D-Serine Regulation: A Possible Therapeutic Approach for Central Nervous Diseases and Chronic Pain

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Abstract: D-Serine, an endogenous modulator of NMDA receptors has been shown to play a vital role in many neuropsychiatric functions such as learning, memory, nociception and implicated in pathological conditions like schizophrenia and Alzheimer's disease. We propose possible therapeutic approaches for some CNS diseases and chronic pain, targeting the D-serine levels by manipulating its uptake, biosynthesis and metabolism.

Key words: D-Serine, NMDA receptors, CNS diseases, chronic pain, serine racemase, D-amino acid oxidase, amino acid transporters.

INTRODUCTION

In mammals, D-amino acids have received little attention as compared to their L-amino acid counterparts; however, all this has changed following the detection of substantial amount of D-serine in the mammalian brain, especially in *N*-methyl-D-aspartate (NMDA) receptor rich brain regions. D-Serine has now been identified to be the endogenous ligand for the glycine binding site of the NMDA receptors [1] and as an important gliotransmitter involved in glia-neuron cross talk [2]. Different techniques and assays are now available to distinguish the D- and L- isomers in various biological fluids and in mammalian tissues, and D-serine has been suggested to play an important role in the pathophysiology of various central nervous system (CNS) diseases including schizophrenia, Alzheimer's disease and also in chronic pain [3-6]. In this review we shall highlight the potential use of D-serine regulators, to control D-serine levels in the CNS by regulating its biosynthesis and metabolism; this may lead to the development of novel therapeutic approaches for CNS diseases and chronic pain.

ANALYSIS METHOD

Analysis of D-amino acids in biological samples is challenging, as it is affected by the presence of large amount of L-amino acids and other biological substances like amines and peptides. Hence a highly sensitive and selective method is necessary for quantitative analysis of D-amino acids. Different analytical methods including gas chromatography, high-performance liquid chromatography, capillary electrophoresis and enzymatic methods have been reported so far [3, 7-9].

D-Serine analysis by gas chromatography has been performed using chiral stationary phases - the most common one being the Chirasil-L-Val. This chiral column separates the enantiomers of amino acids as their *N,O*-pentafluoropropionyl isopropyl esters. Hashimoto *et al*. first reported the presence of substantial quantities of free D-serine in mammalian brain tissue using this method [10]. This method has also been used to determine the enantiomers of 14 amino acids including D-serine in human urine and blood serum samples [11].

HPLC is the most popular technique for D-serine analysis in mammalian tissues, CSF and blood samples. The technique can be subdivided into 2 types. The first type employs chiral derivatization (e.g. *o*-phthalaldehyde (OPA) derivatization in the presence of chiral thiols such as cysteine analogs) followed by HPLC separation on a nonchiral stationary phase and nonchiral derivatization with fluorescent reagents, followed by HPLC separation with chiral columns. Chiral derivatization was performed using *N-tert*.-butyloxy-carbonyl-L-cysteine (Boc-L-Cys) as a chiral thiol in combination with OPA and D-serine was determined along with other amino acid enantiomers like D-aspartate in rat frontal brain [10]. Morikawa *et al*. analyzed D-serine with D-alanine and D-aspartate by the same procedure in seven brain areas and serum [12]. 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) also known as Marfey's reagent has also been used for D-amino acids quantification. The other approach uses chiral stationary phases in conjunction with fluorescence labelling of amino acids for quantitative D-serine analysis. Hamase *et al*. have determined 17 D-amino acids in rat brain using Pirkle-type chiral stationary phases. The amino acids were labelled with a fluorescent derivatizing reagent 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), separated by reversed-phase HPLC followed by enantiomeric separation on a Pirkle-type chiral stationary phase [13]. Their results showed that a large amount of D-serine was present in the cerebrum, hippocampus and hypothalamus. Another method for simultaneous determination of D- and L- serine in rat brain microdialysates was developed by Fukushima *et al*. using NBD-F for fluorescence derivatization followed by separation using a column-switching HPLC system comprising of an octadecylsilica column and a series of two chiral columns [8]. An analysis method for quantitative analysis of D- and L-serine in cerebrospinal fluid (CSF) using 4-(dimethylamino) azobenzene-4-sulfonyl (dabsyl) chloride labelling and HPLC separation techniques using a simple

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UV-visible detector has recently been standardized [3]. The dabsyl derivatives of the amino acids are very stable when compared to the above mentioned fluorescent derivatives and can be easily detected by visible detection at 438 nm [14]. Very recently two mass spectrometry based methods for quantitative analysis of serine enantiomers and glycine in CSF have been reported – one employed a nonchiral derivatization and a chiral (Chirasil-L-Val column) separation in a GC-MS system and other used chiral derivatization using Marfey's reagent and a LC-MS analysis [15].

DISTRIBUTION IN THE CNS

D-Serine has been reported to be heterogeneously distributed throughout the brain. In the brain of different mammalian species, D-serine has been found in the frontal lobe, thalamus, striatum, hippocampus, hypothalamus and cerebral cortex with the highest concentration found in the latter. Small amounts of D-serine have also been found in the cerebellum, medulla oblongata and spinal cord, and also in the peripheral tissues including various endocrine glands [10, 12, 16]. Immunohistochemical studies also showed high D-serine localization in the forebrain and the developing cerebellum and low levels in the hind brain, with the distribution resembling that of the NMDA receptors [17, 18]. Endogenous D-serine and serine racemase (SR) have similar distribution in the CNS with the highest expression reported in forebrain [19, 20], with both expressed mainly by the glial fibrillary acidic protein (GFAP) positive astrocytes [19,21] and to some extent by the quiescent and activated microglia [21, 22]. In addition to the glial cells, D-serine has also been found in neurons of the cerebral cortex [21, 23] and in the glutamatergic neurons of brain stem and the olfactory bulb [21]. D-serine like immunoreactivity has also been reported in the dendrites and axons of cortical neurons [24]. The presence of SR in some cortical neurons supports the notion that neurons are not only involved in D-serine clearance at the synapse but are also an additional source of D-serine [2].

BIOSYNTHESIS AND METABOLISM

The only source of endogenous D-serine in the brain is L-serine and its synthesis is catalyzed by the enzyme SR. The activity of SR has been shown to be increased by various physiological cofactors including pyridoxal 5'-phosphate (PLP), Mg^{2+} and ATP [25]. Binding of the Ca^{2+} to the SR enzyme causes an increase in the intracellular Ca^{2+} concentration in astrocytes, which in turn leads to an increase in D-serine levels. This suggests that Ca^{2+} could be an important SR cofactor [26]. In addition, glycine, L-aspartic acid, L-asparagine and α,β-threo-3-hydroxyaspartic acid have all been reported to inhibit SR competitively [27].

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor, a glutamate receptor, has been identified as a major stimulating pathway for the release of D-serine from glial cells. It has been shown that AMPA or kainate and metabotropic receptors activation can trigger a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and Ca^{2+} dependent release of D-serine from astrocytes [28]. There have been few reports that strongly suggest a vesicular storage and release mechanism for D-serine and support the existence of specific storage organelles for D-serine [2]. D-Serine immunoreactivity has been identified in glutamate vesicular transporter bearing vesicles [28] and such co-storage of glutamate and D-serine in the same vesicles seems most favourable for NMDA receptor activation. Hashimoto *et al*. studied the endogenous free D-serine in different brain regions using an *in vivo* microdialysis based analysis and their results showed that the basal extracellular D-serine level was not affected by Ca^{2+} deprivation or Na^{+} blockers [29]. These results suggest the presence of a specific membrane carrier for the release of D-serine. Quite a few D-serine membrane transporters have been identified so far. These include a Na^{+}-independent neutral amino acid transporter – the alanine-serine-cysteine transporter (Asc-1). Asc-1 has been identified in dendrites and somata of neurons at the presynaptic terminals and has a high affinity for D-serine (Km ~10μM). D-serine uptake by this transporter relies on the release of amino acid substrates of this Asc-1 [30]. A Na^{+}-dependent transporter in glial cells with low affinity for D- and L- serine is similar to the ASCT2 system [31]. In addition, a Na^{+}/Cl^{-} sensitive serine transporter with low affinity for other neutral amino acids has also been reported in rat brain synaptosomes [32]. Studies using Asc-1 knockout mice on ^{3}H D-serine uptake by the forebrain and cerebellar synaptosomes suggests that the major portion of the total synaptic D-serine is regulated by the high affinity Na^{+}-independent transporter while a smaller portion is taken up by the low-affinity Na^{+}-dependent transporters [33]. Taken together, existing evidence support the presence of multiple transport mechanisms including both vesicular and non-vesicular pathways for D-serine transport and release, and they help to regulate D-serine concentrations at the synapses.

The half-life of D-serine in brain is about 16 hours but the metabolic pathway responsible for its degradation remains elusive [34]. Mammalian D-serine is metabolized by an enzyme present in astrocytes of the hindbrain and cerebellum - peroxisomal flavoprotein D-amino acid oxidase (DAAO) [17, 35, 36]. In adult mice deficient in DAAO, increased D-serine concentrations have been observed in areas of the brain where its levels are generally low; in contrast, its levels in the forebrain (rich in D-serine) remained unchanged [37]. In the forebrain, DAAO enzyme is almost undetectable thus suggesting the existence of other mechanisms that regulate D-serine concentrations. SR catalyzes α,β-elimination of water from both the D-serine and L-serine, although the process is less effective for D-serine as compared to L-serine. Astrocytes could regulate their D-serine concentrations physiologically by SR activity and this mechanism could be the alternate pathway for D-serine removal in brain regions where DAAO is absent [34, 38]. In addition, SR activity in astrocytes is enhanced by D-serine and inhibited by nitric oxide (NO) while DAAO activity is enhanced by NO [39, 40]. Thus NO can play a role in regulating D-serine level by accelerating D-serine elimination *via* SR inhibition and DAAO activation. A more recent study supports the existence of a model of feedback inhibition of D-serine formation in presynaptic cells through the *S*-nitrosylation of SR caused by postsynaptic stimulation of NO formation [41].

PHYSIOLOGICAL ACTIVITY AND ROLES

D-Serine has been identified as a neuromodulator and as a co-agonist for the NMDA receptors. NMDA receptor is one of the subtypes of glutamate receptors and is known to play important roles in several physiological processes including learning, memory and nociception [42, 43] and pathophysiological conditions like schizophrenia and epilepsy [44, 45]. The NMDA receptor is a tetramer composed of two NR1 and two NR2 subunits and has a glycine binding site located on the NR1 subunit [46]. The receptor activation requires binding of glutamate to the NR2 subunit and a simultaneous binding of co-agonist to the glycine binding site in NR1 subunit. The co-agonist was initially proposed to be glycine but D-serine is about 3 times more potent than glycine at this binding site [47, 48]. Hence, D-serine is the key endogenous co-agonist on the strychnine-insensitive glycine binding site on the NR1 subunit of the heteromeric NMDA receptor to enhance the flux of Ca^{2+} current through this ligand-gated channel and mediate diverse functions like learning and memory, long-term changes in synaptic plasticity and neural development [2, 49].

Addition of exogenous DAAO that metabolizes D-serine, impaired the astrocytic D-serine and NMDA receptor dependent long-term potentiation (LTP) in hippocampal slices suggesting that D-serine may be the major endogenous glycine binding site modulator for NMDA receptor functions in the hippocampal and supraoptic neurons [2, 50]. Further evidence for the vital role of D-serine in memory process was provided by Maekawa *et al*., when a point mutation of the DAAO in mice led to an enhanced NMDA-dependent hippocampal LTP [51]. Mothet *et al*. in their recent study using senescent rats, also reported that deficient LTP in these rats is primarily due to the significant impairment of D-serine metabolism secondary to reduced D-serine production in the hippocampus. Moreover, the deficient LTP is not associated with a decrease in glycine level and could be reversed by supplying D-serine to these tissues [52]. Additional studies also showed that NMDA receptor-mediated neuronal death in hippocampus slices can be prevented by treatment with D-serine degrading enzyme but not with glycine degrading enzyme [53]. D-Serine (600 mg/kg) has been shown to improve spatial reversal learning in mice in the Morris water maze [54]. Also in cognitively deficient mice, the hippocampal slices exhibit both long-term depression (LTD) and LTP deficits and these can be overcome by D-serine addition. D-Serine thus improves cognitive flexibility (improvement in reversal learning and LTD) and NR-2B-dependent synaptic plasticity in the hippocampus of NR2 glycine binding site deficient mice. Hence, D-serine is the key player in NMDA receptor regulation in hippocampus and has a vital role in NMDA receptor-mediated neurotoxicity in this region.

POSSIBLE ROLES IN CHRONIC PAIN AND CNS DISEASES

D-Serine has been shown to be involved in the mechanisms of nociception *via* the glycine binding site of NMDA receptors in the spinal cord. Intrathecal administration of D-serine has been reported to be pronociceptive in tail-flick assay and this is blocked by co-administration of 7-chlorokynurenic acid, a selective NMDA receptor antagonist of the glycine site [55, 56]. Pretreatment with D-serine has also been shown to block the antinociceptive action of nocistatin (low dose) in formalin test and a chronic constriction injury model [57, 58]. In mutant mice lacking DAAO, the second phase of formalin-induced pain response is significantly increased [59]. Spinally administered D-serine significantly facilitated C-fibre responses of wide dynamic range neurons in the spinal dorsal horn evoked by noxious cutaneous electrical stimulation [60]. In contrast, intracerebroventricular administration of D-serine produces a dose-dependent antinociceptive effect in the tail-flick test and potentiates the antinociceptive effect of morphine when co-administered with it [61]. CSF obtained from patients suffering from chronic knee pain due to degenerative osteoarthritis showed higher concentrations of D-serine compared to an age matched no-pain group, suggesting important roles for D-serine in chronic pain mechanisms [3].

D-Serine analysis in CSF from schizophrenic patients has shown reduced D-serine levels as compared to control subjects [4, 5]. Studies using post-mortem schizophrenic brains have shown altered SR immunoreactivity and increased DAAO activity [62]; lower Asc-1 immunoreactivity [63] and increased DAAO activity in the prefrontal cortex region [64]. Thus low D-serine level can result from a reduction in D-serine synthesis, lowered D-serine uptake, and enhanced degradation, or any combination of these processes may lead to NMDA receptor inactivation and the clinical state of schizophrenia.

Free D-serine in the ventricular CSF obtained from patients with Alzheimer's disease has been reported to be significantly higher than in normal control subjects [6]. D-Cycloserine, a partial agonist of the glycine binding site of the NMDA receptor improved the cognition and performance of memory-related tasks in patients with Alzheimer's disease [65, 66]. Also the percentage of D-serine in the total serine (D + L) in the serum of Alzheimer's disease patients has been shown to be significantly lower than the age and gender matched control subjects [67]. Furthermore Wu *et al*. have reported that amyloid β-peptide increases D-serine levels and mRNA of SR in cultured microglia cells [22]. These studies put together with the established role for NMDA receptors mediated glutamatergic signalling in the pathophysiology of Alzheimer's disease strongly suggest that D-serine as a co-agonist of the NMDA receptors has a pathological role in Alzheimer's disease.

IMPLICATIONS FOR THERAPEUTICS

There have been extensive studies emphasizing the roles of up or down regulation of NMDA receptor mediated neurotransmission in the pathophysiology of a number of neurodegenerative disorders such as stroke, chronic pain, schizo-phrenia, Alzheimer's disease, amyotrophic lateral sclerosis (Table **1**). To date effective treatment for most of these diseases remains far from satisfactory. NMDA antagonists like Gabapentin and Ketamine have been effective against some of these diseases but they can cause serious side effects. D-Serine related molecules could be developed as novel therapeutic agents for these challenging CNS dis-

Table 1. Possible CNS Diseases for Therapeutic Approaches by Regulation of D-Serine

Diseases with Therapeutic Potential for D-Serine Regulators
A) Conditions with decreased D-serine level
1. Schizophrenia
2. Alzheimer's disease
3. D-Serine deficient syndrome (infant) [80]
B) Conditions with elevated D-serine level
1. Nerve cell death caused by stroke [53]
2. Chronic pain
3. Amyotrophic lateral sclerosis (ALS) [81]
C) Conditions with possible D-serine involvement
1. Ataxia caused by spinocerebellar degeneration [71]
2. Bipolar disease [82]
3. Anxiety [83]
4. Posttraumatic stress disorder (PTSD) [84]
5. Cognitive disorder in aging [85]

NOTE: The topics not described in the text are referenced here.

eases, as these compounds would not only be clinically effective but also have less adverse effects. As discussed in earlier sections, D-serine levels are controlled physiologically by many factors and some of these mechanisms still remain unknown. We propose to refer such therapeutic molecules as D-serine regulators as they increase or decrease D-serine levels by affecting the D-serine regulatory systems in different areas of the CNS. Since there are numerous mechanisms involved in D-serine regulation, the possible strategies and approaches including the existing known compounds that have promising therapeutic potential are summarized here:

1. D-Serine

Clinical efficacy of D-serine in the treatment of schizophrenia has been reported but this requires a high dose (30 mg/kg/day for 6 weeks) because of its low permeability at the blood-brain barrier (BBB) [68]. Furthermore combination therapy of D-serine together with approved antipsychotics has been reported to be beneficial for schizophrenic patients but additional clinical studies are required to confirm this [69].

2. D-Cycloserine

Many reports have been published showing that D-cycloserine is partially effective for the positive symptoms of schizophrenia. However, D-cycloserine reportedly did not improve the cognitive and negative symptoms clinically [70]. Its administration to ataxia patients with spinocerebellar degeneration showed significant improvement in ataxia symptoms and was well-tolerated with no serious adverse effects [71]. Similarly short-term D-cycloserine treatment at a dose of 100mg/day has been shown to have cognitive enhancing effect for patients with Alzheimer's disease [66].

3. Synthetic Agonists and Antagonists at the D-Serine Binding Site of NMDA Receptors

Phencyclidine is well known to induce the positive symptoms in schizophrenia and a glycine binding site agonist, D-serine and glycine reverse these PCP effects without exhibiting any serious side effects. Such agonists and antagonists seem to be the most promising therapeutic candidates among the D-serine regulators. The crystal structures of NMDA receptor NR1 ligand-binding core with the agonist glycine and D-serine, the partial agonist D-cycloserine and the antagonist 5, 7-dichlorokynurenic acid have been published [72]. This information would be valuable for designing the most suitable agonists and antagonists although no such compounds have been reported so far. The chemical structures of some of these agonists and antagonists at the NR1 glycine binding site are shown in Fig. (**1**).

4. Serine Racemase Inhibitors

Modulators of serine racemase activity constitute another group of promising candidates for D-serine regulation since the only known pathway for D-serine biosynthesis is from L-serine and is catalyzed by this enzyme serine racemase. Serine racemase is activated by pyridoxal phosphate and inhibited by aminooxyacetic acid, a potent inhibitor of pyridoxal dependent enzymes. This enzyme is thus a key player in the regulation of D-serine levels associated with various pathophysiological conditions in CNS as reported by an *in vivo* microdialysis study [73].

5. D-Serine Release Inhibitors or Stimulators

D-Serine has been detected by the *in vivo* microdialysis method in the brain and its presence in CSF has been confirmed. The extracellular D-serine is released from the nerve and glial cells but the biosynthesis of D-serine and its neuromodulator-like and/or neurotransmitter activity might be different in neurons and glial cells. The extracellular D-serine levels could thus be controlled by compounds that stimulate or inhibit its release from these cells. Kanematsu *et al.* published an interesting report that the continuous-cortical infusion of a glia toxin, fluorocitrate, caused a decrease in the cortical extracellular D-serine and an increase in L-serine and glycine [74].

6. D-Serine Transporter Inhibitors

The concentration of D-serine in nerve and glial cells are regulated by neutral amino acid transporters and/or glycine transporter-1. Recently sarcosine and its derivative N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)-propyl]sarcosine, inhibitors of glycine transporter-1 have been reported to improve the cognitive deficits observed in schizophrenia, a disease associated with NMDA receptor hypofunction [75, 76].

7. D-Amino Acid Oxidase (DAAO) Inhibitors and Activators

Although the metabolic pathways of D-serine have been suggested to be different in glial astrocytes and the forebrain, DAAO is the only D-serine degrading enzyme known so far in brain. Hence this is one of the most active fields of research currently and is targeted in the discovery of new therapeutic drugs for D-serine related diseases like schizo-

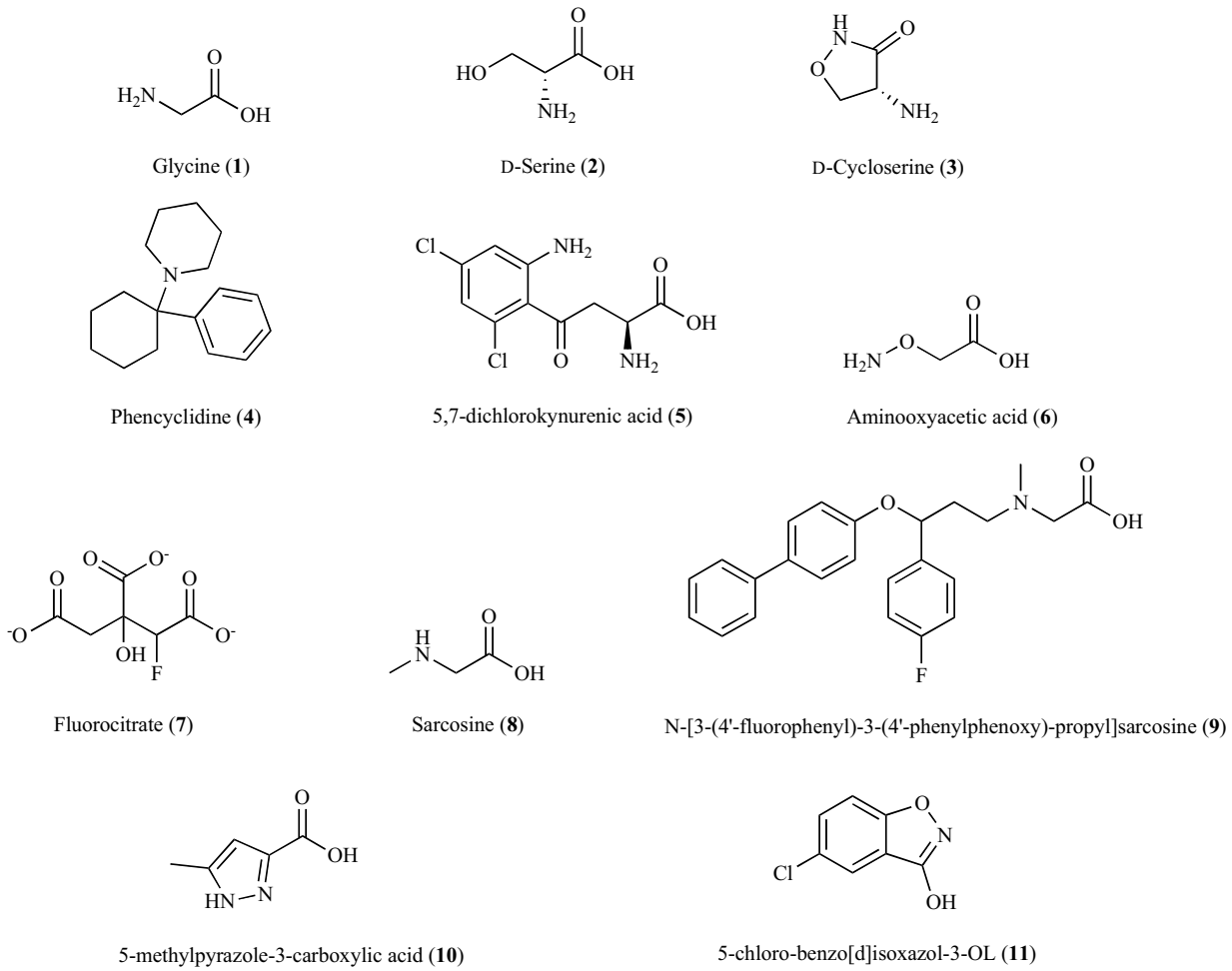

Fig. (1). Structures of D-serine regulatory molecules.

phrenia and Alzheimer's disease. Especially after the identification of the gene product of G72 as an activator of DAAO and its role in schizophrenia [77], many pharmaceutical companies are focussing on novel DAAO activators and inhibitors. An interesting and promising report was published by a Merck group on the DAAO inhibitor, AS057278 (5-methylpyrazole-3-carboxylic acid) which improves cognitive and positive symptoms of schizophrenia [78]. Ferraris *et al*. have also recently reported a new, promising DAAO inhibitor - 5-chloro-benzo[d]isoxazol-3-ol (CBIO) [79]. Although these D-serine regulators are promising novel therapeutic drugs for a variety of CNS diseases and chronic pain, for them to be clinically effective they should be developed to possess high specificity to their target sites with reasonable permeability at the BBB so that they could be devoid of serious side effects.

CONCLUSION

The important pathophysiological roles for D-serine as an endogenous agonist at the glycine binding site of NMDA receptor NR1 subunit, in CNS diseases and psychological disorders have been recently identified and hence intensely investigated over the past few years. Many factors and mechanisms involved in the regulation of D-serine levels have been identified, though some of these still remain to be elucidated. In this review we have summarized few possible strategies and approaches for the design of D-serine regulators which have the potential to develop into novel therapeutics against certain challenging CNS diseases.

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