serine racemase by AMPA receptors. Serine racemase was strongly stimulated by GRIP-1 in the presence of AMPA, which promotes the dissociation of GRIP-1 from AMPA receptors [39]. Such dissociation of GRIP-1 is presumably responsible for the physiological activation of the serine racemase.

Another role for GRIP-1 and serine racemase interaction is to modulate neuronal migration in the developing cerebellum. Granule cell migration along Bergmann glia is blocked by degradation of D-serine or serine racemase inhibition, suggesting a role D-serine in mediating NMDA-dependent granule cell migration [39]. The migration appears to involve GRIP-1 influences on serine racemase because GRIP-1 viral infection to intact mice augments granule cell migration [39].

The molecular mechanism by which GRIP-1 binding regulates serine racemase activity is unknown. Nevertheless, it raises the possibility that the C-terminal region of serine racemase plays a regulatory role in its activity. This notion is strengthened by recent data showing that the C terminus of serine racemase also interacts with the scaffold protein interacting with C-kinase-1 (PICK-1), although no effects on serine racemase activity were reported for this interaction [63]. Curiously, whereas the human and the mouse serine racemase display the C-terminal PDZ binding motif, the rat [64] and the bovine protein (GenBank accession number XM_603692.2) lack this motif. This indicates that the regulation of serine racemase by GRIP-1 may differ among species.

Another recently identified regulator serine racemase is the Golgin subfamily A member 3 (Golga3) protein, which

acts by modulating the degradation of serine racemase by the ubiquitin–proteasome system [62]. Serine racemase turnover is mediated by its ubiquitylation and proteasomal degradation. The half-life of the overexpressed serine racemase protein is relatively short (4 h), much shorter than that of NMDA receptors (20 h) or D-serine itself (12 h) [62]. Golga3 (also known as golgin-160) decreases the ubiquitylation of serine racemase and significantly increases its half-life in pulse-chase experiments. It is plausible that Golga3, a Golgi and cytosolic protein involved in trafficking of proteins to the membrane, interferes with the action of a still unidentified E3 ubiquitin protein ligase to serine racemase. These results led us to propose that the ubiquitin system is a putative main regulator of serine racemase and D-serine levels. The ubiquitin system has been implicated in protein turnover at synaptic sites, but the mechanisms regulating synaptic proteins by degradation only started to be unveiled [65]. Local modulation of serine racemase degradation, such as that promoted by Golga3, provides a new mechanism for regulating synaptic D-serine levels with implications for NMDA receptor activity and neurotoxicity as well. Further studies will be required to identify the E3 ubiquitin protein ligase that targets serine racemase to the proteasome, as well as signals that might affect serine racemase half-life.

In addition to the actions of interacting proteins and the proteasomal system, serine racemase is also regulated at the transcription level. Hashimoto and colleagues demonstrated that in vivo injection of blockers of NMDA receptors [66, 67] elicit an increase in serine racemase mRNA, providing a link between synaptic activity and D-serine synthesis. Barger and colleagues demonstrated that activation of microglia by lipopolysaccharide or β-amyloid increases serine racemase mRNA by enhancing its transcription [68, 69].

Altogether, the above studies suggest that D-serine levels are dynamically regulated by extracellular signals transduced by glutamate receptors, protein degradation machinery, and gene transcription.

Neuronal Serine Racemase and D-serine

The specific release of D-serine from astrocytes was a central assumption in the "unidirectional model" of D-serine action (Fig. 1), which supports the role of glia in affecting neuronal activity by releasing gliotransmitters [70, 71]. Previous papers demonstrating the role of D-serine in mediating NMDA receptor responses assumed that the effects observed were caused by glial and not neuronal D-serine [32–35]. This assumption was correct in the light of the known disposition of D-serine at the time, although a brief study reported the presence of D-serine in some neurons of the cerebral cortex when using an amplification system [72]. We recently explored a possible neuronal localization of D-serine and serine racemase utilizing new antibodies against D-serine and serine racemase [31]. The results provided strong evidence that D-serine is also synthesized in neurons both in vitro and in vivo (Fig. 3). This led us to propose a new model in which a bidirectional flux of D-serine exists between neurons and astrocytes, and accounts for the activation of NMDA receptors (Fig. 4, see next sections).

We initially observed that virtually pure neuronal cultures synthesize as much D-serine as glial cultures [31]. The amount of D-serine synthesized was not dependent on the amount of glia, which was monitored in all cultures. The presence of serine racemase in neurons was further verified by immunocytochemistry both in cultures and in situ (Fig. 3) [31]. The use of a new antibody against serine racemase was key for our observation of substantial quantities of serine racemase in neurons. The previous serine racemase antibody was reported to be devoid of immunoreactivity against paraformaldehyde-fixed tissue and was only suitable for methanol-fixed sections [49] known to generate artifacts in slice sections. Although this technique was useful to reveal serine racemase in glia, it might have negatively affected the antigenicity of serine racemase and precluded the observation of the neuronal staining. Using a new antibody suitable for paraformaldehyde fixation, we detected serine racemase in neurons in situ, at levels comparable or higher than those found in glia (Fig. 3). Recently, Hashimoto and colleagues reported that serine racemase protein and mRNA are higher in cultured neurons than astrocytes, confirming our recent findings [67].

In the light of the presence of serine racemase in neurons, we reexamined D-serine staining and observed D-serine in neurons using two different antibodies. 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 previously employed. We found that neuronal D-serine is more prominent in the cerebral cortex, whereas glial D-serine predominated in other regions.

Glial and neuronal D-serine may be differentially regulated during development. Puyal et al. observed that the localizations of D-serine in the rat vestibular nuclei shifts from glia to neurons in older rats [73]. The developmental decrease in glial D-serine was associated to an increase in D-amino acid oxidase enzyme, which is mostly expressed in glia. Neuronal staining was more prominent in the vestibular nuclei of adult rats, indicating that glial and neuronal D-serine may have distinct functional roles depending on the developmental stage of the vestibular network. Recently, Pow and colleagues also observed D-serine in a subset of neurons by employing paraformaldehyde as a fixative for D-amino acids, instead

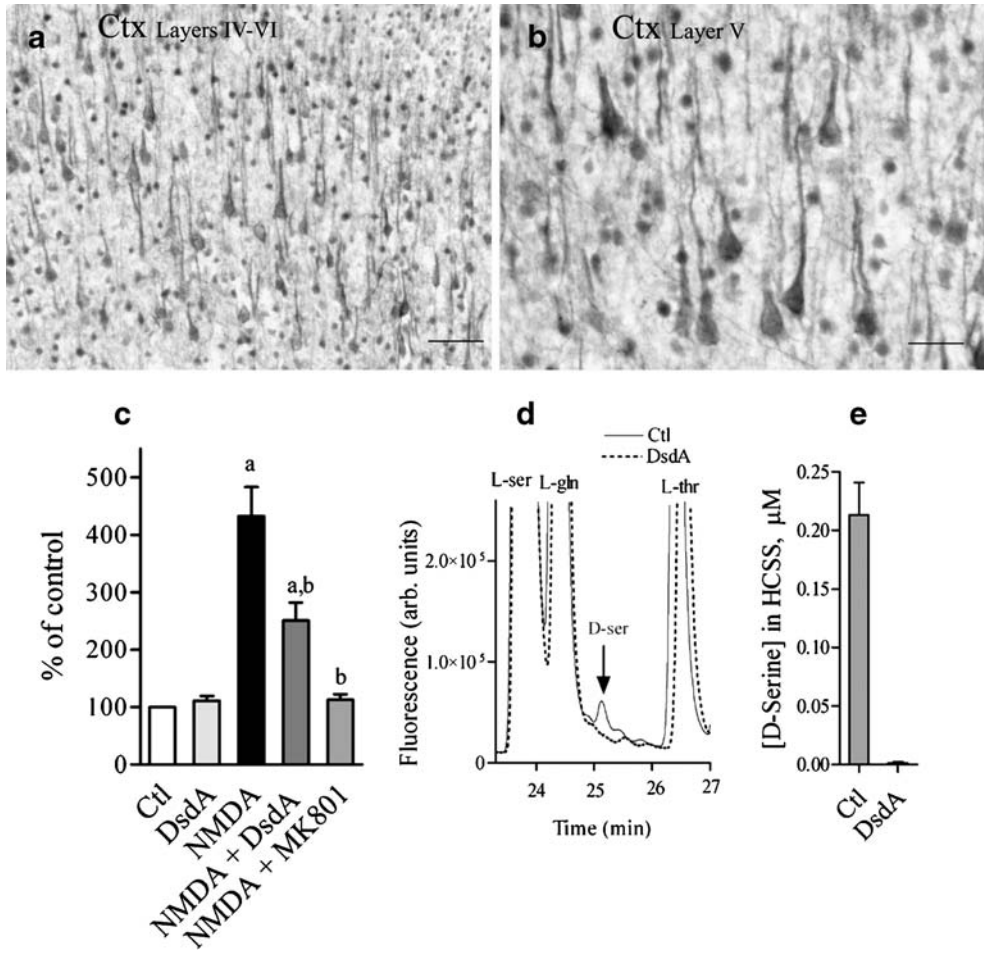

Fig. 3 Presence of serine racemase in neurons and role of neuron-derived D-serine in NMDA receptor-elicited neurotoxicity. **a** and **b** Serine racemase is present in the cerebral cortex of rats as revealed by immunohistochemistry. **c** Removal of D-serine by D-serine deaminase (*DsdA*) significantly decreased NMDA-elicited cell death in virtually pure cortical neuronal cultures (*NMDA+DsdA*). Blockage of the NMDA receptors by MK801 abolished the cell death promoted by NMDA (*NMDA+MK801*). Cultures were treated with 500 µM NMDA and cell death was assayed 24 h later by release of lactate dehydrogenase. **d** HPLC analysis of culture medium reveals a discrete D-serine peak (*continuous line*) that was completely destroyed by DsdA treatment (*dashed line*). **e** D-serine is released into the conditioned medium (*HCSS*) of virtually pure neuronal cultures. Adapted from Kartvelishvily et al. [31]

of the classic glutaraldehyde fixation [74]. Different from our study [31], they did not report D-serine in cortical neurons, although they observed some neuronal staining for D-serine in the hindbrain. The reasons for this apparent discrepancy are unclear, but it may be related to the different fixatives and antibodies employed.

Role of Neuronal D-serine

A major question regarding the presence of serine racemase and D-serine in neurons relates to its role relative to glial D-serine. We recently demonstrated that neurons exhibit a regulated D-serine release and that neuron-derived D-serine mediates a significant fraction of NMDA receptor-elicited neurotoxicity in primary cultures (Fig. 3) [31]. Instead of the unidirectional flow of D-serine from astrocytes to neurons (Fig. 1), we propose a model in which NMDA stimulation may be carried out by both neuronal and glial D-serine, which flows in opposite directions (termed "bidirectional model", Fig. 4).

We initially investigated the role of neuronal D-serine by employing an in vitro model of NMDA-elicited neurotoxicity [31]. In virtually pure neuronal cultures, the neuron-derived D-serine accounts for a significant fraction of the NMDA-elicited excitotoxicity (Fig. 3). Thus, treatment of the cultures with D-serine deaminase to remove endogenous D-serine led to a significant decrease in the cell death caused by NMDA addition (Fig. 3). This result suggests that neurons are an important source of D-serine with implications for the regulation of NMDA receptor transmission. In this context, it is conceivable that neuronal D-serine exerts an autocrine or paracrine activation of NMDA receptors.

Our recent result highlights the importance of neuron-derived D-serine in mediating the NMDA receptor activation that occurs in excitotoxicity. On the other hand, as in vivo neurons do not occur in the absence of glia, additional studies in slices and in situ will be important to determine the relative importance of glial vs neuronal D-serine in neurotoxicity. To directly answer this question, it will be crucial to develop reliable techniques to knockdown D-serine synthesis and release from specific cell populations.

The availability of L-serine, the substrate for D-serine synthesis, may also affect the neuronal synthesis of D-serine. In the cerebral cortex, pyramidal neurons exhibit high densities for L-serine, but in other brain regions

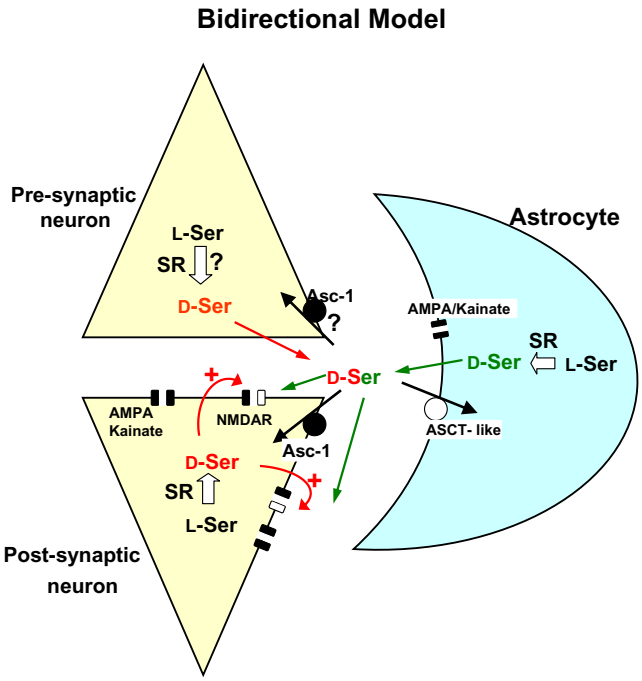

Fig. 4 Bidirectional model of D-serine signaling. The proposed model includes a bidirectional flow of D-serine from neurons and astrocytes in opposite directions. The model accounts for the recently reported presence of D-serine [31, 72, 73, 74] and serine racemase [31] in neurons. D-Serine is released from astrocytes upon AMPA/kainate receptor stimulation either by a nonvesicular [86, 106] or vesicular [42] mechanism and act in concert with glutamate to stimulate NMDA receptors (*green*) [24]. Reuptake of released D-serine by glia is mediated by a neutral amino acid transporter similar to the alanine, serine, cysteine, threonine transported (ASCT-like) [86, 107]. Neurons expressing serine racemase also synthesize D-serine (*red*) and release D-serine upon ionotropic glutamate receptor activation and depolarization by KCl [31]. It is not known if neuron-derived D-serine stimulates synaptic, extrasynaptic receptors or both. Moreover, neuronal serine racemase immunoreactivity occurs in neuronal somata and dendrites, whereas its presence in presynaptic sites has not been investigated. Neuronal uptake of D-serine is carried out by the Asc-1 transporter [84, 85]. Asc-1 immunoreactivity has been observed both at the presynaptic [84] and at postsynaptic sites [85], but other D-serine transporters may exist. As both neuronal and glial D-serine are released by glutamate receptor stimulation, the dynamic fluxes of glutamate from astrocytes and neurons (not depicted in the model) will likely determine the direction and amount of D-serine release

L-serine levels are higher in astrocytes [75]. Astrocytes were shown to actively export L-serine to neurons [76–78]. Thus, the supply of L-serine to neurons may also play an important role in the regulation of D-serine synthesis. Future studies will be important to define the L-serine threshold for D-serine synthesis by neurons and astrocytes.

Neuronal Release of D-serine

Neurons are also able to mediate regulated release of D-serine. In virtually pure neuronal cultures, we observed the production and release of D-serine in the conditioned medium (Fig. 3) [31]. Ionotropic glutamate receptor activation promotes the release of D-[^{3}H]serine from neurons in a Ca^{2+}- and Na^+-dependent manner [31]. This effect is more evident with AMPA and kainate receptor stimulation. The release promoted by AMPA was not inhibited by bafilomycin A_1, which blocks the vesicular uptake of transmitters. In addition, D-serine uptake in purified brain synaptic vesicles was also not detectable under conditions that are optimal for the uptake of known neurotransmitters [31]. Although these data do not definitively exclude vesicular release of D-serine from a small population of cells, the neuronal D-serine release under our experimental conditions was most probably from a cytosolic nonvesicular pool.

Cortical neurons expressing serine racemase also contain NMDA receptors in situ and NMDA receptor activation also promotes an increase in neuronal D-serine release [31]. This provides a functional link between NMDA receptors and the neuronal pool of D-serine. Our demonstration that NMDA can induce neuronal D-serine release shed light on 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 [79]. 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+}. AMPA/kainate receptor activation also increases intracellular Ca^{2+}, either through opening Ca^{2+} permeable receptors [80, 81] or by favoring the reverse mode of the Ca^{2+}/Na^+ exchanger [82]. 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. Further studies will be required to unravel the pathway(s) for D-serine release from neurons.

The demonstration of neuronal D-serine also imply a role for the Asc-1 transporter, recently shown to possess a relatively high-affinity for D-serine [83, 84]. This neutral amino acid transporter is exclusively found in neurons and the presence of both serine racemase and D-serine in neurons indicates that Asc-1 plays a role in D-serine transport. Whereas the selective localization of Asc-1 in neurons has been clearly demonstrated, there is no consensus on it cellular localization. Thomsen and colleagues reported an exclusive presynaptic localization by electron microscopy [84], whereas Kanai and colleagues found ASC-1 mainly in neuronal cell bodies and dendrites [85].

The mechanisms of D-serine release from neurons display some similarities with glia, but are not identical. Both are induced by AMPA receptor stimulation, but may display different molecular mechanisms. The glial release of D-serine has been shown to be of both nonvesicular [86] and vesicular origin [42]. On the other hand, KCl and NMDA induce release of D-serine from neurons, but are ineffective in glia [31]. Mothet and coworkers have shown that cultured astrocytes and C6 glioma cells can mediate

vesicular release of D-serine when stimulated by AMPA [42]. The vesicular release of D-serine from glia would provide a fast regulation of NMDA receptors and strengthen the possibility that D-serine is a gliotransmitter. On the other hand, there are only few vesicles in astrocyte processes in vivo [71]. Thus, it is not clear if the vesicular mechanism could surpass the release from the cytosolic route and if it takes place in more physiological preparations, such as slices. Future studies will be important to elucidate the relative contributions of cytosolic vs vesicular D-serine, as well as the roles of neuronal vs glial D-serine. It will be also important to define the nature and regulation of D-serine release, i.e., as to whether it is dependent on astrocytic/neuronal activation or released in a continuous fashion.

Pathological Aspects

The prominent role of D-serine in mediating the NMDA receptor-elicited neurotoxicity has important pathological implications. Stroke is a leading cause of death and disability in the world. Nevertheless, therapies to prevent stroke damage are not yet very effective. The NMDA receptor is a major pharmacological target to prevent or decrease stroke damage, as its overactivation is a main culprit in the post ischemic cell death that occurs after stroke [43]. But unfortunately, blockers of the coagonist site of NMDA receptors were effective in animal models of stroke, but little tolerated in clinical trials because of adverse effects, such as hallucinations [87]. This has been attributed to excessive blockage of the NMDA receptors and alteration of normal neurotransmission [88].

Recently, there has been a paradigm shift toward the development of more gentle inhibitors, with lower affinity for the NMDA receptors and therefore less side effects. Memantine, a low-affinity open-channel blocker of NMDA receptors has been approved by the European Union and FDA for use in dementia, as it shows beneficial effects in Alzheimer's disease [88]. In this context, it is possible that inhibitors of serine racemase would offer a more gentle and indirect way to decrease NMDA receptor function, with less unwanted side effects. As removal of D-serine protects against NMDA receptor-elicited cell death [31, 38], serine racemase inhibitors are promising drug candidates to prevent stroke damage [89]. In addition, D-serine has been shown to be involved in spinal cord NMDA receptor-mediated pain sensation [90]. Thus, inhibitors of D-serine production could be useful against chronic pain management as well.

Another important pathological aspect of D-serine relates to NMDA hypofunction thought to occur in schizophrenia [91]. Blockers of NMDA receptors, such as phencyclidine and ketamine, mimic many of the symptoms of schizophrenia [92, 93]. Likewise, genetically engineered mice that display reduced expression of NR1 subunit of NMDA receptors exhibit increased locomotor activity, stereotypy and deficits in social and sexual behavior that resembles schizophrenia [94]. These data support the notion that NMDA receptor hypofunction play a role in the disease. Schizophrenia exhibits three classes of symptoms: positive, cognitive and negative symptoms. The positive symptoms, such as delusions and hallucinations are well controlled by antipsychotic agents, which act by blocking D2 dopamine receptors [95]. On the other hand, the patients display decreased cognitive ability (cognitive symptoms) and social interaction deficits (negative symptoms) that are more refractory to therapy.

Based on the NMDA hypofunction hypothesis, several clinical trials were carried out to evaluate the efficacy of stimulation of NMDA receptors in schizophrenia. Administration of D-serine greatly ameliorates the positive, negative and cognitive symptoms of schizophrenia when associated with conventional neuroleptics [95–97]. It is interesting to note that schizophrenic patients display a higher ratio of L- to D-serine in the blood and cerebrospinal fluid [98–101]. The possible involvement of D-serine in schizophrenia was also raised by genetic studies. Cohen and colleagues identified a linkage between polymorphisms in D-amino acid oxidase and its activator gene (G72) in familial forms of the disease [102]. Nevertheless, despite the significant association of schizophrenia with polymorphisms in D-amino acid oxidase gene, the genetic data should be interpreted with caution. Schizophrenia is a complex and multifactorial disease, in which several genes are likely to be involved and the contribution of a single gene may be very difficult to ascertain. Additional studies with larger populations will be required to establish a role of D-amino acid oxidase in schizophrenia.

D-Serine may also play a role in the cognitive modifications that occur in aging. D-Serine enhances the long-term potentiation of synaptic plasticity in CA1 subfield of the hippocampus of aged senescence-accelerated mice [35]. In old rats, D-serine rescues the age-related impairment of hippocampal long term potentiation and NMDA receptor mediated synaptic potentials [103]. The relative impairment of the NMDA receptor transmission in old rats occurs along with a decrease in hippocampal D-serine levels and serine racemase expression [103]. As old rats exhibit considerable increase in glia (gliosis) and loss of neurons, it is possible that the age-related reduction in D-serine levels is attributable to a loss of neuronal D-serine, as neurons express significant levels of serine racemase [31].

Conclusions and Perspectives

In the last few years, accumulating evidence indicate that D-serine is a relevant chemical transmitter in the brain. It

possesses a target receptor, biosynthetic and degradative enzymes, transport mechanisms and regulated release routes. D-Serine acts as a physiological ligand at the glycine site of NMDA receptors and may be the dominant agonist in several brain regions. Moreover, D-serine mediates NMDA neurotoxicity, and serine racemase is a potential target for drugs to decrease NMDA-mediated cell death. New data indicate that D-serine is not a specific glial transmitter, but is also produced and released from neurons as well. In light of the recent evidence that D-serine is a glial/neuronal coagonist, we now proposed a "bidirectional model" for D-serine actions, in which the activation of NMDA receptors results from the flow of D-serine from both neurons and glia. Thus, it seems appropriate to propose that some previous conclusions about the role of D-serine as a specific glia-derived coagonist should be reevaluated considering the actions of D-serine originated from neurons. Future studies will be important to elucidate the molecular mechanisms of D-serine release and the relative roles of glia and neurons in D-serine signaling.

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