

# Neurobiology through the looking-glass: D-serine as a new glial-derived transmitter

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## Abstract

D-Amino acids have been known to be present in bacteria for more than 50 years, but only recently they were identified in mammals. The occurrence of D-amino acids in mammals challenge classic concepts in biology in which only L-amino acids would be present or thought to play important roles. Recent discoveries uncovered a role of endogenous D-serine as a putative glial-derived transmitter that regulates glutamatergic neurotransmission in mammalian brain. Free D-serine levels in the brain are about one third of L-serine values and its extracellular concentration is higher than many common L-amino acids. D-Serine occurs in protoplasmic astrocytes, a class of glial cells that ensheath the synapses and modulate neuronal activity. Biochemical and electrophysiological studies suggest that endogenous D-serine is a physiological modulator at the co-agonist site of NMDA-type of glutamate receptors. We previously showed that D-serine is synthesized by a glial serine racemase, a novel enzyme converting L- to D-serine in mammalian brain. The enzyme requires pyridoxal 5'-phosphate and it was the first racemase to be cloned from eucaryotes. Inhibitors of serine racemase have therapeutic implications for pathological processes in which over-stimulation of NMDA receptors takes place, such as stroke and neurodegenerative diseases. Here, we review the role of endogenous D-serine in modulating NMDA neurotransmission, its biosynthetic apparatus and the potential usefulness of serine racemase inhibitors as a novel neuroprotective strategy to decrease glutamate/NMDA excitotoxicity.

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## 1. Introduction

D-Amino acids are mirror, looking-glass images of their L-counterparts. Though their chemical properties are identical, they play distinct roles in living organisms. In most bacteria, D-amino acids are components of the cell wall peptidoglycan, making them more resistant to glycopeptide antibiotics (Roper et al., 2000). Despite a few reports of D-amino acids in invertebrates, it took five decades to begin uncovering the roles of D-amino acids in mammals. The neurobiology of such odd chiral molecules reveals new mechanisms to modulate neurotransmission and also established the conservation of D-amino acid metabolism in mammals. Lajtha and coworkers first detected the presence of high levels of free D-aspartate in the rat and human brain (Dunlop et al., 1986). Subsequently, Hashimoto et al. discovered remarkable quantities of D-serine in mammalian brain at levels even higher than some L-amino acids (Hashimoto et al., 1992).

D-Aspartate occurs in neurons in the early stages of the development (Wolosker et al., 2000) and is enriched in neuroendocrine tissues in the adulthood (Schell et al., 1997a). By contrast, high densities of D-serine are found in astroglial cells in gray matter areas of the brain (Schell et al., 1995); its levels in neurons are much lower, barely detectable with special immunohistochemical techniques (Yasuda et al., 2001a).

In the past few years, several roles for D-amino acids in mammals have been proposed. For instance, D-aspartate accumulates over time in PC12 cell cultures (Long et al., 1998) and is synthesized from L-aspartate in primary neuronal cultures (Wolosker et al., 2000). D-Aspartate was shown to influence the secretion of several hormones, such as growth and luteinizing hormones, testosterone, melatonin and oxytocin (D'Aniello et al., 1996; Ishio et al., 1998; Nagata et al., 1999; D'Aniello et al., 2000; Wang et al., 2000). Pharmacological studies have shown that D-serine activates NMDA receptor responses by binding to the "glycine site" of the receptor with identical or even higher affinity than the co-agonist glycine (Danysz et al., 1990; Matsui et al., 1995; Danysz and Parsons, 1998). This led Hashimoto and

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co-workers to propose that D-serine would be an endogenous agonist of NMDA receptors (Hashimoto et al., 1993a). Subsequently, immunohistochemical experiments demonstrated that D-serine is enriched in astrocytes in regions containing highest levels of NMDA receptors (Schell et al., 1995). Direct evidence that endogenous D-serine regulates NMDA receptors came from experiments in which NMDA receptor activity in slices and cell cultures diminished after the selective removal of endogenous D-serine by adding D-amino acid oxidase, an enzyme that destroys D-serine (Mothet et al., 2000). Release of D-serine from astrocytes to regulate postsynaptic NMDA receptors represents a new form of glial-neuronal communication to modulate neurotransmission.

Because enzymes that convert L- to D-amino acids, namely racemases, were thought to be restricted to bacteria, the presence of D-serine in the brain was regarded by many as of exogenous origin, derived either from the diet or by the activity of intestinal bacteria. Recently, mammalian serine racemases have been purified and cloned from mouse and human brain (Wolosker et al., 1999a,b; De Miranda et al., 2000). Pharmacological inhibition of serine racemase decreases cellular D-serine levels and provides a new strategy to decrease NMDA receptor coactivation (Panizzutti et al., 2001). Additionally, several aspects of the neurobiology of D-serine can be learned from the cloned enzyme and are also subject of our present review.

## 2. D-Serine disposition

D-Serine occurs almost exclusively in the mammalian brain with much lower levels in peripheral tissues (Hashimoto et al., 1992, 1993a). Highest levels of D-serine were observed in forebrain areas, such as cerebral cortex, hippocampus and striatum (Hashimoto et al., 1993a). D-Serine levels in the brain are about two orders of magnitude higher than D-alanine, which is the most prevalent neutral D-enantiomer in bacteria. In the extracellular fluid, D-serine concentration ranges from 5 to 7  $\mu\text{M}$  in forebrain areas, three- to five-fold higher than L-glutamate and L-aspartate as measured by *in vivo* microdialysis (Hashimoto et al., 1995).

To determine its cellular localization and assess its candidacy as a neuromodulator, Schell et al. developed a stereoselective antibody against D-serine and localized D-serine to protoplasmic astrocytes in the gray matter, with a distribution closely resembling that of NR2A/B subtypes of NMDA receptors (Schell et al., 1995, 1997b). D-Serine densities are inversely correlated with the expression of D-amino acid oxidase, an enzyme that robustly deaminates neutral D- (but not L-) amino acids (Schell et al., 1995). D-Amino acid oxidase was discovered more than six decades ago (Krebs, 1935), but the full appreciation of its function was hampered by the lack of knowledge on the existence of D-enantiomers in mammals, especially in the brain. Hence, the inverse localizations of D-serine and D-amino acid oxidase implies

that the enzyme play a role in metabolizing endogenous D-serine. In the developing cerebellum, Bergmann glia stains intensely for D-serine and has no detectable D-amino acid oxidase (Schell et al., 1995). Animals older than 3 weeks begin to express D-amino acid oxidase in the cerebellum (Horiike et al., 1987), which coincides with a large reduction of D-serine levels in Bergmann glia (Schell et al., 1997b). Accordingly, mice expressing an inactive mutant form of D-amino acid oxidase exhibit a 10-fold increase of D-serine levels in the cerebellum and in the brainstem, indicating that D-amino acid oxidase is the catabolic enzyme for D-serine in those regions (Hashimoto et al., 1993b).

## 3. D-Serine as an endogenous agonist of NMDA receptors

NMDA-type of glutamate receptors are a class of ionotropic receptor channels that require binding of more than one agonist to mediate ion influx, i.e. the channel only functions if both a glutamate site and co-agonist site are occupied. From the pioneering work of Ascher and others, it was thought that glycine was the sole co-agonist that physiologically activates NMDA receptors (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Glycine binding to NMDA receptor is insensitive to strychnine and this co-agonist site is generally referred to as strychnine-insensitive site. Though the concentrations of glycine required to fully activate NMDA receptors are in the submicromolar range, the synaptic glycine levels undergo dynamic regulation by a powerful glycine reuptake system and abundant evidence has been accumulated that this site is not saturated *in vivo* (Bergeron et al., 1998; for review see also Danysz and Parsons, 1998). Moreover, the studies demonstrating the requirement of a co-agonist to activate NMDA receptor did not address the identity of the endogenous co-agonist, but rather showed that exogenous applied glycine was sufficient to fully activate NMDA receptor activity (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988).

Several lines of evidence indicate that D-serine is an endogenous ligand of the NMDA receptor. D-Serine is up to three-fold more potent than glycine at the co-agonist site of NMDA receptors (Matsui et al., 1995). Extracellular D-serine concentration is similar or even higher than glycine in some brain areas as measured by *in vivo* microdialysis (Hashimoto et al., 1995). D-Serine localizes to gray matter areas enriched in NMDA receptors, while glycine densities are prominent in the brainstem and spinal cord (Schell et al., 1997b). Moreover, D-serine is functionally 100 times more effective than glycine in potentiating NMDA receptor-mediated spontaneous synaptic currents in hypoglossal motoneurons (Berger et al., 1998). The difference between the relative activities of exogenous D-serine and glycine to activate recombinant and synaptic NMDA

receptors has been attributed to the presence at synapses of a glycine transporter that sets the cleft concentration of glycine well below that required to saturate the glycine site (Berger et al., 1998; Bergeron et al., 1998).

To directly assess the identity of the endogenous co-agonist, Mothet et al. (2000) employed a simple strategy to selectively deplete brain slices and cell cultures from endogenous D-serine. They pre-incubated slices and cell cultures with the enzyme D-amino acid oxidase, which destroyed more than 90% of total D-serine content without affecting other amino acid levels. D-Amino acid oxidase selectively decreased NMDA receptor responses by 50–70%. In hippocampal cell cultures, D-amino acid oxidase reduced the amplitude of spontaneous NMDA receptor-mediated synaptic currents with no effect on the frequency, implying a post-synaptic effect. The inhibitory effect of D-amino acid oxidase was fully reversed by exogenous D-serine. Non-NMDA ionotropic excitatory amino acid receptor function was unaffected by the enzyme. Thus, in the context of previous studies showing that D-serine is a potent agonist ligand for the strychnine-insensitive glycine site of NMDA receptors (Matsui et al., 1995; Danysz and Parsons, 1998), the data strongly support a role for D-serine as an endogenous co-agonist of NMDA receptors (Mothet et al., 2000). NMDA responses were not fully blocked by addition of D-amino acid oxidase. The D-amino acid oxidase-insensitive varied depending on the experimental conditions, ranging from 30 to 50% of NMDA activity. Thus, endogenous glycine might account for activation of the remaining co-agonist sites of the NMDA receptor and may be the main endogenous ligand in other parts of the brain, such as brainstem and spinal cord.

Even in the spinal cord, in which glycine concentration is higher than in the forebrain, D-serine may also be an endogenous ligand of the NMDA receptor. Accordingly, mice expressing an inactive mutant form of D-amino acid oxidase along with augmented D-serine levels in the spinal cord exhibited an increase on NMDA receptor-mediated excitatory post-synaptic currents recorded from dorsal horn neurons (Wake et al., 2001).

At present, the synaptic concentration of D-serine and glycine are not known. Selective blockers of D-serine uptake system or biosynthetic apparatus should allow the distinction of glutamatergic synapses that are pre-dominantly dependent on D-serine from those modulated by glycine.

#### 4. Serine racemase

The high levels of D-serine in discrete areas of rat brain imply the existence of a biosynthetic pathway. We felt that L-serine was a strong candidate to serve as precursor for D-serine synthesis. Intraperitoneal injection of L-serine promoted accumulation of D-serine in the cerebral cortex (Takahashi et al., 1997) and injection of [<sup>3</sup>H] L-serine into rat brain led to accumulation of labeled D-serine as well (Dunlop and Neidle, 1997). Both L-serine and D-serine are

enriched in astrocytes, which possess the enzymatic apparatus to synthesize L-serine (Yamasaki et al., 2001; Yasuda et al., 2001b).

To purify serine racemase, we added L-serine to brain subfractions and measured the conversion of L- to D-serine (Wolosker et al., 1999a). Like bacterial racemases, the purified mammalian serine racemase requires the cofactor pyridoxal 5'-phosphate (Wolosker et al., 1999a). The enzyme is highly selective toward L-serine, failing to racemize other amino acids (Wolosker et al., 1999a). The cloned enzyme has 30–40% homology to enzymes of the serine/threonine dehydratase family, which like serine racemase, are dependent on pyridoxal 5'-phosphate (Wolosker et al., 1999b; De Miranda et al., 2000). Though several amino acid racemases have been cloned from bacterial sources, no eukaryotic amino acid racemase has been previously cloned. All of the known amino acid racemases have been cloned from archae or eubacteria including alanine, aspartate, glutamate, serine and phenylalanine racemase. However, none of these display significant amino acid sequence homology to serine racemase, implying that the mammalian racemase evolved from an ancestor related to the serine/threonine dehydratase family. Over-expression of serine racemase in cultured HEK293 cells elicits robust synthesis of D-serine and the amounts of D-serine produced greatly increase as L-serine is supplemented to the culture media (Wolosker et al., 1999b). Serine racemase is highly enriched in brain and localizes to astrocytes with a distribution pattern similar to that of D-serine. Co-localization of serine racemase and D-serine in glia indicates that D-serine is formed in the cells that contain endogenous D-serine rather than being synthesized in neurons and transported into glia.

Since D-serine is an endogenous agonist of NMDA receptors, selective inhibitors of serine racemase should be valuable tools for investigating the regulation of NMDA transmission. Moreover, serine racemase can also be conceived as a novel therapeutic target. Massive stimulation of NMDA receptors is implicated in neural damage following stroke (Choi and Rothman, 1990) and inhibitors of serine racemase may be useful to prevent stroke damage. Elevations of extracellular concentrations of D-serine are observed after transient cerebral ischemia in rats (Lo et al., 1998). Drugs that block the “glycine site” of NMDA receptors prevent stroke damage in animal models (Gill et al., 1995), but were not well tolerated in clinical trials or did not attained therapeutic levels in the brain due to poor penetration into the blood-brain barrier (Danysz and Parsons, 1998). Thus, inhibitors of serine racemase provide a new strategy to decrease NMDA receptor coactivation and may be useful in conditions such as stroke and neurodegenerative diseases where over-stimulation of NMDA receptors play a pathological role (Panizzutti et al., 2001). We recently demonstrated that serine racemase activity and D-serine synthesis in primary astrocyte cultures is inhibited by L-serine O-sulphate, which is a model compound for designing selective activity-based inhibitors (Panizzutti et al., 2001).

The study of serine racemase properties and regulation will be important to learn the mechanism by which this unusual enzyme works and also will further shed light on the neurobiology of D-serine.

## 5. D-Serine and schizophrenia

NMDA receptor hypofunction has been implicated in the pathophysiology of schizophrenia. NMDA antagonists, such as phencyclidine, induce positive, negative, and cognitive schizophrenic-like symptoms in healthy volunteers and precipitate thought disorder and delusions in schizophrenia patients (Goff and Coyle, 2001). Animal studies also support this hypothesis. Mice expressing only 5% of normal levels of NMDAR1 (NR1) subunit display behavioral abnormalities, including increased motor activity and stereotypy and deficits in social and sexual interactions (Mohn et al., 1999). As a selective agonist of NMDA receptor, D-serine reverses the hyperactivity and stereotype behavior induced by NMDA receptors antagonists (Contreras, 1990).

Based on the NMDA receptor hypofunction hypothesis, several clinical trials were carried out to evaluate the efficacy of map co-agonists of NMDA receptors in schizophrenic patients. Glycine administration was shown to greatly improve symptoms of schizophrenia (Heresco-Levy et al., 1996, 1999). Likewise, in a study conducted by Coyle and associates, D-serine greatly improved the positive, negative, and cognitive symptoms of schizophrenic subjects when associated to conventional neuroleptics (Tsai et al., 1998). The beneficial effect of D-serine as adjuvant therapy supports

the NMDA hypofunction hypothesis for schizophrenia, but the efficacy of the therapy should be further confirmed in additional clinical trials.

## 6. A putative glial-derived transmitter

In recently years, it has been shown that astrocytes and neurons exhibit a dynamic signaling that profoundly influences neuronal activity and development (Haydon, 2001; Lemke, 2001). It has been shown that astrocytes directly increase neuronal transmission by releasing chemical transmitters, such as glutamate (Haydon, 2001). Moreover, astrocytes undergo spontaneous calcium oscillations that trigger NMDA receptor-mediated inward currents in neurons (Parri et al., 2001). Within this context, we propose that D-serine is a new type of transmitter released from glia to influence nearby neurons (Fig. 1). We have previously shown that D-serine is synthesized by a specific racemase located in glia (Wolosker et al., 1999a,b). D-Serine accumulated in astrocytes cultures is released by kainate, a potent agonist of non-NMDA receptors enriched in glia (Schell et al., 1995). Upon release, D-serine acts on a specific target, the NMDA receptor in nearby neurons (Fig. 1). The concerted action of glutamate and D-serine would be required for full NMDA receptor activation. Thus, D-serine seems to play a role in a new type of glial-neuronal communication.

To clarify the role of D-serine as a modulator of NMDA receptors, one should identify the factors that will promote the termination of D-serine signaling. Though D-amino acid oxidase levels are high in the cerebellum and brainstem,

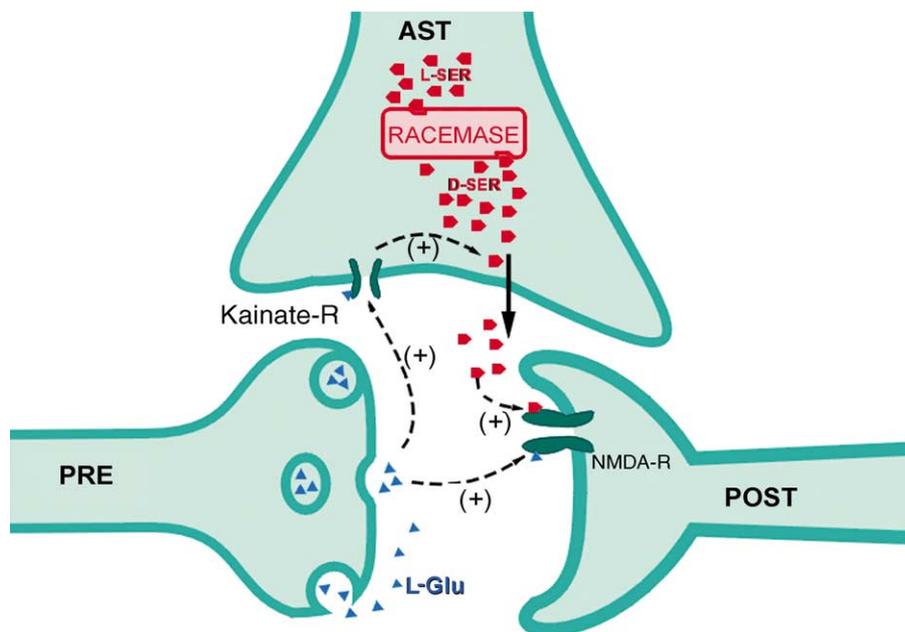


Fig. 1. D-serine: a putative glial-derived transmitter. D-Serine is synthesized from L-serine in astrocytes (AST) by serine racemase. L-Glutamate (L-Glu) released from neurons interacts with kainate-type of glutamate receptors in astrocytes (Ast) to stimulate the release of D-serine. D-Serine acts in concert with glutamate to activate post-synaptic NMDA receptors.

previous studies failed to detect significant amounts of D-amino acid oxidase in the forebrain (Horiike et al., 1987). Thus, it is conceivable that D-serine could be removed from the synaptic cleft by amino acid transporters. A D-serine uptake was demonstrated in C6 glioma cells, with properties resembling ASCT2 neutral amino acid transport system (Hayashi et al., 1997). In cortical astrocytes, we also detected bi-directional transport of D-serine by a similar mechanism (Ribeiro et al., 2002). Recently, a high-affinity transport system for D- and L-amino acids has been cloned, but its presence in astrocytes and localization in the brain have not been determined yet (Fukasawa et al., 2000). Another possibility is that the synaptic concentration of D-serine will be largely regulated by the activity of the biosynthetic enzyme, serine racemase. If this will be the case, D-serine signaling properties will resemble other non-conventional transmitters, such as nitric oxide and carbon monoxide, that lack specific degrading systems and their extracellular levels are regulated mostly by the activities of their biosynthetic enzymes (Snyder and Ferris, 2000).

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