D-Serine in Health and Disease

Sabine Fuchs

ISBN 978-90-8559-027-9

Illustrations by: Sophie Evers

Layout and printing: Optima Grafische Communicatie, Rotterdam, The Netherlands

D-Serine in Health and Disease

D-Serine in Gezondheid en Ziekte

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
dinsdag 2 november 2010 des middags te 2.30 uur

door

Sabine Annemijn Fuchs

geboren op 27 februari 1974, te Heerlen

Promotoren:	Prof. dr. R. Berger
	Prof. dr. J.L.L. Kimpen
Co-promotoren:	Dr. T.J. de Koning
	Dr. L.W.J. Klomp

Dit proefschrift werd mede mogelijk gemaakt met financiële steun van ZonMw (ZonMw programma Agiko stipendia, 920-03-345), het Netherlands Metabolomics Center, Genzyme Europe BV, GlaxoSmithKline BV en Merck BV.

When D-serine seemed right and L-serine was left...

Voor mijn familie: mijn ouders
Coralie en Saskia
Bas
Sophie, Job en Pepijn

Table of Contents

INTRODUCTION

Preface: Outline of the thesis 11

Chapter 1: D-amino acids in the central nervous system in health and disease 17

D-SERINE AND L-SERINE ANALYSIS

Chapter 2a: Editorial clinical chemistry: CSF serine enantiomers and glycine in the study of neurologic and psychiatric disorders 45

Chapter 2b: Two mass-spectrometric techniques for quantifying serine enantiomers and glycine in cerebrospinal fluid: potential confounders and age-dependent ranges 51

D-SERINE IN HUMAN PHYSIOLOGY

Chapter 3: D-serine in the developing human central nervous system 71

Chapter 4: D-serine in the developing human central nervous system: clinical implications 83

Chapter 5: D-serine influences synaptogenesis in a P19 cell model 105

D-SERINE IN HUMAN PATHOLOGY

Chapter 6: Increased concentrations of both NMDA receptor co-agonists D-serine and glycine in global ischemia: a potential novel treatment target for perinatal asphyxia 119

Chapter 7: Cerebrospinal fluid D-serine and glycine concentrations are unaltered and unaffected by olanzapine therapy in male schizophrenic patients 135

DISCUSSION

Chapter 8: General discussion 149

TO CONCLUDE

Summary 177

Summary in Dutch - Nederlandse samenvatting 181

Contributors 185

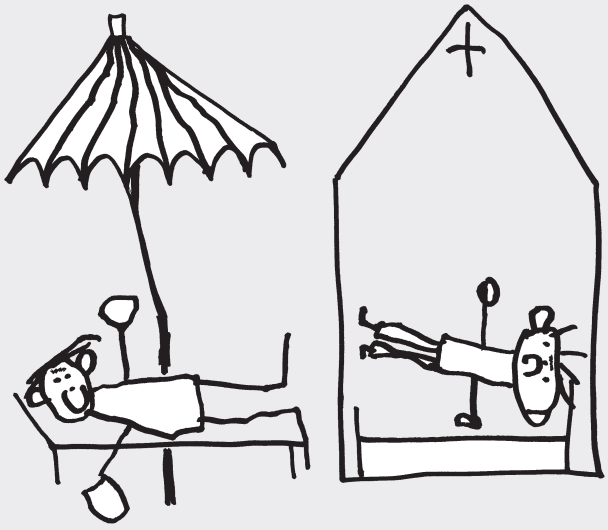
Acknowledgements - Dankwoord 187

Curriculum vitae 191

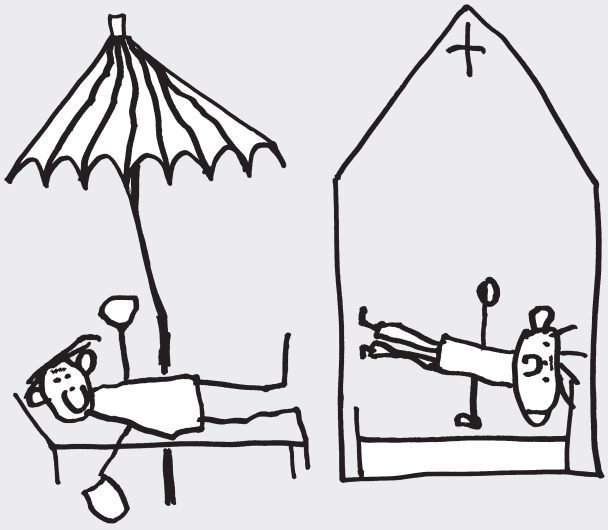
Publications 193

List of abbreviations 195

Introduction



Preface



OUTLINE OF THE THESIS

As building units of peptides and proteins, amino acids are among the most important molecules for living beings. Most amino acids occur as different enantiomers (an L- and a D-form). Although the chemical and physical properties of L- and D-amino acids are almost identical, they differ in their spatial positioning, which plays a major role in structural interactions.

It was long believed that only L-amino acids were selected for formation of peptides and proteins on the primitive earth.(1) Based on this concept, identification of D-amino acids in different organisms, including mammals, was revolutionary. In **chapter 1**, an overview is given of the different D-amino acids occurring in mammals and their putative function, focusing on D-serine. D-Serine was found to be synthesized and metabolized endogenously and to function as an important neurotransmitter, being an endogenous co-agonist of the N-methyl-D-aspartate receptor (NMDAr). The NMDAr is broadly distributed throughout the central nervous system (CNS) and has been implicated in physiological processes such as CNS development, memory and learning (2) and in pathological processes including neurodegenerative conditions (such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and Huntington's disease), stroke, epilepsy, polyneuropathies, chronic pain (3) and psychiatric disorders such as schizophrenia.(4) It had long been known that the NMDAr is an exceptional receptor, requiring binding by two different agonists for its activation. In addition to glutamate, glycine was believed to be the necessary co-agonist. However, by now, it is generally appreciated that D-serine is the endogenous obligatory co-agonist in most regions of the brain. Consequently, D-serine might well be involved in all processes associated with NMDAr (dys-) function.

The first part of the thesis (**D-serine and L-serine analysis**) describes how we developed two different chiral chromatographic separation techniques (liquid and gas chromatography), combined with mass spectrometric detection to determine concentrations of D-serine, glycine and their common precursor L-serine in biological fluids (**chapter 2**). Using these analytical techniques, we created reference ranges in human cerebrospinal fluid, since this represents the most accessible read-out for extracellular CNS concentrations.

The second part of the thesis (**D-serine in human physiology**) starts with our finding of an evident age-dependency in our cerebrospinal fluid D-serine reference values, with very high concentrations directly after birth and a rapid decline over the first 3 years (**chapter 3**). To investigate the physiological relevance of these high D-serine concentrations, we determined D-serine concentrations in cerebrospinal fluid of patients with 3-phosphoglycerate dehydrogenase deficiency, a rare metabolic disorder in the synthesis of L-serine and hence D-serine, characterized by serious neurological

symptoms, including microcephaly, psychomotor retardation and intractable seizures. (5) Furthermore, we analyzed D-serine concentrations in the one patient with complete reversal of the phenotype upon pre- and postnatal treatment with L-serine.(6) In **chapter 4**, we elaborate on the role of D-serine, as an NMDAr co-agonist, in CNS development, focusing on the clinical implications. To gain more specific insight in the role of D-serine in CNS development, we studied a rat P19 cell model as described in **chapter 5**. These cells can be differentiated into glia and neurons, expressing glutamatergic receptors, including the NMDAr. We observed that these differentiated P19 cells express serine racemase and synthesize D-serine, thereby providing a suitable model to study the role of D-serine in early CNS development. We examined the effects of manipulating D-serine concentrations and inhibiting D-serine (or glycine) binding to the NMDAr on P19 cell differentiation.

In the third part of the thesis (**D-serine in human pathology**), we focus on pathological conditions associated with altered NMDAr activity, potentially induced by altered D-serine concentrations. This is of particular interest since these concentrations might by pharmacologically manipulated as the synthesizing and metabolizing enzymes of D-serine are known, thereby providing interesting therapeutic targets. As pediatricians, we chose to investigate perinatal asphyxia, currently one of the greatest causes of neonatal mortality and morbidity worldwide.(7) Perinatal asphyxia is the consequence of disturbed blood circulation between mother and fetus, for example after placental pathology or umbilical cord accidents, leading to insufficient delivery of oxygen, glucose and other blood-borne fuels to the fetal organs, including the brain. In the long term, this can lead to mental retardation, epilepsy and spasticity.(8;9) Treatment is limited to supportive intensive care. The pathophysiology has not been fully elucidated, but overstimulation of the NMDAr appears to play a central role. In **chapter 6**, we describe our D-serine and glycine concentration studies in a rat glioma cell model for perinatal asphyxia, in an experimental piglet model for perinatal asphyxia and in cerebrospinal fluid from human newborns, who experienced perinatal asphyxia.

Another pathological condition strongly associated with NMDAr dysfunction (10) and potentially with altered D-serine concentrations (11-13) is schizophrenia. This is a serious and relatively common psychiatric disorder, characterized by positive symptoms (including hallucinations), negative symptoms (blunted affect, emotional withdrawal) and cognitive defects.(14) In **chapter 7**, we present the results of our analyses of D-serine concentrations in cerebrospinal fluid of patients with schizophrenia before and after treatment with the antipsychotic drug olanzapine, compared with control cerebrospinal fluid.

Finally, in **chapter 8**, the results of the studies in this thesis are discussed, emphasizing on the role of D-serine as an essential human endogenous regulator of NMDAr activity in physiological and pathological processes, with a view towards the future.

REFERENCES

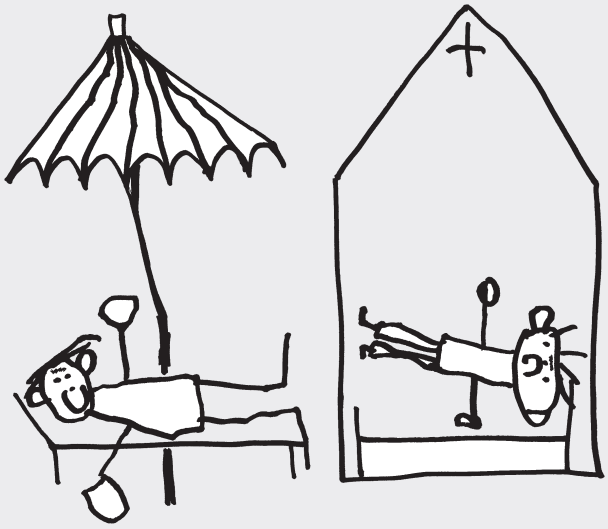
1. Lamzin VS, Dauter Z, Wilson KS. How nature deals with stereoisomers. Curr Opin Struct Biol 1995 December;5(6):830-6.
2. Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C et al. Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. Proc Natl Acad Sci U S A 2003 December 9;100(25):15194-9.
3. Danysz W, Parsons CG. Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol Rev 1998 December;50(4):597-664.
4. Goff DC, Coyle JT. The emerging role of glutamate in the pathophysiology and treatment of schizophrenia. Am J Psychiatry 2001 September;158(9):1367-77.
5. de Koning TJ, Klomp LW. Serine-deficiency syndromes. Curr Opin Neurol 2004 April;17(2): 197-204.
6. de Koning TJ, Klomp LW, van Oppen AC, Beemer FA, Dorland L, van dB, I et al. Prenatal and early postnatal treatment in 3-phosphoglycerate-dehydrogenase deficiency. Lancet 2004 December 18;364(9452):2221-2.
7. Lawn JE, Cousens S, Zupan J. 4 million neonatal deaths: when? Where? Why? Lancet 2005 March 5;365(9462):891-900.
8. Robertson CM, Finer NN, Grace MG. School performance of survivors of neonatal encephalopathy associated with birth asphyxia at term. J Pediatr 1989 May;114(5):753-60.
9. Shankaran S, Woldt E, Koepke T, Bedard MP, Nandyal R. Acute neonatal morbidity and long-term central nervous system sequelae of perinatal asphyxia in term infants. Early Hum Dev 1991 May;25(2):135-48.
10. Goff DC, Coyle JT. The emerging role of glutamate in the pathophysiology and treatment of schizophrenia. Am J Psychiatry 2001 September;158(9):1367-77.
11. Bendikov I, Nadri C, Amar S, Panizzutti R, De MJ, Wolosker H et al. A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. Schizophr Res 2007 February;90(1-3):41-51.
12. Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N et al. Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. Arch Gen Psychiatry 2003 June;60(6):572-6.
13. Hashimoto K, Engberg G, Shimizu E, Nordin C, Lindstrom LH, Iyo M. Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naive schizophrenic patients. Prog Neuropsychopharmacol Biol Psychiatry 2005 June;29(5):767-9.
14. Goldner EM, Hsu L, Waraich P, Somers JM. Prevalence and incidence studies of schizophrenic disorders: a systematic review of the literature. Can J Psychiatry 2002 November;47(9): 833-43.

Chapter 1

D-Amino acids in the central nervous system in health and disease

S.A. Fuchs, R. Berger, L.W.J. Klomp, T.J. de Koning

Mol Genet Metab 2005;85:168-80



ABSTRACT

Recent evidence has shown that D-amino acids are present in animals and humans in high concentrations and fulfill specific biological functions. In the central nervous system (CNS), two D-amino acids, D-serine and D-aspartate, occur in considerable concentrations. D-Serine is synthesized and metabolized endogenously and the same might account for D-aspartate. D-Serine has been studied most extensively and was shown to play a role in excitatory amino acid metabolism, being a co-agonist of the N-methyl D-aspartate (NMDA) receptor. Insight into D-serine metabolism is relevant for physiological NMDA receptor (NMDAr) activation and for all the disorders associated with an altered function of the NMDAr, such as schizophrenia, ischemia, epilepsy and neurodegenerative disorders. D-Aspartate appears to play a role in development and endocrine function, but the precise function of D-aspartate and other D-amino acids in animals and humans requires further investigation. As D-amino acids play biological roles, alterations in the concentrations of D-amino acids might occur in some disorders and relate to the pathogenesis of these disorders. D-Amino acid concentrations may then not only help in the diagnostic process, but also provide novel therapeutic targets. Consequently, the presence and important roles of D-amino acids in higher organisms do not only challenge former theories on mammalian physiology, but also contribute to exciting new insights in human disease.

INTRODUCTION

Amino acids are among the most important molecules in nature and most exist in an L- and a D-form. Despite almost identical chemical and physical properties, in the past it was assumed that only L-amino acids were selected during evolution for formation of polypeptides and proteins.(1) This selection of the L-amino acids was generally considered to be a result of chance,(2) although it was also attributed to stabilization of polypeptides by neutral current interactions, leading to lower energies.(3) The resulting homochirality was considered to be essential for life, as it dictates the spatial architecture of biological polymers and therefore plays a major role in enzymatic specificity and structural interactions.

As early as the beginning of the twentieth century, various studies nevertheless strongly suggested the presence of some D-amino acids in micro-organisms, resulting in the isolation of D-alanine and a derivative of D-glutamine in a peptidoglycan found in the cell walls of virtually all bacteria.(4) Subsequent research has shown that micro-organisms produce, metabolize and utilize D-amino acids.

The first reports on D-amino acids in animal tissue were restricted to amphibians and invertebrates. Using chromatographic techniques, free D-alanine was isolated from the blood of the milkweed bug,(5) followed by many reports on animal D-amino acids (mostly incorporated in proteins) such as D-alanine, D-phenylalanine, D-glutamate, D-ornithine, D-serine, D-asparagine, D-methionine and D-cysteine.(6-9) Development of more sensitive chromatographic techniques also revealed low concentrations of D-amino acids in mammals, including humans. D-amino acid oxidase (DAO) and D-aspartate oxidase (DAOX), two flavoproteins responsible for the oxidative deamination of neutral and dicarboxylic D-amino acids respectively, had long been known to exist in mammals,(10,11) but had largely been neglected, since animals and humans were not thought to possess D-amino acids. The identification of D-amino acids in mammals finally provided a rationale for the widespread occurrence of these enzymes in animals and humans. Mammalian D-amino acids were assumed to arise from endogenous microbial flora, from ingestion with the diet or from spontaneous racemization of L-amino acids incorporated in polypeptides during ageing.(12) It was assumed that the D-amino acids did not possess a specific biological function.

In this context, the concentrations of free D-aspartate in neonatal rat cerebral hemispheres (164 nmol/g, 8.4% of total aspartate), in other tissues of rodents (up to 38% of total aspartate in newborn blood cells) and in human blood, were surprisingly high.(13) A second D-amino acid, D-serine, was then identified in significant amounts in the brains of rodents and man.(14) Subsequent studies confirmed that some D-amino acids exist in the mammalian CNS and peripheral tissues in unexpectedly high concentrations, sometimes even exceeding the concentration of the L-enantiomer.(15) These D-amino

acids fulfill specific biological functions, with D-serine playing an important role in neurotransmission and D-aspartate in development and endocrine regulation. Altered levels of D-amino acids might be involved in various pathological conditions and thereby possibly provide new therapeutic targets. As this might have important clinical implications, we here review the known functions of free D-amino acids in the CNS of higher organisms in health and disease, with a particular emphasis on D-serine.

D-SERINE

Localization of D-serine

Using mostly chromatographic techniques, free D-serine has been localized predominantly to the rodent and human forebrain, with highest levels in the cerebral cortex, hippocampus and striatum, followed by the limbic forebrain, diencephalon and midbrain and low levels in the pons, medulla, cerebellum and spinal cord.(15-17) D-Serine concentrations in the rat brain are about one third of L-serine concentrations, thereby exceeding the concentration of many common L-amino acids.(18) Immunohistochemical localization of D-serine has shown a selective localization to protoplasmic type II astrocytes, a subtype of glial cells that ensheathes nerve terminals and is particularly enriched in cortical gray matter.(19-21) The anatomical distribution of D-serine in the CNS closely mimics that of the NR2 A/B subtypes of the N-methyl-D-aspartate (NMDA) type excitatory amino acid receptor.(16,20)

D-Serine concentrations have been shown to vary with age in different areas of the rodent brain. On the day of birth, D-serine was present in substantial concentrations throughout rat brain.(22) D-Serine concentration increased in the cerebrum by the third postnatal week, remaining rather constant thereafter. In the cerebellum, D-serine increased in the first postnatal week to its highest levels in the second week and thereafter declined dramatically to only trace levels, coinciding well with the drastic increase in DAO activity in the cerebellum during that period.(20,22,23) In humans, a high concentration of D-serine was determined in the frontal cortex at gestational week 14 and this remained rather constant throughout embryonic and early postnatal life.(15) D-Serine concentrations decreased later in life to half of these levels in adolescent and aged brains.(15)

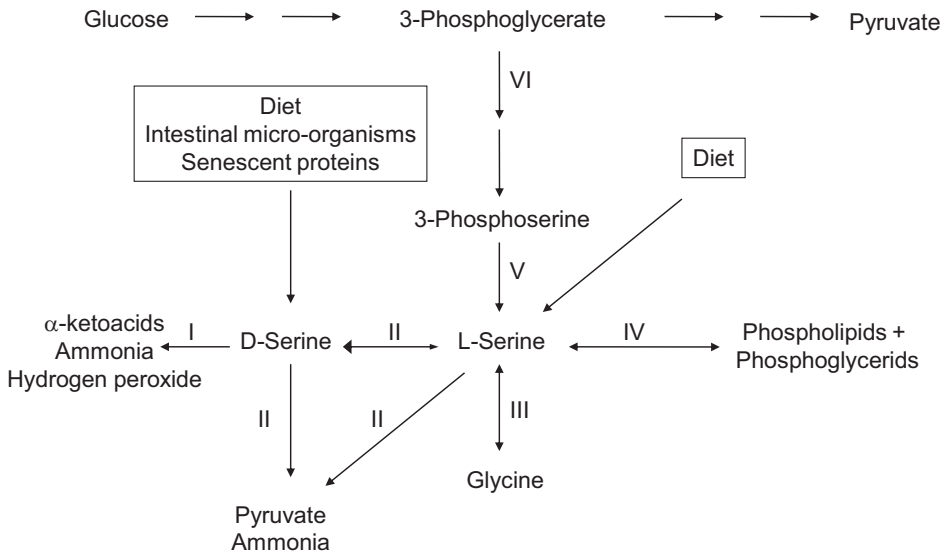
D-Serine has also been identified in peripheral mammalian tissues. Small amounts were detected in human serum, saliva and urine (24-26) and recently also in the retina of various vertebrates.(27) The peripheral concentrations again appear to vary with age, as substantial amounts of D-serine were found in almost all peripheral rat organs in the first few days of life, decreasing to trace or non-detectable levels thereafter.(22)

Synthesis and metabolism of D-serine

Humans can acquire D-serine through ingestion with food, derivation from gastrointestinal bacteria, liberation from metabolically stable proteins, which contain D-amino acids after racemization with ageing, and through biosynthesis from L-serine. Few data are available on the relative contributions of these four sources, but biosynthesis appears to be important. The enzyme serine racemase (SR) directly converts L- to D-serine in the presence of the co-factors pyridoxal 5-phosphate, magnesium and ATP (Fig. 1).(28-30) SR also converts D- to L-serine, albeit with lower affinity.(30) D-Serine concentrations are thus highly related to L-serine concentrations and thereby also to glycine concentrations (Fig. 1).(31) Of the different pathways involved in L-serine biosynthesis, the glucose–3-phosphoglycerate-3-phosphoserine–biosynthesis pathway is essential for normal embryonic development, especially for brain morphogenesis.(32) Consequently, D-serine concentrations in the developing CNS might also depend heavily on this pathway.

SR is highly expressed in the brain, with lower levels in the liver and small or no detectable expression in other tissues. In the brain, SR localizes to protoplasmic astrocytes in a pattern similar to D-serine.(29,30) Physiological synthesis of D-serine by SR in the glia was implicated by the strong spatiotemporal correlation between D-serine and SR (33)

Figure 1. Interdependency of D-serine, L-serine and glycine



Pathways involved in L-serine and D-serine biosynthesis, catabolism and exogenous sources. As glycine and D-serine are both agonists of the glycine site of NMDArs, the possibility of interconversion between D-serine, L-serine and glycine is of particular relevance. I = D-amino acid oxidase, II= serine racemase; III = serine hydroxymethyltransferase; IV = synthesis of phospholipids and phosphoglycerides from cytidine diphosphodiacylglycerol and palmitoyl-coenzyme A; V = 3-phosphoserine phosphatase; VI = 3-phosphoglycerate dehydrogenase.

and by the decrease in D-serine concentrations in astrocytes after pharmacological inhibition of SR.(29) The cDNA encoding human SR has been cloned and D-serine synthesis by SR has been demonstrated in living cells after heterologous overexpression.(34)
Whereas human serine hydratase does not contribute substantially to the degradation of L-serine to pyruvate, SR was found to catalyze, in addition to the racemase activity, the α,β-elimination of water from both L-serine and D-serine to form pyruvate and ammonia.(28,35) Under physiological conditions, pyruvate formation seems to equal or excess D-serine formation. Pyruvate formed by SR may be sufficient for the energy requirements of the astrocytes. This reaction further implies that SR is not only involved in D-serine synthesis, but also in D-serine metabolism as a mechanism to regulate intracellular D-serine levels.(35)
Mammalian D-amino acids can be metabolized by the peroxisomal flavoprotein DAO,(10) with the concomitant reduction of the co-factor flavin adenine dinucleotide (FAD) (36):

$$\text{Neutral D-amino acid} + O_2 + H_2O - \text{DAO} \rightarrow \text{corresponding } \alpha\text{-ketoacid} + NH_3 + H_2O_2$$

The activity of DAO is selectively restricted to the metabolism of neutral D-amino acids, with highest affinity for D-serine, D-alanine, D-proline, D-leucine and D-methionine *in vivo*.(37,38) Glycine is not a substrate at physiological pH values, nor are L-amino acids or dicarboxylic amino acids.(39) The expression of DAO is highest in the kidneys, followed by the liver (with the possible exception of mice livers, where the expression of DAO was not detected (40)) and the CNS, where DAO is particularly concentrated in astrocytes of the hindbrain and cerebellum,(41) with a putative preferential localization to type I astrocytes.(42)
Physiological degradation of D-serine by DAO was suggested by the marked regional and developmental variation in DAO levels in a pattern reciprocal to D-serine levels.(19,33) Furthermore, *Dao*$^{-/-}$ mice manifest an increase in D-serine levels, especially in areas with low levels in wild type animals such as the cerebellum and periphery.(37) The relatively unchanged D-serine levels in the forebrains of *Dao*$^{-/-}$ mice imply that in these areas, other mechanisms might regulate D-serine concentrations.(37,38)

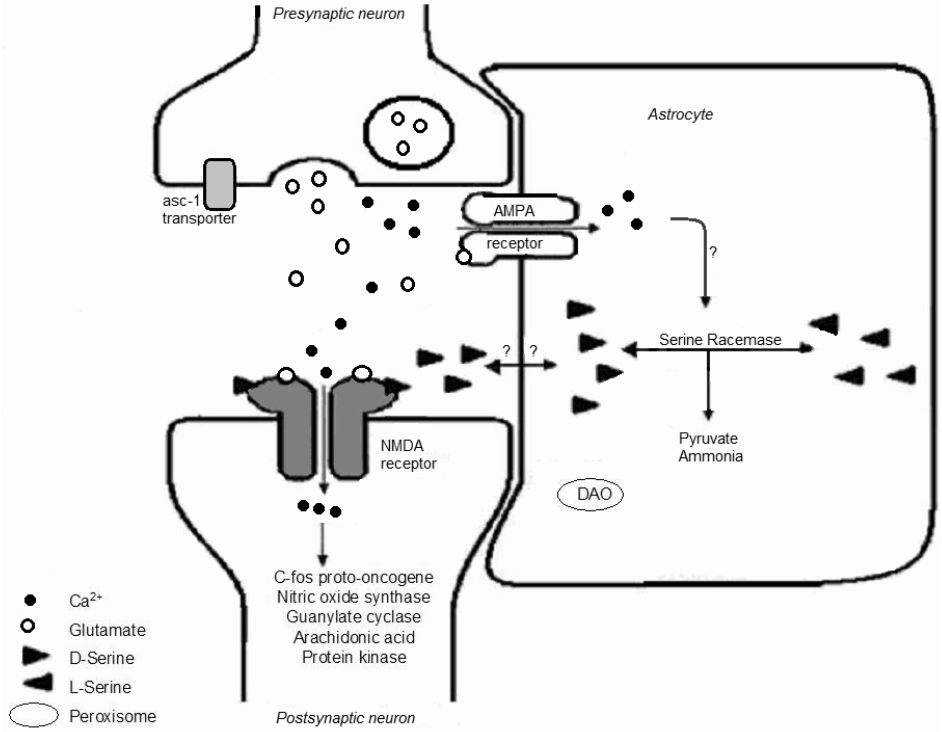
Biological function of D-serine

The close correlation between the anatomical distribution of D-serine and SR with the regional variation of the NMDA receptor (NMDAr) suggests a functional relationship. NMDArs are broadly distributed throughout the CNS and play a major role in glutamatergic synaptic transmission. They are members of a class of ionotropic receptor channels, organized as heteromeric assemblies composed of an NR1 subunit, combined with at least one of four NR2 (A-D) subunits. A third subunit, NR3, can co-assemble with NR1/NR2 complexes. Based on the subunit composition, the properties of NMDArs vary.

NMDArs require simultaneous ligand binding at two sites for activation. Glutamate molecules bind to the NR2 subunit (43) and glycine was assumed to be the necessary co-agonist, reacting with the strychnine-insensitive "glycine site" of the NMDAr NR1 subunit.(44-46) Immunohistochemical localization of glycine, D-serine and NMDArs showed a specific pattern of co-localization of D-serine with the NMDAr in the telencephalon and the developing cerebellum, while glycine co-localized with the NMDAr in the hindbrain, the adult cerebellum and the olfactory bulb.(20) Compared to glycine, D-serine acts as a selective and at least equally potent agonist for this "glycine site" of NMDArs.(47,48) Direct evidence for the regulation of NMDAr activity by endogenous D-serine comes from the decreased NMDAr activity in immature rat cerebellar slices, rat hippocampus slices and in primary hippocampal cell cultures after selective removal of D-serine by adding DAO, the effect of which was fully reversed by application of exogenous D-serine.(49) All these data favor D-serine as the predominant endogenous ligand for most NMDArs. Nevertheless, glycine might well be the principal ligand in some areas, such as the brainstem,(20) the spinal cord (50) and the cerebellum.(20,49) Pharmacological or genetic manipulation of the D-serine biosynthetic or degrading pathways should allow the distinction between predominantly D-serine and glycine dependent glutamatergic receptors. In this context, recent interesting evidence implies that the NR3 subunit of NMDArs, which occurs in cerebrocortical neurons, can also form a complex with NR1 subunits to produce a unique receptor that is excited by glycine and inhibited by D-serine, without being affected by glutamate or NMDA.(51)

Snyder et al. proposed a mechanism for the interdependency of glutamate and D-serine at glutamatergic synapses (Fig. 2).(52) The first stimulus needed is the release of glutamate from presynaptic neurons. Concurrent with binding to the NMDArs on postsynaptic neurons, glutamate binds to the non-NMDA, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid - kainate subtype of glutamate receptors (AMPArs) on astrocytes ensheathing the synapse.(19) This triggers D-serine biosynthesis in the astrocytes through a mechanism that is not yet understood, possibly involving calcium entry through calcium permeable AMPArs.(53,54) Upon biosynthesis, D-serine is released through currently unknown mechanisms into the synaptic space, where it interacts with the NMDAr by binding to its "glycine site". The occupation of both the glutamate and glycine binding sites of the NMDAr results in depolarization and calcium entry through the channel, leading to several possible intracellular metabotropic responses including the activation of guanylate cyclase (55) and neuronal nitric oxide synthase,(49) release of arachidonic acid,(56) translocation and activation of protein kinase C (57) and increased expression of the c-fos proto-oncogene.(58) Activation of NMDArs has been associated with physiological processes, such as memory, learning, development, pain and synaptic plasticity but also with pathological phenomena, including neurotoxicity and neurodegenerative diseases.(59,60)

Figure 2. D-serine biosynthesis and interaction with the NMDAr at a glutamatergic synapse.



Glutamate, released from a presynaptic neuron, can bind to both the NMDAr on a postsynaptic neuron and the AMPAr on an ensheathing astrocyte. The latter triggers D-serine biosynthesis in the astrocyte, possibly after calcium entry. The mechanism is not fully understood. After release of D-serine into the synaptic space, it can bind to the NMDAr. Binding of both D-serine and glutamate to the NMDAr results in activation of the NMDAr and calcium influx in the postsynaptic neuron, leading to several possible intracellular metabotropic responses. The mechanism responsible for removal of D-serine from the synaptic space is unclear and might involve amino acid transporters (possibly the asc1 transporter), peroxisomal DAO or serine racemase. AMPA = amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA = N-methyl D-aspartate; DAO = D-amino acid oxidase; small black dot = calcium; black arrowhead pointing to the right = D-serine; black arrowhead pointing to the left = L-serine, black open dot = glutamate; ? = mechanism that is not fully understood.

The molecular mechanisms of D-serine transport and the factors regulating its synaptic concentration require further elucidation. It would be conceivable that DAO degrades synaptic D-serine and thereby terminates D-serine signaling.(49) However, as DAO is a peroxisomal enzyme,(61) this would require D-serine transport to the peroxisomes. Previous studies failed to detect significant amounts of DAO in the forebrain (23) and D-serine levels in the forebrains of $Dao^{-/-}$ mice were relatively unaffected,(37) further suggesting other means of D-serine removal. Na^+-Dependent (62) or Na^+-independent amino acid transporters (63-66) might participate, with the Na^+-independent alanine-serine-cysteine transporter 1 (asc1) being the most probable candidate. Asc1 transports

D-serine with high affinity (Km=22.8μM, relatively close to the physiological extracellular D-serine concentration of 30μM in adults) and shows high levels of expression in neurons throughout the brain.(63-65) Finally, the extracellular level of D-serine may be regulated predominantly by the activity of its biosynthetic enzyme SR.(35,67) Through degradation of D-serine to pyruvate or conversion to L-serine (figures 1 and 2), SR might contribute to the clearance of synaptic D-serine, especially with increasing D-serine concentrations. Degradation of D-serine to pyruvate specifically appears to participate in the regulation of cellular D-serine concentrations, since cells, transfected with a point mutant of SR and displaying selective impairment of α,β-elimination activity, displayed concentrations of D-serine several fold higher than wild-type cells both in vitro and in vivo.(35) The relative importance of the enzymatic reactions and transporters has yet to be determined.

Ligand binding to the "glycine site" of the NMDAr seems to serve several critical functions. One is to allow glutamate to open the ion channel, while another is to decrease desensitization of the receptor. When external glycine and D-serine are reduced below saturation levels, the NMDArs show a faster rate of desensitization, possibly through allosteric interactions between the two binding sites.(68,69) In addition, binding of D-serine to the NMDAr has been shown to enable long-term potentiation induction in hippocampal slices, thereby playing an important role in long-term synaptic plasticity, which may serve as a cellular mechanism underlying learning and memory.(70) Regulation of the concentration of D-serine might thus serve to set the sensitivity of NMDArs under physiological conditions, thereby modulating the excitability of neurons.(27) The requirement of NMDArs to be stimulated by two endogenous neurotransmitters could provide a protection mechanism against the neurotoxicity associated with excess glutamatergic stimulation of the NMDAr.(52)

Whether the "glycine site" of NMDArs is saturated by glycine or D-serine in physiological conditions is not clear yet, although evidence is pointing towards a differential occupancy in various synapses.(46,71) In the thalamus, prefrontal cortex, neocortex, brainstem and hippocampus, increasing glycine concentration enhanced NMDAr signaling, demonstrating that the glycine site of NMDArs is not constitutively saturated at synapses in these brain regions.(72-76) As adding glycine or D-serine did not influence NMDAr activation at rat cerebellar cell synapses, the glycine sites here might indeed be saturated.(77) In summary, D-serine seems to fully occupy glycine sites of NMDArs at some synapses, while not saturating other NMDArs.(49) Incomplete saturation of the glycine site in vivo would increase the possibilities to modulate neurotransmission.

In the cerebellum, D-serine was localized immunohistochemically to astrocytes in the granular layer and Bergmann glia that regulate granule cell migration.(20) This localization and the transient D-serine increase in the developing cerebellum, which concurs with a transient expression of NMDArs, suggests a role in cerebellar development. Blocking

NMDArs during rodent development resulted in a disrupted cerebellar ontogeny.(78,79) The exact role of D-serine in cerebellar development remains to be determined.

D-Serine and disease

Excessive stimulation of NMDArs has been implicated in a large number of acute and chronic neurodegenerative conditions, including stroke, epilepsy, polyneuropathies, chronic pain, amyotrophic lateral sclerosis, Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD).(80) D-Serine concentrations might thus be altered in these disorders and contribute to their pathogenesis. D-Serine levels rose in animal models of stroke and this was paralleled by an increase in glycine and L-glutamate concentrations.(81) Reperfusion damage following hypoxemia was reduced by administration of glycine site antagonists.(82,83) Therefore, in stroke, D-serine release might be involved in the neuronal damage caused by overstimulation of NMDArs and consequent excitotoxicity and cell death.(84) The same process may play a role in global ischemia, as caused by perinatal asphyxia. The concentration of glycine increased in newborns following perinatal asphyxia, but unfortunately, D-serine concentrations were not determined.(85) However, when compared to AMPAr antagonists, glycine site antagonists and NMDAr antagonists only seemed to provide a small degree of neuroprotection in global cerebral ischemia.(82)

In PD and AD patients, D-serine concentrations in the temporal, parietal and prefrontal cortex were not significantly altered.(86-88) Nevertheless, modulation of the glycine site of NMDArs may influence the severity of symptoms. In PD patients, for instance, glycine site antagonists have shown beneficial effects on the motor abnormalities.(89)

As injection of D-serine in rat periaquaductal gray matter shows anxiogenic effects (90) and because glycine site antagonists reduce anxiety in animal models,(91,92) manipulation of NMDAr activity may constitute a new anxiolytic approach.

NMDAr hypofunction has been implicated in the pathology of schizophrenia, as NMDAr antagonists (93) or reduced numbers of NMDArs (94) induced positive, negative and cognitive schizophrenia-like symptoms, which could be reversed by intraventricular D-serine.(95) Compared to healthy controls, schizophrenic patients displayed lower D-serine and higher L-serine and total serine levels in the prefrontal cortex and serum.(87) Cerebrospinal or serum D- and L-serine levels thus may serve as convenient markers for schizophrenia.(96) Restoring D-serine concentrations might be therapeutic, as addition of oral D-serine to neuroleptics greatly improved positive, negative and cognitive symptoms of schizophrenia, with minimal self resolving side effects.(97) Further clinical trials are needed to corroborate these preliminary results. Interestingly, patients with signs of asphyxia at birth appear to have an increased risk of developing schizophrenia as adults,(98) thus linking two disorders that have been associated with NMDA receptor dysfunction and altered levels of D-serine.

In other disorders, manipulation of the glycine site of NMDArs yielded contrasting findings. Stimulation of the NMDAr by D-serine can induce seizures (99,100) and NMDAr antagonists can suppress convulsions.(101,102) In contrast, D-serine has also been shown to enhance anticonvulsant drug activity (103) and to increase the threshold for induction of seizures.(104) One theory to explain the latter is that epilepsy may originate through disinhibition of central neuronal networks. NMDAr activation is required to stimulate the inhibiting γ-amino butyric acid type A ($GABA_A$) receptors. Failure to activate these main inhibiting receptors results in disinhibition, which may lead to epilepsy. Therefore, NMDAr hypofunction, as induced by reduced D-serine concentrations, might induce seizures. Some of the conflicting effects of NMDAr modulation might be due to the different NMDAr subtypes and their differential anatomical distribution in the CNS. (105,106)

In spite of the relative frequency of disorders of amino acid catabolism in humans, inborn errors of serine degradation resulting in markedly elevated serine levels have not been identified as of yet. In contrast, low serine concentrations caused by disorders of serine biosynthesis have been reported, namely 3-phosphoglycerate dehydrogenase (3-PGDH) deficiency (107) and phosphoserine phosphatase (PSP) deficiency (108) (Fig. 1). 3-PGDH Deficiency is characterized by congenital microcephaly, seizures and severe psychomotor retardation.(107) PSP deficiency has only been identified in one patient, also suffering from Williams disease.(108) Both enzyme deficiencies result in reduced levels of L-serine, with a potential concomitant D-serine deficiency. The significance of reduced D-serine concentrations in these disorders requires further investigation.

Modulation of D-serine levels for clinical use

Clinical use of glycine site agonists and antagonists remains limited. Glycine site antagonists were badly tolerated in clinical trials or failed to attain therapeutic levels in the brain due to poor penetration into the blood-brain barrier.(80) These problems might be circumvented by modulating NMDAr activity through manipulation of D-serine levels, with SR and DAO as possible targets. Modifying human D-serine levels might not be without risk. One could speculate that disruption of D-serine metabolism would induce abnormal behavior, but $Dao^{-/-}$ rodents, expressing higher peripheral and central D-serine concentrations, exhibit seemingly normal behavior, development and reproducibility. (41,109) However, exaggerated pain-related behavior has been observed in $Dao^{-/-}$ mice and other complex behaviors such as learning and memory are currently under investigation.(110) Exogenous application of large doses of D-serine, as opposed to L-serine, have been found to induce a reversible acute necrosis of the terminal portions of the proximal renal tubules.(111,112) The mechanism has not been clarified. However, when used orally in a clinically effective dose that was less than 4% of the renal toxic dose, no effect on renal function was observed.(97)

Peripherally administered D-serine appears to reach the CNS. Intraperitoneal administration of D-serine (10 mmol/kg bodyweight) increased D-serine concentrations significantly in all the studied brain areas and peripheral tissues of infant and adult rats.(113) Concordantly, recent experiments with oral D-serine administration to schizophrenic patients and various animal studies suggest that low doses of peripherally administered D-serine induce central effects.(97,114) Peripheral and central administration of L-serine might also induce elevations in D-serine concentrations in specific brain areas (highest in the forebrain areas, followed by the cerebellum and pons-medulla).(67)
Taken together, modulation of D-serine concentrations appears promising for clinical use and might have advantages over glycine site antagonists, as central bioavailability after peripheral administration seems feasible and side effects are expected to be limited.

OTHER D-AMINO ACIDS

Localization of D-amino acids

Besides D-serine, D-aspartate is the only other D-amino acid that occurs in significant concentrations in the CNS. During embryonic development in the rat CNS, D-aspartate first appeared in the cortex, striatum, midbrain, diencephalon and cerebellum, with lower levels in the pons and medulla, later extending over the whole brain, before disappearing almost completely postnatally.(115,116) Remarkably, D-aspartate concentrations in the human frontal cortex at gestational week 14 exceeded those of the L-form.(15) A similar transient occurrence of free D-aspartate during distinct periods of early development and subsequent decrease to small amounts in adult tissue has been observed in the blood, retina, adrenals, pineal gland, and testes.(13,22,117,118) In addition to D-aspartate, nanomolar quantities of its N-methyl derivative NMDA have been described in rat CNS and endocrine glands.(119)
Several other D-amino acids have been identified in the CNS, including D-alanine, D-leucine, D-proline and D-glutamate (38,118,120,121) (Table 1). D-Glutamate was also isolated from the liver and kidneys, where D-glutamate concentrations of individual animals always exceeded those of D-aspartate.(121) Various D-amino acids have been identified in human plasma, urine, cerebrospinal fluid and amniotic fluid, with highest levels in urine and lowest levels in amniotic fluid or cerebrospinal fluid. D-Amino acid levels were almost always less than 1% of the corresponding L-amino acid.(122)

Synthesis and metabolism of D-amino acids

Analogous to D-serine, the different mechanisms to recruit D-amino acids in mammals involve dietary ingestion, derivation from intestinal bacteria, racemization with ageing and biosynthesis (table 1). D-Alanine is mainly derived from intestinal bacteria,(123,124) while D-methionine principally comes from the diet.(123,125) Biosynthesis might be of

Table 1. D-amino acids in mammals

D-amino acid	Animal	Localization	Origin	Putative functions / associated disease	Reference
D-serine	Rodents, humans	CNS, CSF, serum, saliva, urine, retina, almost all neonatal organs	Biosynthesis, intestinal bacteria, diet	Neuromodulator via the NMDAr / all disorders associated with NMDAr dysfunction and renal disease	See references in D-serine paragraph
D-aspartate	Rodents, humans	CNS, CSF, testes, adrenals, pituitary, hypothalamus, pineal gland, enamel, dentine, eye lens, retina	Biosynthesis, intestinal bacteria, diet	Function in developmental and endocrine regulation / theoretically in disorders associated with NMDAr dysfunction, ageing and renal disease	See references in other D-amino acids paragraph
D-glutamate	Rats	Brain, liver, kidneys			(121)
D-alanine	Rats, *Dao*$^{-/-}$ mice	Pituitary gland			(38)
	Humans	CNS, serum, saliva, urine	Intestinal bacteria	Alzheimer's disease, ageing, renal disease	(25,123,124,154, 155, 157-159)
D-leucine	Rats, mice, *Dao*$^{-/-}$ mice	CNS (pineal gland, hippocampus)			(118,120)
D-proline	Rats, mice, *Dao*$^{-/-}$ mice	CNS (pineal and pituitary gland)			(118,120)
	Humans	Serum, saliva, urine		Renal disease	(25,157-159)
D-methionine	Humans	Serum, saliva, urine	Diet		(123,125)
D-asparagine + D-aspartate	Humans	Serum, saliva, urine		Renal disease	(157-159)
D-arginine				Role in the urea cycle	(151)

Anatomical localization, origin, function and possible association with disease of some mammalian D-amino acids. CNS = central nervous system; CSF = cerebrospinal fluid.

importance for D-aspartate. Although D-aspartate racemase has solely been identified in bacteria,(126,127) it seems plausible that it has been conserved in mammals. This is supported by the accumulation of D-aspartate over time in cultured cells derived from adrenal medulla and pituitary tumor (128,129) and by the synthesis of D-aspartate from L-aspartate in embryonic neuronal primary culture cells.(115) This activity could be inhibited by amino-oxyacetic acid, an inhibitor of pyridoxal phosphate dependent enzymes. All known racemases require pyridoxal phosphate for their activity.

Similar to the oxidative deamination of neutral D-amino acids by DAO, the peroxisomal flavoprotein DAOX selectively degrades dicarboxylic amino acids, including D-aspartate, D-glutamate and N-alkyl-derivatives such as NMDA (130-132):

$$\text{Dicarboxylic D-amino acids} + O_2 + H_2O - \text{DAOX} \rightarrow \text{oxaloacetate} + NH_3 + H_2O_2$$

DAOX protein and mRNA are ubiquitous in mammals, with highest expression in the kidneys, the liver and the CNS and low expression in other peripheral tissues.(133)

DAOX appears to participate at least in the metabolism of mammalian endogenous D-aspartate.(134-137)

Biological function of D-amino acids

In the CNS, D-aspartate might potentate NMDAr mediated transmission through the glutamate binding site of the NMDAr,(138,139) but the affinity of NMDArs for D-aspartate is ten times lower than for L-glutamate (139) and localization of D-aspartate does not correlate with the NMDAr.(140) D-Aspartate might however enhance NMDAr activity after enzymatic methylation by a methyltransferase to generate NMDA in the brain.(119,141) The properties of storage, release and uptake of D-aspartate have not been well characterized. D-Aspartate may be stored in secretory granules and secreted through a Ca^{2+} dependent exocytotic mechanism, at least in the adrenals.(142) Several studies, mostly using the metabolically inert [^{3}H]D-aspartate, have suggested that the biological activity of D-aspartate is terminated after removal from the synaptic cleft by high–affinity transporters.(143-146) Synaptically released D-aspartate might also be inactivated by DAOX,(140) but DAOX is present in peroxisomes and not in axon terminals and synaptic complexes.(132)

On the basis of the developmental correlation between the periods of maximal D-aspartate contents in many different organs and the periods of morphological and functional maturation of the organ,(15,22,117) it has been suggested that D-aspartate may play a role in the maturation and differentiation of cells in tissues where it transiently appears, such as the CNS, the retina, the adrenals and the testes.(22,117) In mammalian endocrine cells, D-aspartate also appears to influence the secretion of several hormones. D-Aspartate stimulated the release of testosterone,(147,148) oxytocin,(149) growth and luteinizing hormones (119) and prolactin (141) while inhibiting the secretion of melatonin. (150) D-Aspartate is likely to participate in the steroidogenesis in the testes,(147,148) possibly through cholesterol translocation into the inner mitochondrial membrane, the rate-limiting process in steroidogenesis.(148)

D-Arginine is the only other D-amino acid that has been implicated in a biological function. It has been proposed to play a role in the urea cycle.(151)

D-Amino acids and disease

Alterations in D-aspartate concentrations have been reported in patients with AD, although these data are conflicting and further studies are warranted to corroborate these results. D-Aspartate levels were found to be significantly higher in the cerebrospinal fluid of patients with AD than in normal cerebrospinal fluid,(152) while in contrast, free D- aspartate content was significantly lower in the brain regions with neuropathological changes of AD (153) and in the white matter of AD brains when compared to normal brains.(154) Some of these discrepancies might relate to the facile degradation of D-

asparagine to D-aspartate during storage of samples or analysis, leading to overestimations of D-aspartate concentrations. Elevated levels of free and protein bound D-alanine have also been reported in the gray matter of AD brains, the significance of which requires clarification.(154,155)

After perinatal asphyxia, D-aspartate was synthesized specifically in the CNS, probably from its precursor alanine.(156) The function of D-aspartate in hypoxia remains to be determined. Elevated levels of serum D-serine, D-alanine, D-proline and D-asparagine and/or D-aspartate have further been associated with ageing and renal disease,(25,157-159) with a positive correlation between D-amino acid levels and markers for renal disease.

D-Amino acids and clinical use

Although modulation of the concentration of D-aspartate does not have an obvious clinical purpose yet, exogenously applied D-aspartate has been evaluated in animals. Addition of 5% D,L-aspartate to the diets of rats depressed growth to a greater extent than the L-isomer alone,(160,161) as did addition of 2% D-aspartate in the soybean protein diets of chickens, as opposed to 2% D-serine.(162) Rats receiving 50 mg/kg D-aspartate in their drinking water, on the other hand, did not show any signs of toxicity in their physiological status, nor in specific organs such as liver and kidneys.(163) The threshold value for toxicity of D-aspartate in mice after intramuscular injection was relatively high (LD_{50} =1000mg/kg bodyweight).(164) Clearly, after unraveling the function of D-aspartate in health and disease, toxicity will need to be addressed before considering clinical use. Inclusion of D-amino acids in antibiotics and synthetic vaccines against infectious or autoimmune diseases may be advantageous in terms of both specificity of the immune response and efficacy through decreased digestion.(165) Use of D-amino acids might also provide new strategies in cancer therapy. As administration of neutral D-amino acids induces DAO activity, it increases the formation of its reaction products, including hydrogen peroxide. This reactive oxygen species can cause oxidative stress and cytotoxicity. Transfection of tumor cells with a DAO-construct and subsequent D-alanine administration resulted in cytotoxicity to tumor cells without causing toxicity to parental cells.(166)

CONCLUSIONS

Up to fifty years ago, it was generally believed that D-amino acids did not occur in living organisms with the exception of micro-organisms and some peptides. The studies from the last few decades have undeniably established the presence of D-amino acids in higher organisms, including humans. The specific functions of the individual D-amino acids are far from being unraveled, despite the growing understanding provided by recent work, which has focused on D-serine and D-aspartate. D-Serine functions as an

important neuromodulator, whereas D-aspartate has been implicated in developmental and endocrine functions. The role of D-amino acids in pathologic conditions requires further investigation. Determination of D-amino acid concentrations in biological fluids and measurement of the activity of the synthesizing and metabolizing enzymes and mutations in their genes might be of use as diagnostic tools in some disorders. This is of particular interest since D-amino acid concentrations and their function may be manipulated through exogenous application, modulation of biosynthesis and degradation and pharmacological manipulation of target receptors, thus providing new therapeutic strategies. Finally, use of exogenous D-amino acids, free or incorporated in peptides and proteins, might bring valuable new treatment possibilities thanks to their specific interactions and high efficacy.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from The Netherlands Organisation for Health Research and Development (grant 920-03-345). We thank L. Dorland and M. de Sain for helpful discussions on the topic.

REFERENCES

1. V.S. Lamzin, Z. Dauter, K.S. Wilson, How nature deals with stereoisomers, Curr. Opin. Struct. Biol 5 (1995) 830-836.
2. V. Prelog, Chirality in chemistry, Science 193 (1976) 17-24.
3. S.F. Mason, Origins of biomolecular handedness, Nature 311 (1984) 19-23.
4. J.J. Corrigan, D-amino acids in animals, Science 164 (1969) 142-149.
5. J. Auclair, R. Patton, Rev Can Biol 9 (1950) 3.
6. I. Beatty, D. Magrath, A. Ennor, Occurrence of D-serine in lombricine, Nature 183 (1959) 591.
7. J.J. Corrigan, N.G. Srinivasan, The occurrence of certain D-amino acids in insects, Biochemistry 5 (1966) 1185-1190.
8. G. Kreil, Peptides containing a D-amino acid from frogs and molluscs, J. Biol Chem. 269 (1994) 10967-10970.
9. R.L. Preston, Occurrence of D-amino acids in higher organisms: a survey of the distribution of D-amino acids in marine vertebrates., Comp. Biochem. Physiol. 87B (1987) 55-62.
10. H.A. Krebs, Metabolism of amino acids. III. Determination of amino acids., Biochem. J. 29 (1935) 1620-1644.
11. J.L. Still, M.V. Buell, W.E. Knox, D.E. Green, Studies on the cyclphorase system VII. D-Aspartate oxidase, J. Biol. Chem. 179 (1949) 831-837.
12. P.M. Helfman, J.L. Bada, M.Y. Shou, Considerations on the role of aspartic acid racemization in the aging process, Gerontology 23 (1977) 419-425.
13. D.S. Dunlop, A. Neidle, D. McHale, D.M. Dunlop, A. Lajtha, The presence of free D-aspartic acid in rodents and man, Biochem. Biophys. Res. Commun. 141 (1986) 27-32.
14. A. Hashimoto, T. Nishikawa, T. Hayashi, N. Fujii, K. Harada, T. Oka, K. Takahashi, The presence of free D-serine in rat brain, FEBS Lett. 296 (1992) 33-36.
15. A. Hashimoto, S. Kumashiro, T. Nishikawa, T. Oka, K. Takahashi, T. Mito, S. Takashima, N. Doi, Y. Mizutani, T. Yamazaki, ., Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex, J. Neurochem. 61 (1993) 348-351.
16. A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, Endogenous D-serine in rat brain: N-methyl-D-aspartate receptor-related distribution and aging, J. Neurochem. 60 (1993) 783-786.
17. Y. Nagata, K. Horiike, T. Maeda, Distribution of free D-serine in vertebrate brains, Brain Res. 634 (1994) 291-295.
18. A. Hashimoto, T. Oka, T. Nishikawa, Extracellular concentration of endogenous free D-serine in the rat brain as revealed by in vivo microdialysis, Neuroscience 66 (1995) 635-643.
19. M.J. Schell, M.E. Molliver, S.H. Snyder, D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release, Proc. Natl. Acad. Sci U. S. A 92 (1995) 3948-3952.
20. M.J. Schell, R.O. Brady, Jr., M.E. Molliver, S.H. Snyder, D-serine as a neuromodulator: regional and developmental localizations in rat brain glia resemble NMDA receptors, J. Neurosci. 17 (1997) 1604-1615.
21. K. Wako, N. Ma, T. Shiroyama, R. Semba, Glial uptake of intracerebroventricularly injected D-serine in the rat brain: an immunocytochemical study, Neurosci. Lett. 185 (1995) 171-174.
22. A. Hashimoto, T. Oka, T. Nishikawa, Anatomical distribution and postnatal changes in endog-

enous free D-aspartate and D-serine in rat brain and periphery, Eur. J. Neurosci. 7 (1995) 1657-1663.

23. K. Horiike, H. Tojo, R. Arai, T. Yamano, M. Nozaki, T. Maeda, Localization of D-amino acid oxidase in Bergmann glial cells and astrocytes of rat cerebellum, Brain Res. Bull. 19 (1987) 587-596.
24. H. Bruckner, S. Haasmann, A. Friedrich, Quantification of D-amino acids in human urine using GC-MS and HPLC, Amino Acids 6 (1994) 211.
25. Y. Nagata, R. Masui, T. Akino, The presence of free D-serine, D-alanine and D-proline in human plasma, Experientia 48 (1992) 986-988.
26. J. Rotgans, R. Wodarz, W. Schoknecht, K. Drysch, The determination of amino-acid enantiomers in human saliva with Chirasil-Val, Arch. Oral Biol 28 (1983) 1121-1124.
27. E.R. Stevens, M. Esguerra, P.M. Kim, E.A. Newman, S.H. Snyder, K.R. Zahs, R.F. Miller, D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors, Proc. Natl. Acad. Sci U. S. A 100 (2003) 6789-6794.
28. J. De Miranda, R. Panizzutti, V.N. Foltyn, H. Wolosker, Cofactors of serine racemase that physiologically stimulate the synthesis of the N-methyl-D-aspartate (NMDA) receptor coagonist D-serine, Proc. Natl. Acad. Sci U. S. A 99 (2002) 14542-14547.
29. H. Wolosker, S. Blackshaw, S.H. Snyder, Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission, Proc. Natl. Acad. Sci U. S. A 96 (1999) 13409-13414.
30. H. Wolosker, K.N. Sheth, M. Takahashi, J.P. Mothet, R.O. Brady, Jr., C.D. Ferris, S.H. Snyder, Purification of serine racemase: biosynthesis of the neuromodulator D-serine, Proc. Natl. Acad. Sci U. S. A 96 (1999) 721-725.
31. T.J. de Koning, L.W. Klomp, Serine-deficiency syndromes, Curr Opin Neurol 17 (2004) 197-204.
32. K. Yoshida, S. Furuya, S. Osuka, J. Mitoma, Y. Shinoda, M. Watanabe, N. Azuma, H. Tanaka, T. Hashikawa, S. Itohara, Y. Hirabayashi, Targeted disruption of the mouse 3-phosphoglycerate dehydrogenase gene causes severe neurodevelopmental defects and results in embryonic lethality, J. Biol Chem. 279 (2004) 3573-3577.
33. L.Z. Wang, X.Z. Zhu, Spatiotemporal relationships among D-serine, serine racemase, and D-amino acid oxidase during mouse postnatal development, Acta Pharmacol. Sin. 24 (2003) 965-974.
34. J. De Miranda, A. Santoro, S. Engelender, H. Wolosker, Human serine racemase: moleular cloning, genomic organization and functional analysis, Gene 256 (2000) 183-188.
35. V.N. Foltyn, I. Bendikov, J. De Miranda, R. Panizzutti, E. Dumin, M. Shleper, P. Li, M.D. Toney, E. Kartvelishvily, H. Wolosker, Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity, J. Biol Chem. 280 (2005) 1754-63.
36. L. Pollegioni, C.M. Harris, G. Molla, M.S. Pilone, S. Ghisla, Identification and role of ionizing functional groups at the active center of Rhodotorula gracilis D-amino acid oxidase, FEBS Lett. 507 (2001) 323-326.
37. A. Hashimoto, T. Nishikawa, R. Konno, A. Niwa, Y. Yasumura, T. Oka, K. Takahashi, Free D-serine, D-aspartate and D-alanine in central nervous system and serum in mutant mice lacking D-amino acid oxidase, Neurosci. Lett. 152 (1993) 33-36.

38. A. Morikawa, K. Hamase, T. Inoue, R. Konno, A. Niwa, K. Zaitsu, Determination of free D-aspartic acid, D-serine and D-alanine in the brain of mutant mice lacking D-amino acid oxidase activity, J. Chromatogr. B Biomed. Sci Appl. 757 (2001) 119-125.
39. A. D'Aniello, A. Vetere, L. Petrucelli, Further study on the specificity of D-amino acid oxidase and D-aspartate oxidase and time course for complete oxidation of D-amino acids, Comp Biochem. Physiol B 105 (1993) 731-734.
40. M. Tada, K. Fukui, K. Momoi, Y. Miyake, Cloning and expression of a cDNA encoding mouse kidney D-amino acid oxidase, Gene 90 (1990) 293-297.
41. Y. Nagata, R. Konno, Y. Yasumura, T. Akino, Involvement of D-amino acid oxidase in elimination of free D-amino acids in mice, Biochem. J. 257 (1989) 291-292.
42. Y. Urai, O. Jinnouchi, K.T. Kwak, A. Suzue, S. Nagahiro, K. Fukui, Gene expression of D-amino acid oxidase in cultured rat astrocytes: regional and cell type specific expression, Neurosci. Lett. 324 (2002) 101-104.
43. R. Dingledine, K. Borges, D. Bowie, S.F. Traynelis, The glutamate receptor ion channels, Pharmacol. Rev 51 (1999) 7-61.
44. J.W. Johnson, P. Ascher, Glycine potentiates the NMDA response in cultured mouse brain neurons, Nature 325 (1987) 529-531.
45. N.W. Kleckner, R. Dingledine, Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes, Science 241 (1988) 835-837.
46. A.M. Thomson, Glycine is a coagonist at the NMDA receptor/channel complex, Prog. Neurobiol. 35 (1990) 53-74.
47. W. Danysz, E. Fadda, J.T. Wroblewski, E. Costa, [3H]D-serine labels strychnine-insensitive glycine recognition sites of rat central nervous system, Life Sci 46 (1990) 155-164.
48. T. Matsui, M. Sekiguchi, A. Hashimoto, U. Tomita, T. Nishikawa, K. Wada, Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration, J. Neurochem. 65 (1995) 454-458.
49. J.P. Mothet, A.T. Parent, H. Wolosker, R.O. Brady, Jr., D.J. Linden, C.D. Ferris, M.A. Rogawski, S.H. Snyder, D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor, Proc. Natl. Acad. Sci U. S. A 97 (2000) 4926-4931.
50. S. Ahmadi, U. Muth-Selbach, A. Lauterbach, P. Lipfert, W.L. Neuhuber, H.U. Zeilhofer, Facilitation of spinal NMDA receptor currents by spillover of synaptically released glycine, Science 300 (2003) 2094-2097.
51. J.E. Chatterton, M. Awobuluyi, L.S. Premkumar, H. Takahashi, M. Talantova, Y. Shin, J. Cui, S. Tu, K.A. Sevarino, N. Nakanishi, G. Tong, S.A. Lipton, D. Zhang, Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits, Nature 415 (2002) 793-798.
52. D. Boehning, S.H. Snyder, Novel neural modulators, Annu. Rev Neurosci. 26 (2003) 105-131.
53. S.P. Cook, I. Galve-Roperh, d.P. Martinez, I. Rodriguez-Crespo, Direct calcium binding results in activation of brain serine racemase, J. Biol Chem. 277 (2002) 27782-27792.
54. H. Tanaka, S.Y. Grooms, M.V. Bennett, R.S. Zukin, The AMPAR subunit GluR2: still front and center-stage, Brain Res. 886 (2000) 190-207.
55. J.T. Wroblewski, E. Fadda, J. Mazzetta, J.W. Lazarewicz, E. Costa, Glycine and D-serine act as positive modulators of signal transduction at N-methyl-D-aspartate sensitive glutamate receptors in cultured cerebellar granule cells, Neuropharmacology 28 (1989) 447-452.
56. J.W. Lazarewicz, J.T. Wroblewski, M.E. Palmer, E. Costa, Activation of N-methyl-D-aspartate-

sensitive glutamate receptors stimulates arachidonic acid release in primary cultures of cerebellar granule cells, Neuropharmacology 27 (1988) 765-769.

57. F. Vaccarino, A. Guidotti, E. Costa, Ganglioside inhibition of glutamate-mediated protein kinase C translocation in primary cultures of cerebellar neurons, Proc. Natl. Acad. Sci U. S. A 84 (1987) 8707-8711.
58. A.M. Szekely, M.L. Barbaccia, E. Costa, Activation of specific glutamate receptor subtypes increases C-fos proto-oncogene expression in primary cultures of neonatal rat cerebellar granule cells, Neuropharmacology 26 (1987) 1779-1782.
59. G.L. Collingridge, R.A. Lester, Excitatory amino acid receptors in the vertebrate central nervous system, Pharmacol. Rev 41 (1989) 143-210.
60. D.T. Monaghan, R.J. Bridges, C.W. Cotman, The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system, Annu. Rev Pharmacol. Toxicol. 29 (1989) 365-402.
61. S. Moreno, R. Nardacci, A. Cimini, M.P. Ceru, Immunocytochemical localization of D-amino acid oxidase in rat brain, J. Neurocytol. 28 (1999) 169-185.
62. C.S. Ribeiro, M. Reis, R. Panizzutti, J. De Miranda, H. Wolosker, Glial transport of the neuromodulator D-serine, Brain Res. 929 (2002) 202-209.
63. Y. Fukasawa, H. Segawa, J.Y. Kim, A. Chairoungdua, D.K. Kim, H. Matsuo, S.H. Cha, H. Endou, Y. Kanai, Identification and characterization of a Na(+)-independent neutral amino acid transporter that associates with the 4F2 heavy chain and exhibits substrate selectivity for small neutral D- and L-amino acids, J. Biol Chem. 275 (2000) 9690-9698.
64. L. Helboe, J. Egebjerg, M. Moller, C. Thomsen, Distribution and pharmacology of alanine-serine-cysteine transporter 1 (asc-1) in rodent brain, Eur. J. Neurosci. 18 (2003) 2227-2238.
65. H. Matsuo, Y. Kanai, M. Tokunaga, T. Nakata, A. Chairoungdua, H. Ishimine, S. Tsukada, H. Ooigawa, H. Nawashiro, Y. Kobayashi, J. Fukuda, H. Endou, High affinity D- and L-serine transporter Asc-1: cloning and dendritic localization in the rat cerebral and cerebellar cortices, Neurosci. Lett. 358 (2004) 123-126.
66. J. Nakauchi, H. Matsuo, D.K. Kim, A. Goto, A. Chairoungdua, S.H. Cha, J. Inatomi, Y. Shiokawa, K. Yamaguchi, I. Saito, H. Endou, Y. Kanai, Cloning and characterization of a human brain Na(+)-independent transporter for small neutral amino acids that transports D-serine with high affinity, Neurosci. Lett. 287 (2000) 231-235.
67. A. Hashimoto, Effect of the intracerebroventricular and systemic administration of L-serine on the concentrations of D- and L-serine in several brain areas and periphery of rat, Brain Res. 955 (2002) 214-220.
68. R.A. Lester, G. Tong, C.E. Jahr, Interactions between the glycine and glutamate binding sites of the NMDA receptor, J. Neurosci. 13 (1993) 1088-1096.
69. L. Vyklicky, Jr., M. Benveniste, M.L. Mayer, Modulation of N-methyl-D-aspartic acid receptor desensitization by glycine in mouse cultured hippocampal neurones, J. Physiol 428 (1990) 313-331.
70. Y. Yang, W. Ge, Y. Chen, Z. Zhang, W. Shen, C. Wu, M. Poo, S. Duan, Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine, Proc. Natl. Acad. Sci U. S. A 100 (2003) 15194-15199.
71. P.L. Wood, The co-agonist concept: is the NMDA-associated glycine receptor saturated in vivo?, Life Sci 57 (1995) 301-310.

72. A.J. Berger, S. Dieudonne, P. Ascher, Glycine uptake governs glycine site occupancy at NMDA receptors of excitatory synapses, J. Neurophysiol. 80 (1998) 3336-3340.
73. R. Bergeron, T.M. Meyer, J.T. Coyle, R.W. Greene, Modulation of N-methyl-D-aspartate receptor function by glycine transport, Proc. Natl. Acad. Sci U. S. A 95 (1998) 15730-15734.
74. L. Chen, M. Muhlhauser, C.R. Yang, Glycine tranporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons in vitro and in vivo, J. Neurophysiol. 89 (2003) 691-703.
75. T.E. Salt, Modulation of NMDA receptor-mediated responses by glycine and D-serine in the rat thalamus in vivo, Brain Res. 481 (1989) 403-406.
76. A.M. Thomson, V.E. Walker, D.M. Flynn, Glycine enhances NMDA-receptor mediated synaptic potentials in neocortical slices, Nature 338 (1989) 422-424.
77. D. Billups, D. Attwell, Active release of glycine or D-serine saturates the glycine site of NMDA receptors at the cerebellar mossy fibre to granule cell synapse, Eur. J. Neurosci. 18 (2003) 2975-2980.
78. H. Komuro, P. Rakic, Modulation of neuronal migration by NMDA receptors, Science 260 (1993) 95-97.
79. S. Rabacchi, Y. Bailly, N. Delhaye-Bouchaud, J. Mariani, Involvement of the N-methyl D-aspartate (NMDA) receptor in synapse elimination during cerebellar development, Science 256 (1992) 1823-1825.
80. W. Danysz, A.C. Parsons, Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications, Pharmacol. Rev 50 (1998) 597-664.
81. E.H. Lo, A.R. Pierce, K. Matsumoto, T. Kano, C.J. Evans, R. Newcomb, Alterations in K+ evoked profiles of neurotransmitter and neuromodulator amino acids after focal ischemia-reperfusion, Neuroscience 83 (1998) 449-458.
82. C.A. Hicks, M.A. Ward, N. Ragumoorthy, S.J. Ambler, C.P. Dell, D. Dobson, M.J. O'Neill, Evaluation of glycine site antagonists of the NMDA receptor in global cerebral ischaemia, Brain Res. 819 (1999) 65-74.
83. M.A. Petty, C. Neumann-Haefelin, J. Kalisch, S. Sarhan, J.G. Wettstein, H.P. Juretschke, In vivo neuroprotective effects of ACEA 1021 confirmed by magnetic resonance imaging in ischemic stroke, Eur. J. Pharmacol. 474 (2003) 53-62.
84. D.W. Choi, S.M. Rothman, The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death, Annu. Rev Neurosci. 13 (1990) 171-182.
85. A. Roldan, J. Figueras-Aloy, R. Deulofeu, R. Jimenez, Glycine and other neurotransmitter amino acids in cerebrospinal fluid in perinatal asphyxia and neonatal hypoxic-ischaemic encephalopathy, Acta Paediatr. 88 (1999) 1137-1141.
86. M.L. Chouinard, D. Gaitan, P.L. Wood, Presence of the N-methyl-D-aspartate-associated glycine receptor agonist, D-serine, in human temporal cortex: comparison of normal, Parkinson, and Alzheimer tissues, J. Neurochem. 61 (1993) 1561-1564.
87. S. Kumashiro, A. Hashimoto, T. Nishikawa, Free D-serine in post-mortem brains and spinal cords of individuals with and without neuropsychiatric diseases, Brain Res. 681 (1995) 117-125.
88. Y. Nagata, M. Borghi, G.H. Fisher, A. D'Aniello, Free D-serine concentration in normal and Alzheimer human brain, Brain Res. Bull. 38 (1995) 181-183.
89. M. Carlsson, A. Carlsson, Interactions between glutamatergic and monoaminergic systems

within the basal ganglia--implications for schizophrenia and Parkinson's disease, Trends Neurosci. 13 (1990) 272-276.
90. M.L. Schmitt, W. Coelho, A.S. Lopes-de-Souza, F.S. Guimaraes, A.P. Carobrez, Anxiogenic-like effect of glycine and D-serine microinjected into dorsal periaqueductal gray matter of rats, Neurosci. Lett. 189 (1995) 93-96.
91. E.W. Anthony, M.E. Nevins, Anxiolytic-like effects of N-methyl-D-aspartate-associated glycine receptor ligands in the rat potentiated startle test, Eur. J. Pharmacol. 250 (1993) 317-324.
92. J.H. Kehne, T.C. McCloskey, B.M. Baron, E.M. Chi, B.L. Harrison, J.P. Whitten, M.G. Palfreyman, NMDA receptor complex antagonists have potential anxiolytic effects as measured with separation-induced ultrasonic vocalizations, Eur. J. Pharmacol. 193 (1991) 283-292.
93. D.C. Goff, J.T. Coyle, The emerging role of glutamate in the pathophysiology and treatment of schizophrenia, Am. J. Psychiatry 158 (2001) 1367-1377.
94. A.R. Mohn, R.R. Gainetdinov, M.G. Caron, B.H. Koller, Mice with reduced NMDA receptor expression display behaviors related to schizophrenia, Cell 98 (1999) 427-436.
95. P.C. Contreras, D-serine antagonized phencyclidine- and MK-801-induced stereotyped behavior and ataxia, Neuropharmacology 29 (1990) 291-293.
96. K. Hashimoto, T. Fukushima, E. Shimizu, N. Komatsu, H. Watanabe, N. Shinoda, M. Nakazato, C. Kumakiri, S. Okada, H. Hasegawa, K. Imai, M. Iyo, Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia, Arch. Gen. Psychiatry 60 (2003) 572-576.
97. G. Tsai, P. Yang, L.C. Chung, N. Lange, J.T. Coyle, D-serine added to antipsychotics for the treatment of schizophrenia, Biol Psychiatry 44 (1998) 1081-1089.
98. C. Dalman, H.V. Thomas, A.S. David, J. Gentz, G. Lewis, P. Allebeck, Signs of asphyxia at birth and risk of schizophrenia. Population-based case-control study, Br. J. Psychiatry 179 (2001) 403-408.
99. B.S. Meldrum, Possible therapeutic applications of excitatory amino acid neurotransmitters, Clin. Sci. 68 (1985) 113-122.
100. L. Singh, R.J. Oles, M.D. Tricklebank, Modulation of seizure susceptibility in the mouse by the strychnine-insensitive glycine recognition site of the NMDA receptor/ion channel complex, Br. J. Pharmacol. 99 (1990) 285-288.
101. G. De Sarro, G.R. Trimarchi, S. Sinopoli, Y. Masuda, A. De Sarro, Anticonvulsant effects of U-54494A and U-50488H in genetically epilepsy-prone rats and DBA/2 mice: a possible involvement of glycine/NMDA receptor complex, Gen. Pharmacol. 24 (1993) 439-447.
102. S.L. Peterson, Infusion of NMDA antagonists into the nucleus reticularis pontis oralis inhibits the maximal electroshock seizure response, Brain Res. 702 (1995) 101-109.
103. S.L. Peterson, Anticonvulsant drug potentiation by glycine in maximal electroshock seizures is mimicked by D-serine and antagonized by 7-chlorokynurenic acid, Eur. J. Pharmacol. 199 (1991) 341-348.
104. W. Loscher, P. Wlaz, C. Rundfeldt, H. Baran, D. Honack, Anticonvulsant effects of the glycine/NMDA receptor ligands D-cycloserine and D-serine but not R-(+)-HA-966 in amygdala-kindled rats, Br. J. Pharmacol. 112 (1994) 97-106.
105. H. Mori, M. Mishina, Structure and function of the NMDA receptor channel, Neuropharmacology 34 (1995) 1219-1237.

106. S. Nakanishi, Molecular diversity of glutamate receptors and implications for brain function, Science 258 (1992) 597-603.
107. J. Jaeken, M. Detheux, L. Van Maldergem, M. Foulon, H. Carchon, E. Van Schaftingen, 3-Phosphoglycerate dehydrogenase deficiency: an inborn error of serine biosynthesis, Arch. Dis. Child 74 (1996) 542-545.
108. J. Jaeken, M. Detheux, J.P. Fryns, J.F. Collet, P. Alliet, E. Van Schaftingen, Phosphoserine phosphatase deficiency in a patient with Williams syndrome, J. Med. Genet. 34 (1997) 594-596.
109. R. Konno, Y. Yasumura, Mouse mutant deficient in D-amino acid oxidase activity, Genetics 103 (1983) 277-285.
110. K. Wake, H. Yamazaki, S. Hanzawa, R. Konno, H. Sakio, A. Niwa, Y. Hori, Exaggerated responses to chronic nociceptive stimuli and enhancement of N-methyl-D-aspartate receptor-mediated synaptic transmission in mutant mice lacking D-amino-acid oxidase, Neurosci. Lett. 297 (2001) 25-28.
111. C. Artom, W.H. Fishman, R.P. Morehead, The relative toxicity of L- and D,L-serine in rats, Proc. Soc. Expl. Biol. Med. 60 (1945) 284-287.
112. M. Wachstein, M. Besen, Electron microscopy of renal coagulative necrosis due to DL-serine, with special reference to mitochondrial pyknosis, Am. J. Pathol. 44 (1964) 383-400.
113. A. Hashimoto, Y. Chiba, Effect of systemic administration of d-serine on the levels of d- and l-serine in several brain areas and periphery of rat, Eur. J. Pharmacol. 495 (2004) 153-158.
114. A. Hashimoto, T. Oka, Free D-aspartate and D-serine in the mammalian brain and periphery, Prog. Neurobiol. 52 (1997) 325-353.
115. H. Wolosker, A. D'Aniello, S.H. Snyder, D-aspartate disposition in neuronal and endocrine tissues: ontogeny, biosynthesis and release, Neuroscience 100 (2000) 183-189.
116. K. Sakai, H. Homma, J.A. Lee, T. Fukushima, T. Santa, K. Tashiro, T. Iwatsubo, K. Imai, Emergence of D-aspartic acid in the differentiating neurons of the rat central nervous system, Brain Res. 808 (1998) 65-71.
117. A. Neidle, D.S. Dunlop, Developmental changes in free D-aspartic acid in the chicken embryo and in the neonatal rat, Life Sci 46 (1990) 1517-1522.
118. K. Hamase, H. Homma, Y. Takigawa, T. Fukushima, T. Santa, K. Imai, Regional distribution and postnatal changes of D-amino acids in rat brain, Biochim. Biophys. Acta 1334 (1997) 214-222.
119. A. D'Aniello, M.M. Di Fiore, G.H. Fisher, A. Milone, A. Seleni, S. D'Aniello, A.F. Perna, D. Ingrosso, Occurrence of D-aspartic acid and N-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release, FASEB J. 14 (2000) 699-714.
120. K. Hamase, T. Inoue, A. Morikawa, R. Konno, K. Zaitsu, Determination of free D-proline and D-leucine in the brains of mutant mice lacking D-amino acid oxidase activity, Anal. Biochem. 298 (2001) 253-258.
121. Y. Kera, H. Aoyama, H. Matsumura, A. Hasegawa, H. Nagasaki, R. Yamada, Presence of free D-glutamate and D-aspartate in rat tissues, Biochim. Biophys. Acta 1243 (1995) 283-286.
122. D.W. Armstrong, M. Gasper, S.H. Lee, J. Zukowski, N. Ercal, D-amino acid levels in human physiological fluids, Chirality 5 (1993) 375-378.

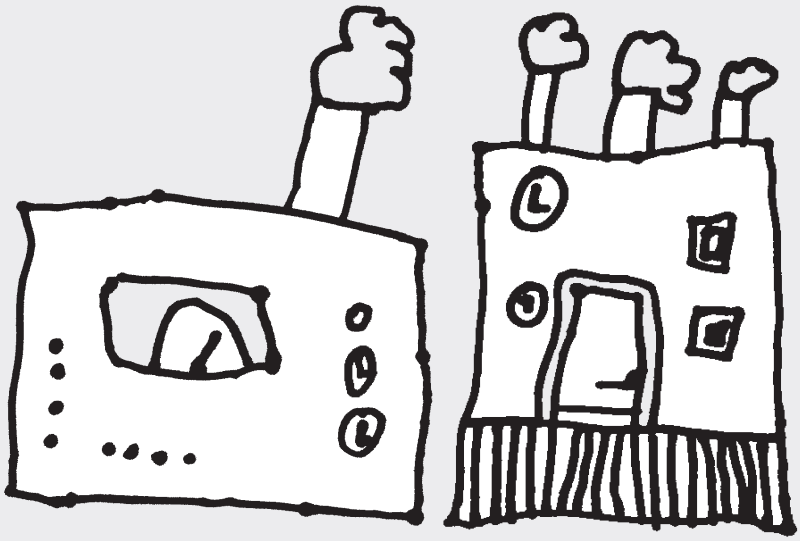
123. R. Konno, A. Niwa, Y. Yasumura, Intestinal bacterial origin of D-alanine in urine of mutant mice lacking D-amino-acid oxidase, Biochem. J. 268 (1990) 263-265.
124. R. Konno, T. Oowada, A. Ozaki, T. Iida, A. Niwa, Y. Yasumura, T. Mizutani, Origin of D-alanine present in urine of mutant mice lacking D-amino-acid oxidase activity, Am. J. Physiol 265 (1993) G699-G703.
125. R. Konno, K. Isobe, A. Niwa, Y. Yasumura, Excessive urinary excretion of methionine in mutant mice lacking D-amino-acid oxidase activity, Metabolism 37 (1988) 1139-1142.
126. M. Yohda, H. Okada, H. Kumagai, Molecular cloning and nucleotide sequencing of the aspartate racemase gene from lactic acid bacteria Streptococcus thermophilus, Biochim. Biophys. Acta 1089 (1991) 234-240.
127. M. Yohda, I. Endo, Y. Abe, T. Ohta, T. Iida, T. Maruyama, Y. Kagawa, Gene for aspartate racemase from the sulfur-dependent hyperthermophilic archaeum, Desulfurococcus strain SY, J. Biol Chem. 271 (1996) 22017-22021.
128. Z. Long, J.A. Lee, T. Okamoto, N. Nimura, K. Imai, H. Homma, d-Aspartate in a prolactin-secreting clonal strain of rat pituitary tumor cells (GH(3)), Biochem. Biophys. Res. Commun. 276 (2000) 1143-1147.
129. Z. Long, H. Homma, J.A. Lee, T. Fukushima, T. Santa, T. Iwatsubo, R. Yamada, K. Imai, Biosynthesis of D-aspartate in mammalian cells, FEBS Lett. 434 (1998) 231-235.
130. G.A. Hamilton, Peroxisomal oxidases and suggestions for the mechanism of action of insulin and other hormones, Adv. Enzymol. Relat Areas Mol. Biol 57 (1985) 85-178.
131. J.L. Still, M.V. Buell, W.E. Knox, D.E. Green, Studies on the cyclphorase system VII. D-Aspartate oxidase, J. Biol. Chem. 179 (1949) 831-837.
132. K. Zaar, H.P. Kost, A. Schad, A. Volkl, E. Baumgart, H.D. Fahimi, Cellular and subcellular distribution of D-aspartate oxidase in human and rat brain, J. Comp Neurol. 450 (2002) 272-282.
133. R. Yamada, H. Nagasaki, Y. Wakabayashi, A. Iwashima, Presence of D-aspartate oxidase in rat liver and mouse tissues, Biochim. Biophys. Acta 965 (1988) 202-205.
134. A. D'Aniello, G. D'Onofrio, M. Pischetola, G. D'Aniello, A. Vetere, L. Petrucelli, G.H. Fisher, Biological role of D-amino acid oxidase and D-aspartate oxidase. Effects of D-amino acids, J. Biol Chem. 268 (1993) 26941-26949.
135. H. Nagasaki, Gender-related differences of mouse liver D-aspartate oxidase in the activity and response to administration of D-aspartate and peroxisome proliferators, Int. J. Biochem. 26 (1994) 415-423.
136. P.P. Van Veldhoven, C. Brees, G.P. Mannaerts, D-aspartate oxidase, a peroxisomal enzyme in liver of rat and man, Biochim. Biophys. Acta 1073 (1991) 203-208.
137. R. Yamada, H. Nagasaki, Y. Nagata, Y. Wakabayashi, A. Iwashima, Administration of D-aspartate increases D-aspartate oxidase activity in mouse liver, Biochim. Biophys. Acta 990 (1989) 325-328.
138. G.E. Fagg, A. Matus, Selective association of N-methyl aspartate and quisqualate types of L-glutamate receptor with brain postsynaptic densities, Proc. Natl. Acad. Sci U. S. A 81 (1984) 6876-6880.
139. H.J. Olverman, A.W. Jones, K.N. Mewett, J.C. Watkins, Structure/activity relations of N-methyl-D-aspartate receptor ligands as studied by their inhibition of [3H]D-2-amino-5-phosphonopentanoic acid binding in rat brain membranes, Neuroscience 26 (1988) 17-31.

140. M.J. Schell, O.B. Cooper, S.H. Snyder, D-aspartate localizations imply neuronal and neuroendocrine roles, Proc. Natl. Acad. Sci U. S. A 94 (1997) 2013-2018.
141. G. D'Aniello, A. Tolino, A. D'Aniello, F. Errico, G.H. Fisher, M.M. Di Fiore, The role of D-aspartic acid and N-methyl-D-aspartic acid in the regulation of prolactin release, Endocrinology 141 (2000) 3862-3870.
142. S. Nakatsuka, M. Hayashi, A. Muroyama, M. Otsuka, S. Kozaki, H. Yamada, Y. Moriyama, D-Aspartate is stored in secretory granules and released through a Ca(2+)-dependent pathway in a subset of rat pheochromocytoma PC12 cells, J. Biol Chem. 276 (2001) 26589-26596.
143. J. Ferkany, J.T. Coyle, Heterogeneity of sodium-dependent excitatory amino acid uptake mechanisms in rat brain, J. Neurosci. Res. 16 (1986) 491-503.
144. J.T. Greenamyre, D.S. Higgins, A.B. Young, Sodium-dependent D-aspartate 'binding' is not a measure of presynaptic neuronal uptake sites in an autoradiographic assay, Brain Res. 511 (1990) 310-318.
145. J. Healy, A. Downes, G.J. McBean, Stimulation of synaptosomal D-[(3)H]aspartate transport by substance P in rat brain, Neurosci. Lett. 290 (2000) 113-116.
146. Y. Kanai, M.A. Hediger, Primary structure and functional characterization of a high-affinity glutamate transporter, Nature 360 (1992) 467-471.
147. A. D'Aniello, A. Di Cosmo, C. Di Cristo, L. Annunziato, L. Petrucelli, G. Fisher, Involvement of D-aspartic acid in the synthesis of testosterone in rat testes, Life Sci 59 (1996) 97-104.
148. Y. Nagata, H. Homma, J.A. Lee, K. Imai, D-Aspartate stimulation of testosterone synthesis in rat Leydig cells, FEBS Lett. 444 (1999) 160-164.
149. H. Wang, H. Wolosker, J. Pevsner, S.H. Snyder, D.J. Selkoe, Regulation of rat magnocellular neurosecretory system by D-aspartate: evidence for biological role(s) of a naturally occurring free D-amino acid in mammals, J. Endocrinol. 167 (2000) 247-252.
150. S. Ishio, H. Yamada, M. Hayashi, S. Yatsushiro, T. Noumi, A. Yamaguchi, Y. Moriyama, D-aspartate modulates melatonin synthesis in rat pinealocytes, Neurosci. Lett. 249 (1998) 143-146.
151. A. Saavedra-Molina, E. Pina, Stimulation of L-ornithine uptake and L-citrulline and urea biosynthesis by D-arginine, Biochem. Int. 24 (1991) 349-357.
152. G.H. Fisher, L. Petrucelli, C. Gardner, C. Emory, W.H. Frey, L. Amaducci, S. Sorbi, G. Sorrentino, M. Borghi, A. D'Aniello, Free D-amino acids in human cerebrospinal fluid of Alzheimer disease, multiple sclerosis, and healthy control subjects, Mol. Chem. Neuropathol. 23 (1994) 115-124.
153. A. D'Aniello, J.M. Lee, L. Petrucelli, M.M. Di Fiore, Regional decreases of free D-aspartate levels in Alzheimer's disease, Neurosci. Lett. 250 (1998) 131-134.
154. G.H. Fisher, A. D'Aniello, A. Vetere, L. Padula, G.P. Cusano, E.H. Man, Free D-aspartate and D-alanine in normal and Alzheimer brain, Brain Res. Bull. 26 (1991) 983-985.
155. A. D'Aniello, A. Vetere, G.H. Fisher, G. Cusano, M. Chavez, L. Petrucelli, Presence of D-alanine in proteins of normal and Alzheimer human brain, Brain Res. 592 (1992) 44-48.
156. M. Puka-Sundvall, M. Sandberg, H. Hagberg, Brain injury after hypoxia-ischemia in newborn rats: relationship to extracellular levels of excitatory amino acids and cysteine, Brain Res. 750 (1997) 325-328.
157. H. Bruckner, M. Hausch, Gas chromatographic characterization of free D-amino acids in the

blood serum of patients with renal disorders and of healthy volunteers, J. Chromatogr. 614 (1993) 7-17.

158. T. Fukushima, T. Santa, H. Homma, R. Nagatomo, K. Imai, Determination of D-amino acids in serum from patients with renal dysfunction, Biol Pharm. Bull. 18 (1995) 1130-1132.
159. Y. Nagata, T. Akino, K. Ohno, Y. Kataoka, T. Ueda, T. Sakurai, K. Shiroshita, T. Yasuda, Free D-amino acids in human plasma in relation to senescence and renal diseases, Clin. Sci (Lond) 73 (1987) 105-108.
160. C.E. Graham, S.W. Hier, H.K. Waitkoff, S.M. Saper, W.G. Bibler, E.I. Pentz, Studies on natural and racemic amino acids with rats, J. Biol Chem. 185 (1950) 102.
161. H.E. Sauberlich, Studies on the toxicity and antagonism of amino acids for weanling rats, J. Nutr. 75 (1961) 61-72.
162. K. Maruyama, M.L. Sunde, A.E. Harper, Effect of D-alanine and D-aspartic acid on the chick, J. Nutr. 102 (1972) 1441-1451.
163. A. Schieber, H. Bruckner, M. Rupp-Classen, W. Specht, S. Nowitzki-Grimm, H.G. Classen, Evaluation of D-amino acid levels in rat by gas chromatography-selected ion monitoring mass spectrometry: no evidence for subacute toxicity of orally fed D-proline and D-aspartic acid, J. Chromatogr. B Biomed. Sci Appl. 691 (1997) 1-12.
164. Y. Inoue, Y. Zama, M. Suzuki, «D-amino acids» as immunosuppressive agents, Jpn. J. Exp. Med. 51 (1981) 363-366.
165. M. Sela, E. Zisman, Different roles of D-amino acids in immune phenomena, FASEB J. 11 (1997) 449-456.
166. L.D. Stegman, H. Zheng, E.R. Neal, O. Ben Yoseph, L. Pollegioni, M.S. Pilone, B.D. Ross, Induction of cytotoxic oxidative stress by D-alanine in brain tumor cells expressing Rhodotorula gracilis D-amino acid oxidase: a cancer gene therapy strategy, Hum. Gene Ther. 9 (1998) 185-193.

D-serine and L-serine analysis

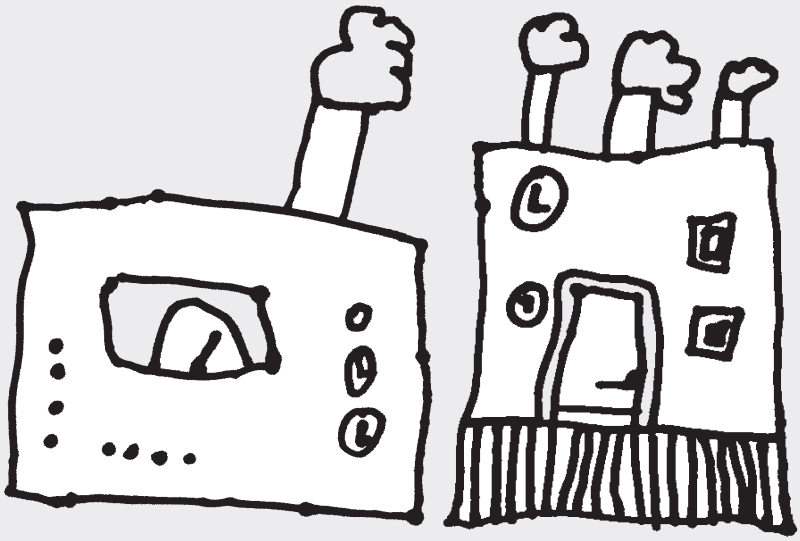


Chapter 2a

CSF serine enantiomers and glycine in the study of neurologic and psychiatric disorders

K. Hashimoto

Editorial in *Clin Chem* 2008;54:1413-14



It was long believed that only the L-isomer of amino acids existed in mammals, and D-amino acids were regarded as laboratory artefacts and categorized as "unnatural" isomers. This term was widely used in textbooks of biochemistry. D-Amino acids were known to be prominent in bacteria, and there were occasional reports of D-amino acids found in invertebrates.(1) Hans Krebs accidentally discovered in kidney tissue an enzyme, D-amino acid oxidase (DAAO), which recognized unnatural D-amino acids (but not their L-counterparts).(2) DAAO was found to degrade D-amino acids produced by bacteria from foods in the gut.

With the advance of chromatographic analysis techniques, small amounts of D-amino acids can now be measured in lower and higher animals, plants, and foods. By the use of 2-dimensional thin-layer chromatography and HPLC, Nagata et al. (3) found free D-amino acids, including D-serine, in kidney and blood of mutant mice lacking DAAO. Subsequently, Hashimoto and colleagues (4) demonstrated that D-serine was present in rat brain at high concentrations that were up to one-third those of L-serine, and that D-serine is heterogeneously distributed throughout rat brain with a pattern resembling that of the N-methyl D-aspartate (NMDA) subtype of glutamate receptors. Glutamate cannot activate the NMDA receptor in the absence of the coagonist glycine, and D-serine is up to 3 times more potent than glycine at the glycine site of the NMDA receptors. D-serine is likely to be the predominant endogenous ligand for the NMDA receptors in most areas of the brain. Although glycine serves this purpose in some sites, in most parts of the brain D-serine distribution matches that of NMDA receptors more than does glycine. (5) D-Serine is produced by serine racemase from L-serine in the brain, and D-serine is metabolized by DAAO.(5)

Column-switching HPLC with fluorescence detection (6) and HPLC with ultraviolet-visible detection (7) have been used for quantification of D- and L-serine. The former method includes precolumn fluorescence derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole and separation of the derivatives on a reversed-phase column and then on Sumichiral OA-2500(S) Pirkle-type columns.(6) In this issue of *Clinical Chemistry*, Fuchs et al. (8) report 2 novel mass-spectrometric techniques for separation and quantification of D- and L-serine. To enable simultaneous determination of D-serine, L-serine, and glycine in small volumes of biological fluids, these authors developed stable isotope dilution assays using GC-MS and LC-MS. The GC-MS system they used is a nonchiral derivatization with chiral (Chirasil-L-val column) separation, and the LC-MS system is a chiral derivatization with Marfeys reagent and LC-MS analysis. Quantification limits for D-serine, L-serine, and glycine in cerebrospinal fluid (CSF) from human sample donors were 0.14, 0.44 and 0.14 μmol/L (GC-MS) and 0.20, 0.41, and 0.14 μmol/L (LC-MS), respectively. The concentrations of D-serine and L-serine in human CSF were also consistent with those previously reported by other groups.(9, 10) Sample preparation time was 60 min for the LC-MS system, whereas it was approximately 6-8 h for the GC-MS

system. The relatively short sample preparation time indicates that the LC-MS system is superior to the GC-MS system in its potential to be automated and used to perform high-throughput analysis.

The ability to measure CSF amino acids raises the question of whether CSF D-serine, L-serine, and glycine have enough potential clinical utility to warrant ongoing study. In patients with 3-phosphoglycerate dehydrogenase deficiency (OMIM 606879), a rare disorder of L-serine biosynthesis, CSF concentrations of D-serine, L-serine, and glycine are much lower than those in controls.(11) In these patients postnatal L-serine supplementation normalized CSF D-serine, L-serine, and glycine as well as the clinical phenotype. Interestingly, in neonates whose mothers had undergone prenatal L-serine treatment, CSF concentrations of these amino acids at birth were near reference interval concentrations and the clinical phenotype was normal. Thus, it seems that L-serine biosynthesis, leading to D-serine synthesis and NMDA receptor activation, is crucial for early neuronal development in humans.(11) In the neonatal cerebellum of rodents, D-serine concentrations are high and peak at the time of granule cell migration, a finding that suggests that the principal role of D-serine in the developing cerebellum is to serve as a coagonist for NMDA receptor-dependent granule cell migration.(12) Taken together, these findings suggest a pivotal role of both isomers of serine in normal and aberrant human brain development.

Alterations in blood or CSF concentrations of D-serine and L-serine have been found in patients with schizophrenia,(9, 10, 13, 14) 3-phosphoglycerate-dehydrogenase deficiency,(11) and chronic pain.(7) Furthermore, we reported increased glutamine:glutamate ratios in CSF of first-episode and drug-naïve schizophrenic patients, suggesting that a dysfunction in the glutamate-glutamine cycle between neurons and glia may play a role in the pathophysiology of schizophrenia.(15) Thus the work from multiple groups has provided evidence that serine enantiomers play important roles in neurological and psychological diseases.

In their current report Fuchs et al. confirm their previous finding (11) that in humans CSF concentrations of D-serine, L-serine, and glycine decrease with age. This process was most pronounced for D-serine concentrations which decreased 4.9-fold (GC-MS) or 9.0-fold (LC-MS) during the first 3 years of life and remained constant thereafter. (8) The age-dependent changes in CSF amino acids illustrate the potential need for differing interpretations of neonatal, prenatal, adolescent, and adult CSF concentrations. Fortunately the novel methods reported here (8) enable such differentiation and should be valuable tools for continued evaluation of the role of amino acids, including D-serine and L-serine, in investigating both normal brain function and the pathophysiology of various neuropsychiatric disorders.

REFERENCES

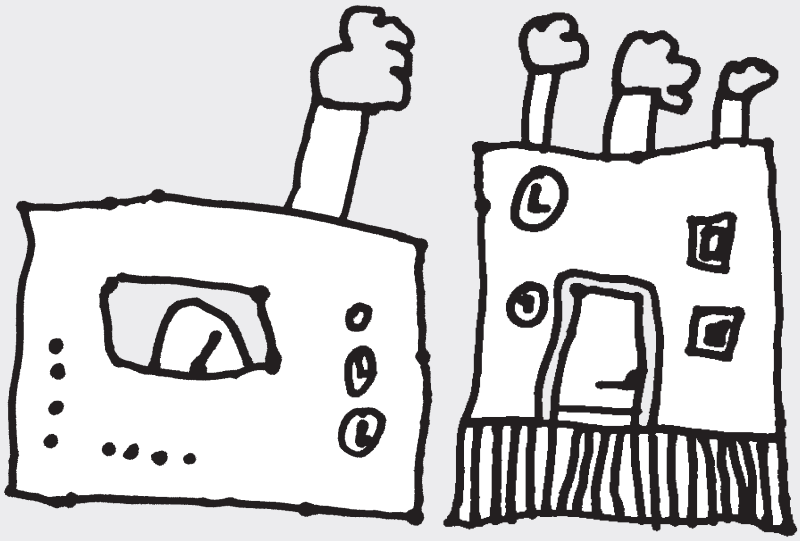
1. Corrigan JJ 1969 D-amino acids in animals. Science 164:142-149
2. Krebs HA 1948 The D- and L-amino-acid oxidases. Biochem Soc Symp 1:2-18
3. Nagata Y, Yamamoto K, Shimojo T, Konno R, Yasumura Y, Akino T 1992 The presence of free D-alanine, D-proline and D-serine in mice. Biochim Biophys Acta 1115:208-211
4. Hashimoto A, Nishikawa T, Oka T, Takahashi K 1993 Endogenous D-serine in rat brain: N-methyl-D-aspartate receptor-related distribution and aging. J Neurochem 60:783-786
5. Schell MJ 2004 The N-methyl D-aspartate receptor glycine site and D-serine metabolism: an evolutionary perspective. Philos Trans R Soc Lond B Biol Sci 359:943-964
6. Fukushima T, Kawai J, Imai K, Toyo'oka T 2004 Simultaneous determination of D- and L-serine in rat brain microdialysis sample using a column-switching HPLC with fluorimetric detection. Biomed Chromatogr 18:813-819
7. Sethuraman R, Krishnamoorthy MG, Lee TL, Liu EH, Chiang S, Nishimura W, Sakai M, Minami T, Tachibana S 2007 Simultaneous analysis of D- and L-serine in cerebrospinal fluid by use of HPLC. Clin Chem 53:1489-1494
8. Fuchs SA, de Sain-van der Velden MG, de Barse MM, Roeleveld MW, Hendriks M, Dorland L, Klomp LW, Berger R, de Koning TJ 2008 Two mass-spectrometric techniques for quantifying serine enantiomers and glycine in cerebrospinal fluid: potential confounders and age-dependent ranges. Clin Chem 54:1443-1450
9. Bendikov I, Nadri C, Amar S, Panizzutti R, De MJ, Wolosker H, Agam G 2007 A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. Schizophr Res 90:41-51
10. Hashimoto K, Engberg G, Shimizu E, Nordin C, Lindstrom LH, Iyo M 2005 Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naive schizophrenic patients. Prog Neuropsychopharmacol Biol Psychiatry 29:767-769
11. Fuchs SA, Dorland L, de Sain-van der Velden MG, Hendriks M, Klomp LW, Berger R, de Koning TJ 2006 D-serine in the developing human central nervous system. Ann Neurol 60:476-480
12. Kim PM, Aizawa H, Kim PS, Huang AS, Wickramasinghe SR, Kashani AH, Barrow RK, Huganir RL, Ghosh A, Snyder SH 2005 Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. Proc Natl Acad Sci U S A 102:2105-2110
13. Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, Nakazato M, Kumakiri C, Okada S, Hasegawa H, Imai K, Iyo M 2003 Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. Arch Gen Psychiatry 60:572-576
14. Yamada K, Ohnishi T, Hashimoto K, Ohba H, Iwayama-Shigeno Y, Toyoshima M, Okuno A, Takao H, Toyota T, Minabe Y, Nakamura K, Shimizu E, Itokawa M, Mori N, Iyo M, Yoshikawa T 2005 Identification of multiple serine racemase (SRR) mRNA isoforms and genetic analyses of SRR and DAO in schizophrenia and D-serine levels. Biol Psychiatry 57:1493-1503
15. Hashimoto K, Engberg G, Shimizu E, Nordin C, Lindstrom LH, Iyo M 2005 Elevated glutamine/glutamate ratio in cerebrospinal fluid of first episode and drug naive schizophrenic patients. BMC Psychiatry 5:6

Chapter 2b

Two mass-spectrometric techniques for quantifying serine enantiomers and glycine in cerebrospinal fluid: potential confounders and age-dependent ranges

S.A. Fuchs, M.G.M. de Sain-van der Velden, M.M.J. de Barse, M.W. Roeleveld, M. Hendriks, L. Dorland, L.W.J. Klomp, R. Berger, T.J. de Koning

Clin Chem 2008;54:1143-50



ABSTRACT

Background

The recent discovery and specific functions of D-amino acids in humans are bound to lead to the revelation of D-amino acid abnormalities in human disorders. Therefore, high-throughput analysis techniques are warranted to determine D-amino acids in biological fluids in a routine laboratory setting.

Methods

We developed 2 chromatographic techniques, a nonchiral derivatization with chiral (chirasil-L-val column) separation in a GC-MS system and a chiral derivatization with Marfey's reagent and LC-MS analysis. We validated the techniques for D-serine, L-serine and glycine determination in cerebrospinal fluid (CSF), evaluated several confounders, and determined age-dependent human concentration ranges.

Results

Quantification limits for D-serine, L-serine and glycine in CSF were 0.14, 0.44 and 0.14 µmol/L, respectively, for GC-MS and 0.20, 0.41 and 0.14 µmol/L for LC-MS. Within-run imprecision was <3% for both methods and between-run imprecision was <13%. Comparison of both techniques with Deming regression yielded coefficients of 0.90 (D-serine), 0.92 (L-serine) and 0.96 (glycine). Sample collection, handling and transport is uncomplicated - there is no rostrocaudal CSF gradient, no effect of storage at 4°C for 1 week before storage at -80°C, and no effect of up to 3 freeze/thaw cycles. Conversely, contamination with erythrocytes increased D-serine, L-serine and glycine concentrations. CSF concentrations for 145 apparently healthy controls demonstrated markedly and specifically increased (5 to 9 times) D-serine concentrations during early central nervous system development.

Conclusions

These 2 clinically applicable analysis techniques will help to unravel pathophysiologic, diagnostic, and therapeutic issues for disorders associated with central nervous system abnormalities, NMDA-receptor dysfunction and other pathology associated with D-amino acids.

INTRODUCTION

Amino acids are essential molecules for all living beings. All amino acids except glycine occur in an L- and a D-form, depending on the tetrahedral configuration around the chiral center on the α-carbon atom. Incorporation of a particular amino acid enantiomer in proteins or (poly)peptides determines the spatial architecture of these biological polymers and hereby plays a major role in enzymatic specificity and structural interactions. (1) Consequently, it was assumed that homochirality evolved in nature, with all living organisms being composed only of L-amino acids.(2)

With the advance of chromatographic analysis techniques, improved determination of the different amino acid enantiomers was achieved, revealing the unexpected but undeniable presence of small quantities of D-amino acids in lower and higher animals, plants and foods. (1) Research has largely focused on the D-amino acid D-serine, which has been identified in surprisingly high concentrations in the mammalian central nervous system.(3) Subsequent studies demonstrated endogenous D-serine metabolism and synthesis from L-serine.(4) Like glycine, D-serine functions as a neuromodulator through binding to the N-methyl D-aspartate (NMDA) excitatory amino acid receptor.(5) NMDA-receptors are involved in central nervous system development, brain plasticity, memory and learning. NMDA-receptor dysfunction has been implicated in various pathological conditions, including schizophrenia, epilepsy, stroke and neurodegenerative conditions.(6) Recently, we reported that D-serine might be essential for human central nervous system development and provided the first example of human D-serine deficiency in patients with 3-phosphoglycerate dehydrogenase deficiency.(7) Other studies have implicated altered D-serine concentrations in schizophrenia (8, 9) and amyotrophic lateral sclerosis,(10) and nociception.(11) Together, these studies imply that D-serine is important in human physiology and pathology.

The presence and possible important roles of D-amino acids in higher organisms (12) not only challenge former theories on mammalian physiology, but also contribute to new insights in disease and treatment modalities. Therefore, it is essential to be able to study D-amino acids in biological samples in a clinical setting. Despite technological advances in the analysis of chiral compounds (for a comparison of the different techniques, we refer to reviews on this subject (13-16)), analysis of D-amino acids in biological samples remains challenging, because of interference from high concentrations of L-amino acids and biological substances such as peptides and amines. To enable sensitive and accurate simultaneous determination of different D- and L-amino acids in small volumes of biological fluids, we optimized 2 stable isotope dilution analysis techniques (gas chromatography–mass spectrometry (GC-MS) (17) and liquid chromatography-mass spectrometry (LC-MS) (18) for a clinical laboratory setting. We focused on the simultaneous determination of D-serine, L-serine, and glycine concentrations in human cerebrospinal fluid (CSF), because of their mutual involvement in glutamatergic neurotransmission.

MATERIALS AND METHODS

Materials

We purchased acetylchloride, 2-propanol, D-serine, Nα-2,4-dinitro-5-fluorophenyl-L-alaninamide (Marfey's reagent), and ammoniumformiate from Sigma-Aldrich Inc.; chloroform, 5-sulfosalicylic acid, L-serine, glycine, di-sodiumtetraborate.10H_2O, formic acid and hydrochloric acid from Merck; pentafluoropropionic anhydride from Pierce; acetonitrile from Rathburn Chemicals Ltd.; acetone from Fluka Chemicals Ltd.; and labeled stable isotopes 3-^{13}C(99%)DL-serine and 1,2-$^{13}C_2$(99%)glycine from Cambridge Isotope Laboratories, Inc. All chemicals were of guaranteed grade.

CSF Samples

For validation studies and evaluation of potential confounding factors, we procured remnants of CSF samples from the laboratory of metabolic and endocrine diseases, the laboratory of microbiology, and the neonatal care unit of our university hospital.
D-serine concentrations were determined in human CSF samples, sent to the laboratory of microbiology in our hospital from July 2004 to November 2007 to exclude meningitis. To avoid interference with the normal routine in the laboratory of microbiology, these samples were kept at 4°C for 1 week, before being stored at -80°C (no difference with our normal routine of immediate storage at -80°C, see Supplemental Data Table 1). We excluded samples with more than 100 erythrocytes/mL, meningitis, HIV, intracranial pathology, perinatal asphyxia, serine biosynthesis disorders, epilepsy, schizophrenia and neurodegenerative disorders. Sample use for our studies was approved by the medical ethics committee of the University Medical Center Utrecht.

Quality control samples

CSF Samples from the neonatal care unit were pooled. To simulate different clinically relevant concentrations, samples of 100, 200 and 300µL (QC1a-c) were derivatized following the normal GC-MS sample preparation procedure described below, which includes drying of the solution after internal standard addition and derivatization of the complete residue. Compared with the 200µL sample, the 100µL and the 300µL samples simulated concentrations that are 0.5 or 1.5 times the concentrations of the pooled CSF batch, respectively. For recovery analysis, CSF from the laboratory of metabolic and endocrine diseases was pooled (QC2) and spiked with aqueous amino acid solutions, theoretically increasing L-serine concentrations with 33.33µmol/L and D-serine and glycine concentrations with 6.66µmol/L, thus doubling the expected concentrations (QC3). Similarly,

we prepared QC4 samples by spiking pooled CSF with aqueous solutions of 15µmol/L D-serine, L-serine and glycine (QC4).

GC-MS analysis

Sample preparation procedure

CSF Samples were thawed at room temperature and derivatized according to the method described by Bruckner et al.(17) After stirring, we transferred 200µL CSF to 1.5 mL microtubes (Sarstedt) and added 50µL of internal standard solution (aqueous solution of 600µmol/L 3-^{13}C-DL-serine and 60µmol/L 1,2-$^{13}C_2$-glycine). The samples were deproteinized by adding 200µL of aqueous 5-sulfosalicylic acid, thoroughly mixing and centrifuging in a Heraeus Biofuge Pico centrifuge (Dijkstra Vereenigde BV) at 10.000tours/min for 4 minutes at room temperature. We applied the supernatant to Durapole microfilters (0.22µm; Millipore) to eliminate remaining (oligo-)peptides and transferred the filtrate to Pyrex culture tubes with screw caps and PTFE-faced rubber lining (Sigma-Aldrich Inc.). The solvents were removed in a nitrogen stream at 40°C. We added 250µL of 2.5mol/L HCl in 2-propanol (acetyl chloride in 2-propanol 1:4 v/v) to the dry residue. After heating for 45 minutes at 70°C in heating blocks, reagents were removed in a nitrogen stream at ambient temperature. We added 400µL chloroform and 100µL pentafluoropropionic anhydride and heated the mixture at 100°C for 20 minutes. Reagents were removed in a nitrogen stream at ambient temperature. We dissolved the residues in 50µL chloroform and subjected 2µL to GC-MS. For each analysis run, a 7-point calibration curve was prepared with aqueous solutions of L-serine (0-112.5µmol/L), D-serine and glycine (0-26.25µmol/L).

Chromatographic conditions

The GC comprised a HP-5890 gas chromatograph and a HP-7673 automatic sampler (Agilent Technologies Netherlands BV). The derivatized amino acids were delivered by automatic injection (split injection port 1:20) over a glass wool liner to the WCOT fused-silica CP chirasil-L-val (*N*-propionyl-L-valine *tert*-butylamide polysiloxane) capillary column (25mx0.25mm internal diameter; 0.12m film thickness; Chrompack). Helium was used as carrier gas (1mL/min), with automatic pressure adaptation. The temperature program started at 80°C (3 min), increased with 3°C/min to 190°C, and held at 190°C for 5 minutes. The injector and detector temperatures were set at 220°C.

Mass spectrometric conditions

In the quadrupole HP-5989B mass spectrometer (Agilent Technologies), the eluted derivatized amino acids were ionized by negative chemical ionization using 5% ammonia in methane (NTG). The ion source and the quadrupole temperatures were set at 250°C

and 150°C respectively, according to the manufacturers' protocol. The MS was run in the selected ion monitoring mode (SIM). The appropriate ion sets were selected, using the following characteristic mass fragments (*m/z*) of the *N(O)* pentafluoropropionyl-2-propanol esters of the amino acids: serine (*m/z* 255), ^{13}C-serine (*m/z* 256), glycine (*m/z* 243), $^{13}C_2$-glycine (*m/z* 245). GC-MS control and data processing were performed with HP G1034C and G1710BA MS Chemstation software respectively (Agilent Technologies).

LC-MS analysis

Sample preparation procedure

CSF Samples were thawed at room temperature and derivatized according to the method described by Goodlett et al. (18) and Berna and Ackermann.(19) We added 50µL internal standard solution (600.5µmol/L 3-^{13}C-DL-serine and 61µmol/L 1,2-$^{13}C_2$-glycine in 0.1mol/L HCl) to 100µL CSF. On drying in a nitrogen stream, the residues were derivatized with 50µL 0.5% Marfey's reagent (wt/vol in acetone) and 100µL 0.125mol/L di-sodiumtetraborate.10H_2O during 30 minutes at 40°C. The reaction was stopped with 25µL 4mol/L HCl. We diluted the resulting solution (1:10) with eluent-buffer (250mg ammonium formiate in 1L milliQ-water; pH adjusted to 4.6 with formic acid) and subjected 10µL to LC-MS. For each analytical run, we prepared a 6-point calibration curve with aqueous solutions of L-serine (0-100µmol/L), D-serine, and glycine (0-20µmol/L).

Chromatographic conditions

The derivatized amino acids were resolved on an Alliance 2795 HPLC system (Waters), with separation on an Atlantis dC18 analytical column (3µm, 3.9mmx150mm) (Waters), using a linear gradient of 100% mobile phase A (250mg ammonium formiate in 1L milliQ-water; pH adjusted to 4.6 with formic acid) to 50% mobile phase A and B (acetonitrile) in 15 minutes. The flow rate was 0.3mL/min.

Mass spectrometric conditions

A Quattro Ultima triple quadrupole mass spectrometer (Waters) was used in the negative electron spray ionization (ESI) mode. The following mass spectrometer settings were used: capillary voltage 3.0kV, cone voltage 40V, cone gas flow 185l/hr, desolvation gas flow 677l/hr, collision gas pressure 1.33e-3 and source temperature 150°C. The appropriate ion sets were selected, using the following characteristic mass fragments *(m/z)* of the dinitrophenyl-L-alanine-amides of the amino acids: serine (*m/z* 356.1), ^{13}C-serine (*m/z* 357.1), glycine (*m/z* 326.1) and $^{13}C_2$-glycine (*m/z* 328.1). Masslynx software, which included Quanlynx (Waters), was used for instrument control, data acquisition and data processing.

Validation studies

For both analysis techniques, validation was performed in accordance with the guidelines from the European Committee for Clinical Laboratory Standards,(20) using CSF samples and the lowest standards of the calibration curves to assess the limit of detection (LOD, concentration at a signal-to-noise ratio=3, n=10) and limit of quantification (LOQ, concentration at a signal-to-noise ratio=10, n=10). We used quality control samples to assess within-run (n=10 of QC1a-c, QC2 and QC3) and between-run (n=10 of QC1a-c, QC2 and Q3) imprecision. We analyzed recovery in QC3 and QC4 samples (n=10), assessed linearity and range of detection in CSF (LOQ to the highest concentration of the calibration curve (r^2>0.99)+10%) for D-serine, L-serine, and glycine and compared the results obtained with the 2 new analysis techniques (n=68). Stability of samples prepared for LC-MS analysis was assessed up to 13 days.

Influence of possible confounding factors

We evaluated the effect of CSF fraction on amino acid concentrations (GC-MS), using different CSF fractions from the same lumbar puncture. These samples were available after diagnostic CSF neurotransmitter analyses, for which CSF was divided in 6 consecutive withdrawal portions: 1)0.5mL directly stored at -80°C; 2)2mL stored at 4°C until analysis, thereafter storage at -80°C; 3)1.5mL directly stored at -80°C; 4)1mL stored at 4°C until analysis, thereafter storage at -80°C; 5)1-2mL stored at 4°C until analysis, thereafter storage at -80°C; 6)1-2mL directly stored at -80°C. We tested the influence of repetitive freeze and thaw cycles by analyzing (LC-MS) 5 different CSF samples upon 1,2 and 3 freeze/thaw cycles (5 samples are required to detect a 15% difference using a technique with a 7% imprecision with a power of 0.8, α=0.05, β=0.1). Finally, we assessed the effect of CSF contamination with blood (>200 erythrocytes/mL) (GC-MS) by comparing concentrations within age groups with CSF containing virtually no blood (<100 erythrocytes/mL).

Statistical analysis

We calculated amino acid concentrations after linear regression analysis of the calibration curve (SigmaStat 3.0), using peak area ratios of the amino acids to their internal standards. We compared the GC-MS and LC-MS techniques using Deming regression, evaluated the influence of storage at 4°C before storage at -80°C with a paired 2-tailed Student *t* test, evaluated the influence of erythrocyte contamination within the corresponding age group with an unpaired 2-tailed Student *t* test, and analyzed CSF fraction and freeze-thaw data with a repeated measurements model. The level of significance

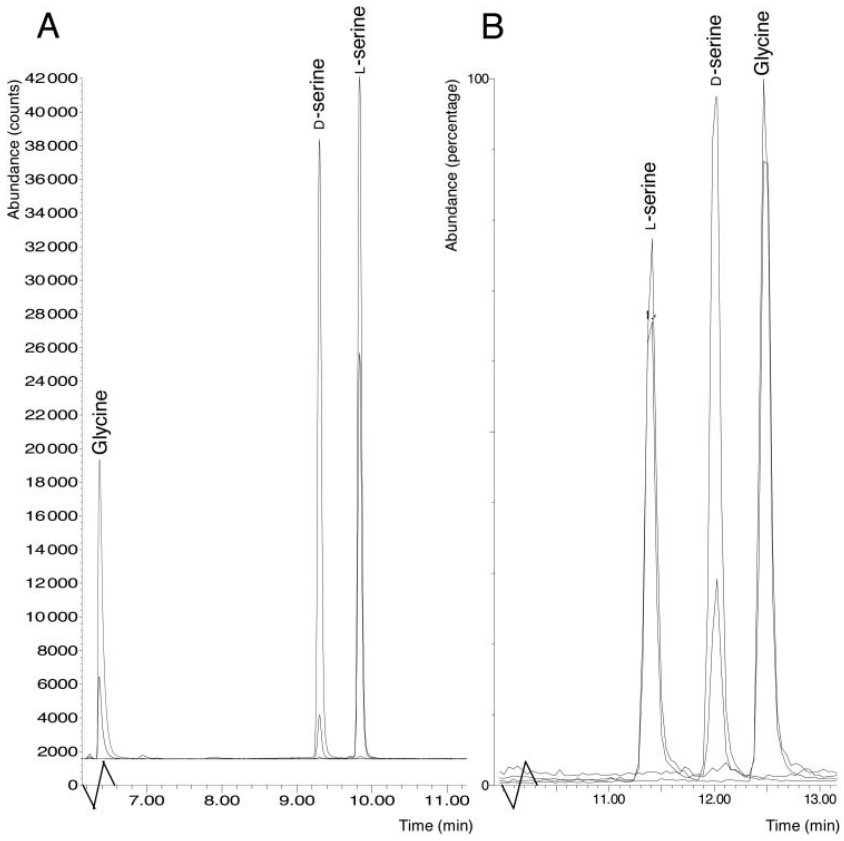
was set at p=0.05. CSF concentrations were categorized by age. For each group the median and confidence interval were determined.

RESULTS

The GC-MS and LC-MS analysis techniques

Both the GC-MS and LC-MS techniques yielded baseline separation of amino acid enantiomers (Figure 1). Whereas GC-MS sample preparation comprised approximately 6-8 hours, LC-MS sample preparation only took 60 minutes. Elution time was similar (GC-MS:6-12 minutes; LC-MS:12-14 minutes).

Figure 1. Chromatographs of D-serine, L-serine, and glycine using GC-MS (A) and LC-MS (B).



The chromatographs represent patient CSF samples, with the internal standard peaks (higher peaks) and endogenous peaks (lower peaks) overlain in the same figure. The x-axis shows the retention time in minutes, the y-axis the abundance in counts (A) or percentage (B; 100% in this case representing 5.89e5 counts).

Validation studies

Both techniques enabled the determination of low D-serine, L-serine, and glycine concentrations in CSF, with good recovery and a range of detection (Table 1) amply covering the physiological range (Table 2). The calibration curves for both methods were reproducible and linear over the range tested (r^2>0.99, Table 1; based on previous GC-MS analyses showing physiological D-serine concentrations varying between 2-14µmol/L, linearity for the LC-MS was only tested up to a concentration of 30µmol/L). Furthermore, analysis with both methods was reproducible, (within-run imprecision<3%, between-run imprecision<13%) (Table 1). Analysis 13 days after sample preparation for LC-MS did not influence D-serine, L-serine, or glycine concentrations (D-serine 8.0µmol/L, after 13 days 8.8µmol/L (equaling 2 times the between-run imprecision of 4.7%), L-serine 46.1µmol/L, after 13 days 48.0µmol/L (within 2 times the between-run imprecision of 5.7%), and glycine 7.4µmol/L, after 13 days: 7.3µmol/L (within 2 times the between-run imprecision of 6.0%)).
GC-MS analysis yielded on average 10% higher D-serine (concentration range tested: 0.04-39.5µmol/L), 8% higher L-serine (10.4-129.6µmol/L) and 4% higher glycine concentrations (4.0-101.5µmol/L) than LC-MS analysis (all within the imprecision resulting from the between-run reproducibility of both analysis techniques for the respective amino acids).

Table 1. Validation studies for the GC-MS and LC-MS technique

	GC-MS			LC-MS		
	D-serine	L-serine	glycine	D-serine	L-serine	glycine
LOD (µmol/L) in CSF	0.04	0.13	0.04	0.06	0.12	0.04
in aqueous solution	0.11	0.10	0.04	0.18	0.32	0.14
LOQ (µmol/L) in CSF	0.14	0.44	0.14	0.20	0.41	0.14
in aqueous solution	0.35	0.34	0.13	0.60	1.07	0.46
Within-run imprecision (%)[a]	2.56	2.88	1.33	0.27	0.60	1.85
Between-run imprecision (%)[a]	12.69	3.09	7.14	4.73	5.68	6.01
Recovery	1.04	0.89	0.96	1.11	1.02	0.97
Range of detection (µmol/L)	0.4-125	0.3-125	0.1-30	0.6-22	1.1-110	0.5-22
Linearity of calibration curve	r^2=0.9967	r^2=0.9963	r^2=0.9990	r^2=0.9983	r^2=0.9990	r^2=0.9953
Slope	0.0099	0.0101	0.0645	0.0080	0.0065	0.0289
Intercept	0.1062	0.1125	0.0573	0.0028	0.0105	0.0152
Comparison LC-MS with GC-MS (95%CI of the proportional bias)	D-serine y=0.90x-0.08 (0.84-0.96)		L-serine y=0.92x-0.86 (0.82-1.02)		glycine y=0.96x-0.37 (0.92-1.00)	

The table represents the limit of detection (LOD, n=10), limit of quantification (LOQ, n=10), within-run imprecision (GC-MS: n=10 (QC1a-c), LC-MS: n=3 (QC2, QC3), between-run imprecision (n=10, QC1a-c, QC2, QC3), recovery (n=10, GC-MS (QC1, QC4), LC-MS (QC2, QC3), range of detection (LOQ - highest concentration of the calibration curve (r^2>0.99)+10%), linearity of the calibration curve (n=10 for r^2, slope and intercept) and comparison of LC-MS with GC-MS (n=68) by Deming regression (CI=Confidence Interval). [a]Only values for QC1b and QC2 are presented here; values for QC1a and c and QC3 were similar.

Table 2. CSF D-serine, L-serine, and glycine concentrations in different age groups

A

GC-MS	N	D-serine (µmol/L)	L-serine (µmol/L)	Glycine (µmol/L)
0 - 3 months	4 (m:2, f:2)	13.7 (4.6 – 33.9)	60.9 (37.3 – 95.5)	12.1 (5.3 – 24.4)
3 months - 3 years	5 (m:1, f:4)	7.1 (3.5 – 13.0)	53.6 (28.0 – 95.4)	7.6 (3.8 – 14.1)
3 - 16 years	5 (m:2, f:3)	2.6 (1.8 – 3.7)	39.1 (28.8 – 52.2)	6.6 (3.7 – 11.2)
16 - 50 years	10 (m:3, f:7)	2.4 (0.6 – 7.3)	32.7 (26.1 – 40.5)	8.3 (4.0 – 15.3)
50+ years	22 (m:12, f:10)	2.8 (0.4 – 11.7)	37.4 (20.4 – 63.4)	9.5 (5.0 – 16.3)

B

LC-MS	N	D-serine (µmol/L)	L-serine (µmol/L)	Glycine (µmol/L)
0 - 1 month	13 (m:5, f:8)	13.5 (9.4 - 19.4)	43.6 (32.4 – 58.9)	6.9 (2.9-16.3)
1 month - 3 months	9 (m:5, f:4)	8.4 (5.2 – 13.6)	32.4 (19.9 – 52.8)	6.8 (4.6 – 9.9)
3 months - 3 years	17 (m:11, f:6)	5.0 (2.9 – 8.7)	34.0 (23.7 – 48.8)	5.6 (1.5 – 21.0)
3 - 16 years	13 (m:9, f:4)	1.9 (0.8 – 4.3)	27.5 (17.2 – 44.0)	6.2 (1.8 – 21.0)
16 - 50 years	21 (m:6, f:15)	1.4 (0.4 – 4.9)	23.0 (11.3 – 46.9)	5.7 (1.5 – 21.7)
50+ years	26 (m:12, f:14)	1.5 (0.4 – 5.7)	22.7 (11.6 – 44.2)	8.4 (2.4 – 29.9)

Amino acid concentrations were determined by GC-MS (A) and LC-MS (B) and are expressed as median in µmol/L (confidence interval) for different age groups because of their non-normal distribution within age groups. N= number of subjects (m=males, f=females).

Influence of possible confounding factors

We evaluated possible clinically relevant confounders by GC-MS and LC-MS. The effect of a rostrocaudal gradient was assessed in CSF from 10 patients from whom 2 (n=8) or 3 (n=2) fractions from the same lumbar puncture were available. Fraction analysis did not yield significant differences between any fraction for any amino acid of interest (See Supplemental Data Table 2). Similarly, up to 3 repetitive freeze/thaw cycles did not affect D-serine, L-serine, or glycine concentrations (freeze/thaw standard error of the mean in the same order of magnitude as the reproducibility standard error of the mean, See Supplemental Data Table 3). We evaluated the effect of traumatic punctures by comparing 25 samples containing >200 erythrocytes/mL with 39 samples containing <100 erythrocytes/mL within the corresponding age group. Contamination with erythrocytes increased D-serine, L-serine, and glycine concentrations significantly in all age groups containing more than 5 samples, except for L-serine concentrations in the age group 3-32 years (Table 3).

Table 3. Influence of erythrocyte contamination on D-serine, L-serine, and glycine concentrations

	Erythrocytes<100		Erythrocytes>200		Student *t* test
D-serine	N	Median (µmol/L)	N	Median (µmol/L)	(p-value)
0-100 days	9	20.8	9	35.5	0.05*
100 days - 1 year	7	6.6	2	17.4	<0.01*
1-3 years	11	7.9	3	6.5	0.37
3-32 years	12	2.9	11	5.5	<0.01*
L-serine					
0-100 days	9	74.6	9	123.2	0.05*
100 days - 1 year	7	61.0	2	70.4	0.46
1-3 years	11	52.0	3	59.0	0.81
3-32 years	12	39.8	11	46.0	0.10
Glycine					
0-100 days	6	9.7	9	22.1	<0.01*
100 days - 1 year	5	10.0	2	19.5	0.05*
1-3 years	9	9.5	3	9.9	0.69
3-32 years	11	6.7	11	10.7	<0.01*

Concentrations of D-serine, L-serine, and glycine (µmol/L) were measured by GC-MS in CSF samples with <100 erythrocytes/mL (n=39) and >200 erythrocytes/mL (n=25, mean number of erythrocytes:6270, range:299-49000). The natural logarithms of the individual values (non-normal distribution) were compared within the corresponding age group using the unpaired 2-tailed Student *t* test, the level of significance set at p=0.05. *=significant difference, N=number of subjects.

CSF concentrations in apparently healthy controls

We measured D-serine, L-serine and glycine concentrations in 145 CSF samples, of which 46 were analyzed by GC-MS and 99 by LC-MS (Table 2). Amino acid concentrations decreased with age, which was most pronounced for D-serine, decreasing 4.9-fold (GC-MS) or 9.0-fold (LC-MS) during the first 3 years of life and remaining constant thereafter. During this same period, L-serine concentrations decreased 1.6-fold (GC-MS) or 1.9-fold (LC-MS), and neonatal glycine concentrations decreased 1.8-fold (GC-MS) or 1.2-fold (LC-MS) to the lowest concentrations in childhood, to increase 1.4-fold (GC-MS) or 1.5-fold (LC-MS) to values >50 years of age.

DISCUSSION

The low LOQs of both methods enable analysis of very small CSF samples. Of further importance for general clinical practice is that CSF can be stored at 4°C for at least a week before analysis, thereby facilitating transport and handling of samples. This concurs with previous studies showing that glycine was stable in CSF for the complete study duration of 8 hours at room temperature and for at least 30 days when stored at -20 or -80°C.(21) Similarly, alanine, a neutral amino acid like glycine and serine, was stable in

CSF at room temperature for at least 72 hours.(22) For longer storage or analysis of less stable amino acids, such as glutamine or asparagine, more stringent storage conditions (-80°C) are advisable.(21, 22) Once prepared for LC-MS analysis, samples were stable for at least 13 days, allowing analysis of long runs and preparation of samples up to 2 weeks before analysis.

Because a CSF rostrocaudal concentration gradient appears to exist for monoamine metabolites,(23, 24) we evaluated the effect of CSF fraction on amino acid concentrations. These data show that any CSF fraction may be used for D-serine, L-serine, and glycine analysis. This finding replicates the absence of a rostrocaudal glycine CSF gradient in the only other study on amino acid CSF gradients we identified.(25) Another possible confounding factor was repetitive freeze/thaw cycles, which might occur in clinical practice, for example upon diagnostic reevaluation, analytical problems or sample transport. Up to 3 freeze/thaw cycles did not influence D-serine, L-serine, and glycine concentrations. However, caution remains warranted after repetitive freezing and thawing when analyzing less stable amino acids, such as glutamine or asparagine. Finally, as might be expected because serum amino acids usually greatly exceed CSF amino acid concentrations,(26) traumatic punctures (>100 erythrocytes/mL) should be either dismissed or mathematically corrected for erythrocyte content, as is done for other blood constituents such as leukocytes or protein.(27)

The LC-MS technique has several advantages. LC-MS sample preparation was definitely less laborious and time-consuming, and theoretically can be automated and performed in 96-wells plates. Separation is achieved on a nonchiral column, concomitantly applicable for a wide variety of polar compounds. Furthermore, the relatively short LC-MS derivatization reaction time in alkaline solution at 40°C might facilitate simultaneous determination of a variety of chiral amino acids, including glutamate and aspartate. These dicarboxylic amino acids are important excitatory amino acids in human physiology and pathology,(28) but are difficult to quantify accurately,(29) because glutamine and asparagine, present in high concentrations in CSF, are very prone to conversion to glutamate and aspartate, respectively. This problem was not encompassed by a milder propylation step in GC-MS sample preparation (data not shown). However, in laboratories without LC-MS, the GC-MS method is a very suitable alternative.

CSF D-serine, L-serine, and glycine concentrations were measured in a human population of varying ages using the 2 different analytical techniques. Because of differences in control sample sizes, we were unable to directly compare values between these methods. Nevertheless, in the validation studies, we showed good correlation between the 2 techniques. Because some deviations are inherent to the use of different analysis techniques, it is advisable for clinical practice to refer to values that have been generated by the same technique. The decrease in concentrations with age underscores the importance of age-specific reference intervals. The median values of the D-serine and

L-serine concentrations we observed here are somewhat lower than those we reported previously.(7) This might be explained by the different and much larger population we studied here. Nevertheless, we were able to replicate our previous finding of 7-fold increased D-serine concentrations during the first few months of life.(7) In addition, the values determined by both techniques correlate well with the recently published values of total serine and glycine in CSF of 77 infants (serine, median 52µmol/L, range 25-105µmol/L; glycine, median 9µmol/L, range 3-19µmol/L) (30) and also with older studies.(31) To our knowledge, age-dependent reference intervals for the separate isomers are not available for comparison with our results. In our previous study,(7) we showed D-serine deficiency in patients with 3-phosphoglycerate dehydrogenase deficiency, illustrating that our analysis technique differentiates between physiology and pathology.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from The Netherlands Organization for Health Research and Development (to S.A.F: 920-03-345). We are indebted to A. Uyttewaal (Department of Metabolic and Endocrine diseases, University Medical Center Utrecht), the laboratory of microbiology, the laboratory of metabolic and endocrine diseases and the neonatal care unit of the University Medical Center Utrecht for their collaboration in cerebrospinal fluid collection and to dr. E. Struys (Department of Clinical Chemistry, VU University Medical Center, Amsterdam) for directing our attention to the HPLC analysis method using Marfey's reagent.

REFERENCES

1. Corrigan JJ 1969 D-amino acids in animals. Science 164:142-149
2. Lamzin VS, Dauter Z, Wilson KS 1995 How nature deals with stereoisomers. Curr Opin Struct Biol 5:830-836
3. Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T, Takahashi K 1992 The presence of free D-serine in rat brain. FEBS Lett 296:33-36
4. Wolosker H, Blackshaw S, Snyder SH 1999 Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. Proc Natl Acad Sci U S A 96:13409-13414
5. Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD, Rogawski MA, Snyder SH 2000 D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 97:4926-4931
6. Danysz W, Parsons AC 1998 Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol Rev 50:597-664
7. Fuchs SA, Dorland L, de Sain-van der Velden MG, Hendriks M, Klomp LW, Berger R, de Koning TJ 2006 D-serine in the developing human central nervous system. Ann Neurol 60:476-480
8. Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, Nakazato M, Kumakiri C, Okada S, Hasegawa H, Imai K, Iyo M 2003 Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. Arch Gen Psychiatry 60:572-576
9. Hashimoto K, Engberg G, Shimizu E, Nordin C, Lindstrom LH, Iyo M 2005 Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naive schizophrenic patients. Prog Neuropsychopharmacol Biol Psychiatry 29:767-769
10. Sasabe J, Chiba T, Yamada M, Okamoto K, Nishimoto I, Matsuoka M, Aiso S 2007 D-serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. EMBO J 26: 4149-4159
11. Sethuraman R, Krishnamoorthy MG, Lee TL, Liu EH, Chiang S, Nishimura W, Sakai M, Minami T, Tachibana S 2007 Simultaneous analysis of D- and L-serine in cerebrospinal fluid by use of HPLC. Clin Chem 53:1489-1494
12. Fuchs SA, Berger R, Klomp LW, de Koning TJ 2005 D-amino acids in the central nervous system in health and disease. Mol Genet Metab 85:168-180
13. Hamase K, Morikawa A, Zaitsu K 2002 D-Amino acids in mammals and their diagnostic value. J Chromatogr B Analyt Technol Biomed Life Sci 781:73-91
14. Maier NM, Franco P, Lindner W 2001 Separation of enantiomers: needs, challenges, perspectives. J Chromatogr A 906:3-33
15. Ward TJ 2000 Chiral separations. Anal Chem 72:4521-4528
16. Ward TJ 2002 Chiral separations. Anal Chem 74:2863-2872
17. Bruckner H, Haasmann S, Friedrich A 1994 Quantification of D-amino acids in human urine using GC-MS and HPLC. Amino Acids 6:211
18. Goodlett DR, Abuaf PA, Savage PA, Kowalski KA, Mukherjee TK, Tolan JW, Corkum N, Goldstein G, Crowther JB 1995 Peptide chiral purity determination: hydrolysis in deuterated acid, derivatization with Marfey's reagent and analysis using high-performance liquid chromatography-electrospray ionization-mass spectrometry. J Chromatogr A 707:233-244

19. Berna MJ, Ackermann BL 2007 Quantification of serine enantiomers in rat brain microdialysate using Marfey's reagent and LC/MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 846:359-363
20. 1986 Guidelines for a User Laboratory to Evaluate and Select a Kit for its own use, European Committee for Clinical Laboratory Standards, part 1: Quantitative Tests. ECCLS document 3:
21. Rizzo V, Anesi A, Montalbetti L, Bellantoni G, Trotti R, Melzi d'Eril GV 1996 Reference values of neuroactive amino acids in the cerebrospinal fluid by high-performance liquid chromatography with electrochemical and fluorescence detection. J Chromatogr A 729:181-188
22. Levine J, Panchalingam K, McClure RJ, Gershon S, Pettegrew JW 2000 Stability of CSF metabolites measured by proton NMR. J Neural Transm 107:843-848
23. Blennow K, Wallin A, Gottfries CG, Mansson JE, Svennerholm L 1993 Concentration gradients for monoamine metabolites in lumbar cerebrospinal fluid. J Neural Transm Park Dis Dement Sect 5:5-15
24. Malm J, Kristensen B, Ekstedt J, Wester P 1994 CSF concentration gradients of monoamine metabolites in patients with hydrocephalus. J Neurol Neurosurg Psychiatry 57:1026-1033
25. Crawford PM, Lloyd KG, Chadwick DW 1988 CSF gradients for amino acid neurotransmitters. J Neurol Neurosurg Psychiatry 51:1193-1200
26. Lam S, Azumaya H, Karmen A 1984 High-performance liquid chromatography of amino acids in urine and cerebrospinal fluid. J Chromatogr 302:21-29
27. Mehl AL 1986 Interpretation of traumatic lumbar puncture. Predictive value in the presence of meningitis. Clin Pediatr (Phila) 25:575-577
28. Thomas RJ 1995 Excitatory amino acids in health and disease. J Am Geriatr Soc 43:1279-1289
29. Schmid DG, der Mulbe FD, Fleckenstein B, Weinschenk T, Jung G 2001 Broadband detection electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry to reveal enzymatically and chemically induced deamidation reactions within peptides. Anal Chem 73:6008-6013
30. Jones CM, Smith M, Henderson MJ 2006 Reference data for cerebrospinal fluid and the utility of amino acid measurement for the diagnosis of inborn errors of metabolism. Ann Clin Biochem 43:63-66
31. Shih V.E. Table B3. Amino acids in CSF. In: Blau N., Duran M., Blaskovics M.E., Gibson K.M. (eds) Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases. Springer-Verlag, 2004:14pp.

Supplemental Data Table 1. Influence of storage at 4°C for one week before storage at -80°C on D-serine, L-serine and glycine concentrations

	D-serine (µmol/L)		L-serine (µmol/L)		Glycine (µmol/L)	
Sample	1week4°C	-80°C	1week4°C	-80°C	1week4°C	-80°C
1	2.75	2.69	25.17	24.43	10.00	10.14
2	2.55	3.17	22.67	24.54	4.76	4.49
3	5.64	5.64	34.44	33.67	7.13	5.81
4	10.41	10.46	31.08	31.04	9.79	11.33
5	1.61	1.61	23.21	22.99	10.16	9.10
6	1.37	1.39	19.88	20.35	4.31	4.86
7	1.87	1.90	17.95	17.57	7.61	7.51
8	2.08	1.81	41.16	40.18	20.94	20.41
9	3.07	3.24	53.72	56.87	29.24	28.98
10	2.50	2.62	20.81	21.43	11.57	11.70
p-value	0.367		0.490		0.655	

10 different fresh CSF samples were divided in two portions, one of which was directly stored at -80°C while the other was first kept at +4°C for one week before storage at -80°C until analysis (LC-MS). D-Serine, L-serine and glycine concentrations were compared using a paired two-tailed Student's t-test. P-values ≤0.05 were considered significant.

Supplemental Data Table 2. Influence of CSF fraction on D-serine, L-serine and glycine concentrations

Patient	Fraction	D-Serine (µmol/L)	L-Serine (µmol/L)	Glycine (µmol/L)
1	1	12.9	71.6	23.8
	2	15.5	75.2	20.7
2	1	2.6	31.6	6.5
	3	2.9	33.2	6.1
	6	2.5	29.5	5.6
3	5	3.2	30.3	4.8
	6	3.4	30.9	4.3
4	1	7.5	53.3	5.9
	3	8.3	58.3	6.5
	4	6.6	45.7	5.0
5	5	10.7	44.4	5.4
	6	9.4	41.6	5.2
6	4	4.1	30.2	4.9
	6	4.5	31.9	7.5
7	4	3.4	38.5	5.7
	6	4.4	39.1	5.8
8	3	16.9	71.8	8.0
	4	16.3	72.8	7.2
9	4	7.4	46.1	5.4
	5	6.9	44.9	5.2
10	1	15.2	53.4	5.1
	3	16.6	59.3	6.0
p-value		0.096	0.083	0.310

Different CSF portions (n=8: 2 portions available, n=2: 3 portions available) from the same diagnostic lumbar puncture, for which 6 consecutive portions of 0.5-2mL were withdrawn, were analyzed (GC-MS). Because of the non-normal age distribution, the natural logarithms were compared with a repeated measurements model, using restricted maximum likelihood (REML) estimation. The level of significance was set at p=0.05.

Supplemental Data Table 3. Influence of repetitive freeze and thaw cycles on D-serine, L-serine and glycine concentrations

	Sample	F/T 1	F/T 2	F/T 3	F/Tsem	Repr.sem
D-serine (µmol/L)	1	2.52	2.14	2.56	0.051	0.055
	2	4.34	3.98	4.10		
	3	1.25	1.21	1.34		
	4	1.86	1.61	1.91		
	5	3.33	3.36	3.51		
L-serine (µmol/L)	1	27.04	31.57	29.67	0.566	0.914
	2	53.18	53.69	50.90		
	3	36.10	39.79	38.84		
	4	30.84	31.85	31.70		
	5	25.16	29.85	27.63		
Glycine (µmol/L)	1	4.86	5.06	4.60	0.233	0.282
	2	5.35	5.90	5.84		
	3	21.51	20.26	19.60		
	4	10.81	10.83	11.03		
	5	10.55	11.21	10.53		

Five different CSF samples were analyzed (LC-MS) upon 1,2 and 3 freeze and thaw cycles. F/T N = number of freeze and thaw cycles. F/Tsem= freeze/thaw standard error of the mean, based on the ANOVA mean residual sum of squares. Repr.sem= reproducibility standard error of the mean, based on the between run variance (LC-MS, Table 1) and average mean concentration of 2.6 µmol/L (D-serine), 36.0 µmol/L (L-serine) and 10.5 µmol/L (glycine). Absence of a freeze/thaw effect was assumed when the freeze/thaw and the reproducibility standard error of the mean were of the same order of magnitude.

D-serine in human physiology

Chapter 3

D-Serine in the developing human central nervous system

S.A. Fuchs, L. Dorland, M.G. de Sain-van der Velden, M. Hendriks, L.W.J. Klomp, R. Berger, T.J. de Koning

Ann Neurol 2006;6-:476-80

ABSTRACT

To elucidate the role of D-serine in human central nervous system, we analyzed D-serine, L-serine, and glycine concentrations in cerebrospinal fluid of normal children and children with a defective L-serine biosynthesis (3-phosphoglycerate dehydrogenase deficiency). Healthy children showed high D-serine concentrations immediately after birth, both absolutely and relative to glycine and L-serine, declining to low values at infancy. D-Serine concentrations were almost undetectable in untreated 3-phosphoglycerate dehydrogenase deficient patients. In one patient treated prenatally, D-serine concentration was nearly normal at birth and the clinical phenotype was normal. These observations suggest a pivotal role for D-serine in normal and aberrant human brain development.

INTRODUCTION

It was long thought that only L-amino acids existed in nature. However, surprisingly high D-serine concentrations were recently detected in mammalian central nervous system (CNS).(1) Subsequent studies demonstrated endogenous D-serine metabolism and synthesis from L-serine.(2) Like glycine, D-serine functions as a neuromodulator, binding to the N-methyl D-aspartate (NMDA) receptor.(3) The NMDA receptor is involved in CNS development, brain plasticity, memory and learning. Immunohistochemical studies have shown elevated D-serine levels in specific rodent and human brain areas during distinct developmental periods,(1, 4, 5) suggesting a specific function for D-serine in brain development. Despite rapid progress in this field, the exact contribution of D-serine to human CNS development and disease remains largely elusive, partly because of the lack of sensitive high-throughput analytical methods.

3-Phosphoglycerate dehydrogenase (3-PGDH) deficiency (OMIM 606879) is a rare disorder of L-serine biosynthesis, characterized by congenital microcephaly, intractable seizures and severe psychomotor retardation.(6) The confinement of clinical features to CNS abnormalities might be explained by the fact that L-serine transport across the blood-brain barrier is limited,(7) except during the end of gestation, when the ASCT-1 amino acid transporter is upregulated.(8) Therefore, CNS L-serine availability depends primarily on L-serine biosynthesis and is particularly affected by L-serine biosynthesis disorders. Patients exhibit low serine and sometimes low glycine concentrations in cerebrospinal fluid (CSF) and blood. Oral L-serine supplementation is very effective in treating the seizures,(9) with marginal effects on head growth and psychomotor development. In addition to these results of postnatal treatment, we demonstrated that prenatal maternal L-serine treatment completely corrected the disease phenotype.(10)

The severe neurological abnormalities in 3-PGDH deficiency and the reversal of these symptoms by early L-serine administration suggest a specific role for L-serine or its metabolites during a specific time-frame of CNS development. Because D-serine is synthesized from L-serine and functions as an NMDA receptor agonist in human CNS, we hypothesized that D-serine plays this role, and that insufficient D-serine is responsible for the severe pathophysiology in 3-PGDH deficiency. Consequently, we investigated the role of D-serine in the human developing brain by analyzing the concentrations of this amino acid together with L-serine and glycine in CSF of control subjects in the first years of life and in patients with 3-PGDH deficiency prior to and during treatment with L-serine. Our results suggest an important role for D-serine in human brain development and plasticity during a critical time window.

METHODS

Study design

CSF was obtained from samples that were sent to the microbiology laboratory in our hospital from July to December 2004 to exclude meningitis. Exclusion criteria were contamination with erythrocytes (> 100/ml), leukocytes (>10/ml) and protein (> age-specific reference values), meningitis, human immunodeficiency virus, intracranial hemorrhage, epilepsy, developmental delay, schizophrenia and perinatal asphyxia. Samples were kept at 4°C for 1 week, before being stored at -80°C. Use for our study was approved by the medical ethics committee of the University Medical Center Utrecht.
We analyzed CSF from 4 of the 10 patients with 3-PGDH deficiency published worldwide. (9-14) These samples had been stored at -80°C on retrieval. Written informed consent was obtained from the patients' parents.

Patient characteristics

The clinical details of patient 1 and 2 are described elsewhere.(6) 3-PGDH deficiency was diagnosed by enzymatic and mutation analysis.(6) L-Serine treatment resolved the seizures and marginally improved head circumference, without improving psychomotor development. Patient 3 was diagnosed prenatally with the same disorder as her brothers. Maternal oral L-serine supplementation from week 27 onward led to the birth of a normocephalic girl. With postnatal L-serine treatment, she develops normally without neurological abnormalities.(10)
Patient 4, the first child from unrelated parents, presented with congenital microcephaly. At 2 months of age, psychomotor developmental delay was evident and he was diagnosed with 3-PGDH deficiency (3-PGDH activity in fibroblasts: 5.18 nmol/min/mg; normal: 19.1 nmol/min/mg). Treatment with the recommended L-serine dosage(9) was decreased at 1 year of age because of side effects (feeding problems, irritability, myoclonus, and acoustic startles) and could not prevent the onset of seizures or normalize psychomotor development or head circumference.

Quantitative determination of D-serine, L-serine and glycine

The concentrations of D-serine, L-serine, and glycine were measured by a stable isotope dilution method according to Bruckner and colleagues' (15) method with modifications. A detailed description of our method will be published elsewhere (Fuchs et al, in preparation).

Statistical analyses

The median and 95% confidence interval of control amino acid concentrations were calculated for specific age groups (Table 1). Patients with 3-PGDH deficiency were compared with the corresponding 95% confidence interval of the control subjects.

RESULTS

D-Serine concentrations in CSF depended greatly on age (Table 1, Figure 1). In the first 100 days, the maximal D-serine concentration was 35.8μM and D-serine concentrations constituted almost one third of L-serine concentrations. D-Serine concentrations rapidly declined during the first year, then remained relatively constant until 3 years of age, when they further decreased to 15% of the initial concentration. L-Serine and glycine concentrations in CSF followed a milder age-related decrease, with levels declining to 52% and 74% of their postnatal values, respectively.

Because D-serine and glycine are synthesized from L-serine, we expressed these data as percentage D-serine of total serine, percentage glycine of the sum of glycine and L-serine, and ratio of D-serine to glycine (Table 1, Figure 1) as indicators of D-serine synthesis, glycine synthesis and the relative quantity of both NMDA receptor co-agonists, respectively. The percentage D-serine of total serine decreased after 3 years to one third

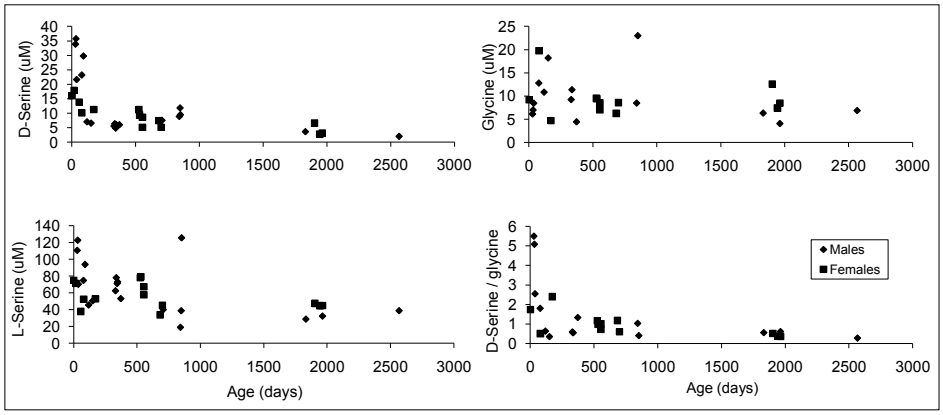


Figure 1. D-Serine, L-serine, and glycine concentrations and ratio of D-serine to glycine in cerebrospinal fluid (CSF) of healthy subjects plotted against their age in days. D-serine and L-serine concentrations (in μM) were determined in CSF of 33 healthy subjects of varying ages (1 day-7 years). Glycine concentrations (in μM) were determined in a subset of 26 healthy subjects (1 day-7 years). After the age of 7 years, the concentrations remained constant (results not shown). Diamonds represent male subjects and squares female subjects.

Table 1. Median and 95% confidence interval of D-serine, L-serine, and glycine concentrations and percentage D-serine of total serine, percentage glycine of the sum of glycine and L-serine and ratio D-serine to glycine in cerebrospinal fluid of healthy subjects.

		D-serine (μM)		L-serine (μM)		glycine (μM)		D-serine/tot serine (%)		glycine/glycine+L-serine (%)		D-serine/glycine	
Age	N	M	95% CI	M	95% CI	M	95% CI	M	95%CI	M	95%CI	M	95%CI
0 - 100 days	9(6)	20.8	8.9–48.8	74.6	36.3–153.6	9.7	4.2–22.9	21.7	15.7-30.1	10.5	3.0-36.5	2.2	0.4-12.6
100 days - 1 year	7(5)	6.6	3.8–11.2	61.0	40.3–92.3	10.0	3.8–26.5	9.6	4.6-20.2	14.7	6.0-35.9	0.7	0.2-3.0
1 - 3 years	11(9)	7.9	4.5–14.1	52.0	19.0–142.4	9.5	4.5–20.1	13.0	5.3-32.2	14.0	7.1-27.6	0.8	0.4-1.7
3 - 7 years	6(6)	3.2	1.4–7.2	39.0	25.1–60.6	7.2	4.0–13.2	7.5	3.7-15.5	15.6	10.2-23.9	0.4	0.3-0.8

Because amino acid concentrations were not normally distributed over the age groups, the median and 95% confidence intervals (CIs) were calculated in different age groups (0-100 days, 100 days–1 year, 1-3 years and 3-7 years). N = number of subjects used for serine analyses, with the number used for glycine analyses between parentheses, M = median.

Table 2. D-serine, L-serine, and glycine concentrations and percentage D-serine of total serine, percentage glycine of the sum of glycine and L-serine and ratio of D-serine to glycine in cerebrospinal fluid of patients with 3-PGDH deficiency before and after L-serine therapy.

	N	Age (days)	D-serine (μM)	L-serine (μM)	Glycine (μM)	D-serine / total serine (%)	Glycine / glycine + L-serine (%)	D-serine / glycine
Patient 1								
Before therapy	0							
After therapy	8	1594 - 3706	8.4 (4.6-16.3)	45.8 (30.7-80.1)	7.7 (4.8-10.3)	15.9 (9.0-22.5)	14.9 (11.0-16.9)	1.1 (0.8-1.6)
Patient 2								
Before therapy	1	2486	0.07	10.7	3.4	0.7	24.1	0.02
After therapy	7	2522 - 4809	5.2 (4.0-8.2)	44.4 (31.5-72.2)	6.6 (4.3-8.4)	10.8 (5.26-13.2)	13.7 (7.9-18.9)	0.8 (0.5-1.3)
Patient 3								
After maternal therapy	1	1	4.6	32.1	6.0	12.6	15.8	0.8
After therapy	4	112-1329	6.4 (4.8-10.0)	32.0 (25.5-41.6)	6.0 (5.1-6.8)	16.5 (12.3-19.3)	16.1 (13.9-19.9)	1.1 (0.7-1.5)
Patient 4								
Before therapy	2	67-95	0.4 (0.4-0.5)	12.0 (11.2-12.9)	3.8 (3.3-4.3)	3.5 (3.1-3.9)	23.9 (22.9-24.9)	0.1 (0.1-0.1)
After therapy	5	202-577	4.7 (3.6-5.8)	41.6 (17.6-66.4)	7.9 (5.2-11.5)	12.0 (7.9-21.6)	18.1 (11.5-30.9)	0.6 (0.5-0.7)

Patient 1, 2 and 4 received oral postnatal L-serine supplementation upon diagnosis. Due to adverse effects, L-serine dosage was lowered in patient 4 at the age of 1 year. Unfortunately, no pretreatment sample of patient 1 was available. Patient 3 received prenatal therapy by maternal supplementation with oral L-serine. The first measurement was performed on her day of birth. N = number of CSF samples available for analysis.

of the postnatal values, and the ratio of D-serine to glycine to one fifth of the postnatal values. In contrast, the percentage glycine of the sum of glycine and L-serine increased 1.5-fold over time. Taken together, D-serine concentrations in CSF of healthy individuals were markedly and specifically elevated early after birth, both absolutely and relative to L-serine and glycine.

D-Serine was virtually absent in CSF from patients with 3-PGDH deficiency, with concentrations of 0.07μM (patient 2) and 0.41μM (patient 4), 100 and 70 times lower than normal, respectively (Tables 1 and 2). In these patients, L-serine concentrations represented 20% and 13% of normal, respectively, whereas glycine concentrations were low to normal. This resulted in decreased percentages D-serine of total serine and D-serine to glycine ratios and normal percentages glycine of the sum of glycine and L-serine. On therapy, all values normalized after an initial D-serine overshoot. No pre-treatment sample was available for patient 1. The similar pattern of normalizing concentrations after a rapid rise and initial overshoot suggests equally low pretreatment concentrations.

After prenatal maternal L-serine treatment (patient 3), immediate postnatal analysis showed the presence of D-serine, albeit in lower concentrations than normal (22% of normal, D-serine/total serine: 58% of normal), whereas glycine and L-serine concentrations and other ratios were normal. Postnatal L-serine supplementation normalized all values.

DISCUSSION

In this study we present three lines of evidence consistent with an important role of D-serine in human brain development and neural plasticity.

First, we observed 7-fold increased D-serine concentrations in the early postnatal period, when compared to values found after 3 years of age. Conceivably, D-serine concentrations peak at distinct developmental stages, as suggested by findings of high D-serine concentrations in the human frontal cortex at gestational week 14.(16) Second, the concomitantly elevated postnatal D-serine fraction of total serine indicates increased perinatal D-serine synthesis from L-serine, conceivably through age-related changes in expression or activity of serine racemase, the enzyme converting L-serine to D-serine. This concurs with the striking correlation between increased serine racemase expression and increased D-serine concentrations in early postnatal mouse cortex, striatum, and cerebellum.(17) Despite postnatally elevated L-serine and glycine concentrations, glycine synthesis from L-serine was not increased, as evidenced by a lower postnatal percentage glycine of the sum of glycine and L-serine when compared with older controls. These data suggest that the proper homeostatic control of the two NMDA receptor co-agonists glycine and D-serine is important during CNS development. Differences in

the ratio of D-serine versus glycine may have relevant functional consequences during specific developmental stages.

These specifically elevated D-serine concentrations during postnatal human CNS development, similar to those seen for other neuromodulators, neurotransmitters and receptors,(18) might be essential for adequate NMDA receptor activity. Pharmacological blockade of NMDA receptors in prenatal and postnatal rats resulted in apoptosis in the developing brain.(19) D-Serine degradation by D-amino acid oxidase and pharmacologic inhibition of serine racemase impeded neuronal migration in neonatal mouse cerebellum, whereas D-serine activated the process.(20) Furthermore, mutant mice lacking D-amino acid oxidase, thereby possessing higher D-serine concentrations, exhibited enhanced long-term potentiation and spatial learning.(21) These studies indicate the importance of D-serine in NMDA receptor activation for proper rodent CNS development and plasticity and support our hypothesis of a similar role for D-serine in humans.

Third, our patients with 3-PGDH deficiency failed to achieve these early elevated D-serine concentrations and exhibited severely disturbed neuronal development. L-Serine therapy restored D-serine concentrations and, when applied during fetal development, corrected the complete phenotype. Obviously, L-serine biosynthesis, leading to D-serine synthesis and NMDA receptor activation, is crucial for early neuronal development. Early severe disturbances in L-serine synthesis lead to structural CNS anomalies, and a lethal phenotype, as demonstrated in a 3-PGDH deficient mouse model.(22) Patients with 3-PGDH deficiency have substantial residual 3-PGDH activity and milder CNS abnormalities. Presumably, in these patients L-serine synthesis may be sufficient to prevent structural anomalies, but insufficient to achieve the elevated D-serine concentrations after 27 weeks of gestation. This would explain the specific window of opportunity during fetal development to treat this disorder, thereby restoring a temporary deficit and not a complete deficiency.

This study does not directly prove that depletion of D-serine, rather than L-serine or one of its metabolites, is responsible for the pathology observed in 3-PGDH deficiency. However, D-serine is the most likely candidate, because of its function as an NMDA receptor co-agonist and the role of NMDA receptors in CNS during late gestation.(19) There is little evidence to suggest that L-serine or other of its metabolites like L-serine derived phospolipids act as neuromodulators in brain plasticity. Glycine, the other potential L-serine metabolite, is not a likely candidate either, because it is not consistently deficient in 3-PGDH deficiency (our patients displayed low to normal concentrations), while CNS pathology is a consistent finding. Despite being theoretically compelling, further studies are warranted to determine the exact role of D-serine during human CNS development. Serine racemase knock-out experiments in transgenic animals and additional analyses of D-serine in CNS disorders are needed to ascertain our supposition.

In conclusion, the specifically elevated D-serine concentrations, both absolutely and relative to L-serine and glycine during early CNS development, the omission of which is associated with severe CNS abnormalities, suggest an important role for D-serine in normal and aberrant human brain development.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from The Netherlands Organization for Health Research and Development (grant 920-03-345). We are indebted to the parents of the affected children; to the laboratory of microbiology of the University Medical Center Utrecht for their collaboration in cerebrospinal fluid collection; to M. Roeleveld and M. de Barse for their contributions to the validation of the analysis technique and to dr. CPMC Peeters for the helpful discussions on the topic.

REFERENCES

1. Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T, Takashima S, Doi N, Mizutani Y, Yamazaki T, . 1993 Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. J Neurochem 61:348-351
2. Wolosker H, Blackshaw S, Snyder SH 1999 Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. Proc Natl Acad Sci U S A 96:13409-13414
3. Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD, Rogawski MA, Snyder SH 2000 D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 97:4926-4931
4. Hashimoto A, Oka T, Nishikawa T 1995 Anatomical distribution and postnatal changes in endogenous free D-aspartate and D-serine in rat brain and periphery. Eur J Neurosci 7: 1657-1663
5. Schell MJ, Brady RO, Jr., Molliver ME, Snyder SH 1997 D-serine as a neuromodulator: regional and developmental localizations in rat brain glia resemble NMDA receptors. J Neurosci 17:1604-1615
6. Jaeken J, Detheux M, Van Maldergem L, Frijns JP, Alliet P, Foulon M, Carchon H, Van Schaftingen E 1996 3-Phosphoglycerate dehydrogenase deficiency and 3-phosphoserine phosphatase deficiency: inborn errors of serine biosynthesis. J Inherit Metab Dis 19:223-226
7. Smith QR, Momma S, Aoyagi M, Rapoport SI 1987 Kinetics of neutral amino acid transport across the blood-brain barrier. J Neurochem 49:1651-1658
8. Sakai K, Shimizu H, Koike T, Furuya S, Watanabe M 2003 Neutral amino acid transporter ASCT1 is preferentially expressed in L-Ser-synthetic/storing glial cells in the mouse brain with transient expression in developing capillaries. J Neurosci 23:550-560
9. De Koning TJ, Duran M, Dorland L, Gooskens R, Van Schaftingen E, Jaeken J, Blau N, Berger R, Poll-The BT 1998 Beneficial effects of L-serine and glycine in the management of seizures in 3-phosphoglycerate dehydrogenase deficiency. Ann Neurol 44:261-265
10. De Koning TJ, Klomp LW, van Oppen AC, Beemer FA, Dorland L, van dB, I, Berger R 2004 Prenatal and early postnatal treatment in 3-phosphoglycerate-dehydrogenase deficiency. Lancet 364:2221-2222
11. Jaeken J, Detheux M, Van Maldergem L, Foulon M, Carchon H, Van Schaftingen E 1996 3-Phosphoglycerate dehydrogenase deficiency: an inborn error of serine biosynthesis. Arch Dis Child 74:542-545
12. Klomp LW, De Koning TJ, Malingre HE, van Beurden EA, Brink M, Opdam FL, Duran M, Jaeken J, Pineda M, Van Maldergem L, Poll-The BT, van dB, I, Berger R 2000 Molecular characterization of 3-phosphoglycerate dehydrogenase deficiency--a neurometabolic disorder associated with reduced L-serine biosynthesis. Am J Hum Genet 67:1389-1399
13. Pind S, Slominski E, Mauthe J, Pearlman K, Swoboda KJ, Wilkins JA, Sauder P, Natowicz MR 2002 V490M, a common mutation in 3-phosphoglycerate dehydrogenase deficiency, causes enzyme deficiency by decreasing the yield of mature enzyme. J Biol Chem 277:7136-7143
14. Pineda M, Vilaseca MA, Artuch R, Santos S, Garcia Gonzalez MM, Aracil A, Van Schaftingen E, Jaeken J 2000 3-phosphoglycerate dehydrogenase deficiency in a patient with West syndrome. Dev Med Child Neurol 42:629-633

15. Bruckner H, Haasmann S, Friedrich A 1994 Quantification of D-amino acids in human urine using GC-MS and HPLC. Amino Acids 6:211
16. Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T, Takashima S, Doi N, Mizutani Y, Yamazaki T, . 1993 Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. J Neurochem 61:348-351
17. Wang LZ, Zhu XZ 2003 Spatiotemporal relationships among D-serine, serine racemase, and D-amino acid oxidase during mouse postnatal development. Acta Pharmacol Sin 24:965-974
18. Herlenius E, Lagercrantz H 2004 Development of neurotransmitter systems during critical periods. Exp Neurol 190 Suppl 1:S8-21
19. Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovska V, Turski L, Olney JW 1999 Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283:70-74
20. Kim PM, Aizawa H, Kim PS, Huang AS, Wickramasinghe SR, Kashani AH, Barrow RK, Huganir RL, Ghosh A, Snyder SH 2005 Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. Proc Natl Acad Sci U S A 102:2105-2110
21. Maekawa M, Watanabe M, Yamaguchi S, Konno R, Hori Y 2005 Spatial learning and long-term potentiation of mutant mice lacking D-amino-acid oxidase. Neurosci Res 53:34-38
22. Yoshida K, Furuya S, Osuka S, Mitoma J, Shinoda Y, Watanabe M, Azuma N, Tanaka H, Hashikawa T, Itohara S, Hirabayashi Y 2004 Targeted disruption of the mouse 3-phosphoglycerate dehydrogenase gene causes severe neurodevelopmental defects and results in embryonic lethality. J Biol Chem 279:3573-3577

Chapter 4

D-Serine in the developing human central nervous system: clinical implications

S.A. Fuchs, L.W.J. Klomp, T.J. de Koning

In: U. Heresco-Levy and D.C. Javitt, editors. Glutamate in neuropsychiatric disorders. Kerala, India: Research Signpost. 2008;chapter 4:75-97

ABSTRACT

Although D-amino acids were long thought to be absent in nature, recently, the D-amino acid D-serine has been identified in high concentrations in the mammalian central nervous system (CNS). D-serine is synthesized and metabolized endogenously and functions as an important N-methyl D-aspartate receptor (NMDAr) co-agonist. NMDAr activation is critical for neuronal migration, maturation, survival, dendritic outgrowth, synaptic formation, brain plasticity and the onset of long-term potentiation, a phenomenon required for memory and learning. Consequently, as an endogenous NMDAr agonist, D-serine is likely to play an essential role in mammalian CNS development. Support for this does not only come from specific increases in D-serine concentrations in human and rodent CNS during the intense period of CNS development in embryonic and early postnatal life and the severe CNS abnormalities observed when these high D-serine concentrations are not reached, as observed in patients with 3-phosphoglycerate dehydrogenase deficiency, a rare inherited disorder in the synthesis of L-serine and hence D-serine. More specifically, there are recent suggestions for a role of D-serine in neuronal migration, maturation and synaptogenesis during early postnatal CNS development. Finally, the enhanced hippocampal long-term potentiation, improved spatial learning and better motor coordination of mutant mice exhibiting increased NMDAr activation through elevated D-serine concentrations underscore the importance of proper D-serine concentrations for normal CNS function. The unexpected finding of D-serine as an important factor in human CNS development and function does not only increase our insight in human physiology, but might also yield novel diagnostic and therapeutic strategies for disorders associated with CNS abnormalities.

INTRODUCTION

As the basic constituents of proteins and peptides, amino acids are among the most important molecules for living beings. All amino acids, with the exception of glycine, exist as different enantiomers (an L- and a D-form), depending on the tetrahedral configuration around the chiral center on the α-carbon atom. Although the chemical and physical properties of L-amino acids and D-amino acids are almost identical, they differ in their spatial positioning. Just like the hands of a person, L- and D-amino acids take a mirror image position in space, hence the derivation of the word chirality from the Greek word for hand (Χειρ). This mirror image position leads to a different spatial architecture of the polymers in which they are incorporated. Therefore, chirality plays a major role in structural interactions and enzymatic specificity of these biological polymers.(1)

The dramatic experience with the chiral drug thalidomide illustrates the physiological relevance and clinical impact of chirality in nature. Thalidomide is a drug that was prescribed to pregnant women between 1957 and 1961 in almost 50 countries worldwide for morning sickness and sleeping problems. Thalidomide use during pregnancy resulted in the birth of approximately 10.000 children with severe malformations,(2) including pronounced shortening of the limbs (phocomelia) and a greatly reduced life expectancy.(3) Subsequent research revealed that the prescribed drug consisted of a racemic mixture of both the L- and the D-form of thalidomide. While the D-form was responsible for the therapeutic effects, only the L-form was the potent teratogen, causing the congenital malformations.(4) This example clearly illustrates that the actions and effects of enantiomers can be strikingly different in the presence of other chiral compounds, like in the human body.

Therefore, it is not surprising that it was long assumed that homochirality existed in nature, with selection of only L-amino acids for polymerization and formation of peptides and proteins in all living organisms.(1) Based on this concept of L-homochirality, detection of D-amino acids in living organisms was initially attributed to laboratory artifacts. However, as early as the beginning of the twentieth century, various studies strongly suggested the presence of D-amino acids in micro-organisms and in some naturally occurring peptides.(5) After the chromatographic detection of free D-alanine in the blood of the milkweed bug in 1950,(6) many reports on animal D-amino acids followed (free or incorporated in proteins), first involving only invertebrates and amphibians,(7-15) but later also mammals, including humans. The D-amino acids in mammalian tissue were assumed to arise from endogenous microbial flora, from ingestion with the diet or from spontaneous racemization with ageing.(16) No specific biological activity was associated with the detected amino acids.

In this context, it was remarkable that in 1986, surprisingly high concentrations of D-aspartate were identified in the brain and other tissues of rodents and in human blood.(17) Some years later, in 1992, a second D-amino acid, D-serine, was found to be present

in significant amounts in the brain of rodents and man.(18) These studies formed the basis for subsequent research on this topic, confirming that some D-amino acids do exist in the mammalian CNS and peripheral tissues in unexpectedly high concentrations, sometimes exceeding the concentration of the L-enantiomer.(19) Furthermore, biological functions were associated with some of these D-amino acids.(20) Most research has focused on D-serine, which was found to play an important role in glutamatergic neurotransmission as an important ligand for the N-methyl-D-aspartate receptor (NMDAr). Recent evidence implies that D-serine is essential for normal human CNS development and that the enzymes in the synthesis and breakdown of D-serine are associated with psychiatric disorders such as schizophrenia and bipolar disorder.(21) The unexpected presence and biological roles of D-serine in the mammalian brain do not only challenge our conventional theories on mammalian development and physiology, but may also have important clinical implications, as altered levels of D-serine might be involved in various pathological conditions and thereby possibly provide new therapeutic targets. In this chapter, we will discuss the role of free D-serine in mammalian CNS development, with a special focus on the clinical implications.

D-SERINE

D-serine localization

Using mostly chromatographic techniques, free D-serine has been localized predominantly to the adult rodent and human forebrain. D-Serine concentrations were highest in the cerebral cortex, hippocampus and striatum, followed by the limbic forebrain, diencephalon and midbrain. In contrast, the pons, medulla, cerebellum and spinal cord contain low D-serine levels.(19;22;23) D-Serine concentrations in the rat brain represent approximately one third of L-serine concentrations, thereby exceeding the concentration of many common L-amino acids.(24) Similarly, in cerebrospinal fluid of human newborns, D-serine concentrations represent one fourth of L-serine concentrations, declining to one thirteenth in adulthood.(20)

The first immunohistochemical localization studies of D-serine have shown a selective localization to the cytoplasm of protoplasmic type II astrocytes. This subtype of glial cells ensheathes nerve terminals and is particularly enriched in cortical gray matter.(25-27) However, the use of a novel D-serine antibody has revealed that D-serine is concentrated into vesicle-like compartments in astrocytes and radial glial cells, rather than being distributed uniformly in the cytoplasm.(28) Interestingly, D-serine was not only identified in type II astrocytes, but also in microglia and in glutamatergic neurons,(29;30) particularly in brainstem regions and in the olfactory bulb,(28) as well as in pyramidal neurons in the

cerebral cortex and in neurons in the nucleus of the trapezoid body.(31) The anatomical distribution of D-serine in the CNS closely mimics that of the NR2 A/B subtypes of the NMDA-type excitatory amino acid receptor.(22;26) (see section "D-serine function") D-Serine is not restricted to the CNS, but has also been identified in peripheral mammalian tissues, albeit in lower concentrations. Small amounts of D-serine were detected in human serum, saliva and urine (32-34) and in the retina of various vertebrates.(35)

D-serine synthesis

Although humans can acquire D-serine through ingestion with food, derivation from gastrointestinal bacteria or liberation from metabolically stable proteins, which contain D-amino acids after racemization with ageing, biosynthesis from L-serine appears to be the most important source.(20) The enzyme serine racemase (SR) directly converts L- to D-serine in the presence of the co-factors pyridoxal 5-phosphate and is activated by divalent cations such as magnesium and calcium as well as by ATP and ADP (Fig. 1).(36-38)

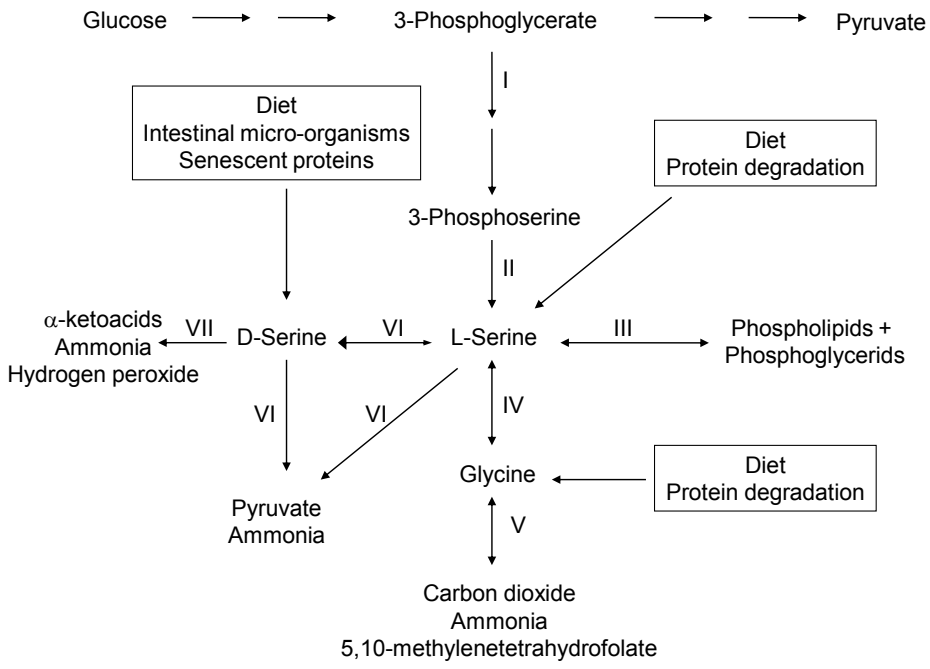


Figure 1. Pathways involved in D-serine, L-serine and glycine acquisition and degradation. As glycine and D-serine are both NMDAr co-agonists, the possibility of interconversion between D-serine, L-serine and glycine is of particular relevance. I = 3-phosphoglycerate dehydrogenase; II = 3-phosphoserine phosphatase; III = synthesis of phospholipids and phosphoglycerides from cytidine diphosphodiacylglycerol and palmitoyl-coenzyme A; IV = serine hydroxymethyltransferase; V = glycine cleavage system; VI = serine racemase; VII = D-amino acid oxidase.

Inversely, SR also converts D- to L-serine, albeit with lower affinity.(38) D-Serine concentrations are thus highly related to L-serine concentrations and thereby also to glycine concentrations (Fig. 1).(39)

SR is highly expressed in the brain, with lower levels in the liver and small or no detectable expression in other tissues. Just like the immunohistochemical localization studies on D-serine, SR was first localized to protoplasmic astrocytes in the brain in a pattern similar to D-serine.(37;38) Recent studies also imply the presence of SR in several neuronal culture types, where D-serine synthesis was found to be comparable to that observed in astrocytes.(29) In primary cultures of rat neurons, SR mRNA and protein levels have even been found to exceed those in astrocytes.(30) These results suggest that not only astrocytes, but also neurons represent a major source of D-serine.

Physiological synthesis of D-serine by SR was implicated by the strong spatiotemporal correlation between D-serine and SR (40) and by the decrease in D-serine concentrations in astrocytes after pharmacological inhibition of SR.(37) The cDNA encoding human SR has been cloned and D-serine synthesis by SR has been demonstrated in living cells after heterologous overexpression.(41) In addition to the racemase activity, SR catalyzes the α,β-elimination of water from both L-serine and D-serine to form pyruvate and ammonia.(36;42) Under physiological conditions, pyruvate formation seems to equal or excess D-serine formation. Pyruvate formed by SR may be sufficient for the energy requirements of the astrocytes. This reaction further implies that SR is not only involved in D-serine synthesis, but also in D-serine metabolism as a mechanism to regulate intracellular D-serine levels and NMDAr activity.(42) In fact, SR also influences intracellular D-serine levels by feedback regulation of its own activity. Upon glutamate and D-serine binding, NMDArs are activated, inducing neuronal nitric oxide formation in postsynaptic neurons, thereby stimulating S-nitrosylation of presynaptic SR. This in turn reduces SR activity and consequently decreases D-serine concentrations.(43) Although the factors regulating SR levels and activity are not completely unraveled, SR appears to be degraded through the ubiquitin-proteasomal system. Ubiquitylation was decreased by the Golgin subfamily A member 3 protein, thereby increasing SR protein half life.(44) We are awaiting the results of the recent generation of a SR knock-out mouse strain,(45) that might further clarify the physiological roles of SR *in vivo*.

D-serine metabolism

D-amino acid oxidase (DAO) and D-aspartate oxidase (DAOX) are two flavoproteins responsible for the oxidative deamination of neutral and dicarboxylic D-amino acids respectively and have long been known to exist in mammals.(46;47) Until recently however, they had largely been neglected, since animals and humans were not thought to possess D-amino acids. The identification of D-amino acids in mammals finally provided

a rationale for the widespread occurrence and high degree of conservation of these enzymes in animals and humans.

DAO is a peroxisomal enzyme that metabolizes neutral D-amino acids (46) with the concomitant reduction of the co-factor flavin adenine dinucleotide (FAD),(48) according to the following reaction:

$$\text{neutral D-amino acid} + O_2 + H_2O - \textit{DAO} \rightarrow \text{corresponding } \alpha\text{-ketoacid} + NH_3 + H_2O_2$$

The affinity of DAO is highest for D-serine, D-alanine, D-proline, D-leucine and D-methionine *in vivo*,(49;50) while glycine is not a substrate at physiological pH values, nor are L-amino acids or dicarboxylic amino acids.(51) In humans, DAO was first identified within the granule fraction of human neutrophilic leukocytes and proposed to be a component of an antibacterial system by generating hydrogen peroxide.(52) Subsequent research revealed that DAO is expressed predominantly in the kidneys, followed by the liver (with the exception of mice livers, where DAO expression has not been detected (53)) and the CNS. In the CNS, DAO is particularly concentrated in astrocytes of the hindbrain, cerebellum, brainstem, medulla and spinal cord (54) with a putative preferential localization to type I astrocytes (55) and to a lesser extent to type II astrocytes, whereas D-serine is known to be localized to type II astrocytes.(25) By now, human DAO has been cloned,(56) produced as a homogeneous protein(57) and its 3-D structure has been characterized.(58) Human DAO differs from other known DAO's by forming dimers in solution.(59) There is no evidence for the existence of different DAO isoforms in the various human (nor rat or pig) tissues.(59)

Physiological degradation of D-serine by DAO was suggested by the marked regional and developmental variation in DAO levels in a pattern reciprocal to D-serine levels.(25;40) Furthermore, *Dao*$^{-/-}$ mice manifest a modest but significant increase in D-serine levels, especially in areas with low levels in wild type animals such as the cerebellum and periphery.(49) However, in the forebrains of *Dao*$^{-/-}$ mice, D-serine concentrations were relatively unchanged, suggesting that in these areas, other mechanisms might regulate D-serine concentrations.(49;50) These results from *Dao*$^{-/-}$ mice contrast with those obtained with a pharmacologic DAO inhibitor, producing increased D-serine concentrations in rat cortex and midbrain and influencing rat behaviour, implying that DAO does reduce D-serine concentrations physiologically in these brain areas.(60) In analogy, a recent study has demonstrated that a naturally occurring mouse strain (ddY/DAO^{-}) lacks DAO activity and has enhanced NMDAr function *in vivo* due to increased occupancy of the NMDAr glycine site by elevated extracellular D-serine levels.(61) Clearly, the exact mechanisms involved in D-serine homeostasis warrant further investigation.

D-serine function

The close correlation between the anatomical distribution of D-serine and SR with the regional variation of the NMDA receptor (NMDAr) suggests a functional relationship. NMDArs are broadly distributed throughout the CNS and play a major role in glutamatergic synaptic transmission. NMDAr activation has been implicated in physiological processes such as CNS development (21) and the induction of long-term potentiation and thereby long-term synaptic plasticity, which may serve as a cellular mechanism underlying learning and memory.(62) Excitotoxicity through NMDAr overstimulation has been implicated in a large number of acute and chronic neurodegenerative conditions, including stroke, epilepsy, polyneuropathies, chronic pain, amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and Huntington's disease.(63) NMDAr hypofunction might play a role in the pathophysiology of schizophrenia, as NMDAr antagonists (64) or reduced numbers of NMDArs (65) induced positive, negative and cognitive schizophrenia-like symptoms, which could be reversed by treatment with D-serine.(66)
NMDArs are members of a class of ionotropic receptor channels, organized as heteromeric assemblies composed of an NR1 subunit, combined with one or more NR2 or NR3 subunits. Although the NR1 subunit is an obligatory component of functional NMDArs, the NR2 subunits determine the biophysical and pharmacological activity of the receptor.(67) NMDArs require simultaneous ligand binding at two sites for activation. Glutamate molecules bind to the NR2 subunit (68) and glycine was assumed to be the necessary co-agonist, reacting with the "glycine site" of the NR1 subunit.(69-71) The first suggestion for a role of D-serine as an endogenous co-agonist of this "glycine site" of NMDArs, comes from immunohistochemical localization of glycine, D-serine and NMDArs. D-Serine showed a specific pattern of co-localization with NMDArs in the telencephalon and the developing cerebellum, while glycine co-localized with NMDArs in the hindbrain, the adult cerebellum and the olfactory bulb.(26) This was reinforced by the finding that D-serine acts as a selective and at least equally potent agonist for this "glycine site" of NMDArs when compared to glycine.(72;73) Direct evidence for regulation of NMDAr activity by endogenous D-serine comes from the decrease in NMDAr activity in immature rat cerebellar slices, rat hippocampus slices and in primary hippocampal cell cultures after selective removal of D-serine by adding DAO, the effect of which was fully reversed by application of exogenous D-serine.(74) All these data favour D-serine as the predominant endogenous ligand for most NMDArs. Nevertheless, glycine might well be the principal ligand in some areas, such as the brainstem,(26) the spinal cord (75) and the cerebellum.(26;74) In part, these differences in NMDAr co-agonist can be explained by the presence of D-amino acid oxidase, which degrades D-serine and not glycine, resulting in areas with low D-serine concentrations and high glycine concentrations. (25;40) Similarly, areas with high D-serine concentrations are enriched with the D-serine

synthesizing enzyme SR,(40) just like the areas with high glycine concentrations are with the glycine synthesizing enzyme serine hydroxymethyltransferase.(76)
Snyder and Kim (77) proposed a model of the interaction between astrocytes and neurons in NMDAr activation. They suggested that, upon release from the presynaptic neuron, glutamate does not only bind to the NMDAr, but also to non-NMDArs (2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors) on nearby astrocytes. In these astrocytes, D-serine is then synthesized by SR and released into the synaptic cleft, where it can bind to the NMDAr complex together with glutamate, leading to NMDAr activation.
As an endogenous co-agonist of the NMDAr, D-serine concentrations are important for all the physiological functions of NMDArs. Similarly, D-serine concentrations might be altered in disorders associated with pathologic NMDAr activity and as such might contribute to their pathogenesis.

CENTRAL NERVOUS SYSTEM DEVELOPMENT

The NMDAr in CNS development

There is substantial evidence implicating an important role for NMDArs in CNS development. Again, the first clue was provided by immunohistochemical localization studies, demonstrating a greater expression and number of NMDArs in the developing brain, relative to the adult brain. In the human brainstem, NMDArs increase to a peak during the infant period to decrease thereafter.(78) A similar pattern was observed the human frontal,(79) temporal (80) and prefrontal cortex.(81) The subunit composition of NMDArs also varies with age. The NR1subunit is widely expressed throughout the CNS at all ages, but the expression profiles of the NR2 subunits are regionally and developmentally regulated.(82) Currently, 4 genetically different NR2 subunits have been identified (NR2A-NR2D).(82;83) In the neonatal rat brain, NR2B and NR2D subunits predominate, but as development proceeds, they are supplemented with, or replaced by the NR2A and NR2C subunits in some brain regions.(83;84) This developmental shift of NR2 subunits, which determine the biophysical and pharmacological properties of NMDArs, might set the optimal conditions for proper CNS development.
In analogy, receptor binding studies have revealed higher NMDAr activity in the developing brain than in the adult brain.(85-87) When comparing the developing brain to the adult brain, differences in NMDAr features include a lower sensitivity to NMDAr blockage by the antagonist magnesium, a higher sensitivity to the NMDAr agonist glycine, differential modulation by polyamines, increased calcium influx through the receptor channel, longer duration of the excitatory postsynaptic potentials after receptor stimulation and

enhanced ability to induce markers of synaptic plasticity such as long-term potentiation. (88-91)

More direct evidence for a role of the NMDAr in specific functions during CNS development is provided by pharmacological studies. Blocking NMDArs with daily injections of an NMDAr antagonist for 2 days in 2-days-old rats significantly increased cell proliferation in the dentate gyrus,(92;93) while activation of NMDArs decreased proliferation of the granule cells of the dentate gyrus.(94) On the other hand, blockage of the NMDAr during late fetal and early neonatal rat life was associated with widespread apoptotic neurodegeneration (95;96). Other studies in which neonatal rats were given NMDAr antagonists during the first 2 postnatal weeks demonstrate that NMDAr blockade is associated with abnormal axonal arborization and apoptotic neurodegeneration.(97) Taken together, all these studies imply an essential role for adequate NMDAr activation in CNS shaping by influencing neuronal proliferation and apoptosis.

During CNS development, neurons in the brain migrate to their mature positions along the processes of radial glia. The NMDAr has also been implicated in this process. Already before undergoing synaptogenesis, migrating cortical neurons possess functional NMDArs (98) and migrating granular cells contain more NMDArs than premigratory neurons,(99) implying optimal functional NMDAr content during neuronal migration. Pharmacological studies demonstrate that blocking NMDArs in cerebellar slices or increasing medium magnesium concentrations slow the rate of granule cell migration, which could be reversed by magnesium removal or addition of the NMDAr agonists glutamate and glycine.(96) Similarly, in cortical slices from the rat and mouse, NMDAr activation was found to stimulate neuronal migration.(100;101)

Synaptic plasticity or synaptogenesis is crucial for shaping the wiring pattern of the brain and is responsible for the mechanism of learning and memory.(102-104) Injecting NMDA into the occipital cortex of 8-day-old rats increased the total number of synapses,(102) while blocking NMDArs decreased the synaptic density (103) and induced deficits in learning and memory.(105) The importance of NMDAr activation for synaptic plasticity is further evidenced by genetically modified mice, exhibiting complete loss of the glycine and D-serine binding NR1 subunit of the NMDAr and displaying impaired long-term potentiation and spatial memory.(106;107)

D-serine in CNS development

A specific function for D-serine in brain development was suggested by immunohistochemical localization studies,(19;26;108;109) showing elevated D-serine levels in specific rodent and human brain areas during distinct developmental periods. On the day of birth, D-serine was present in substantial concentrations throughout rat brain.(108) D-Serine concentration increased in the cerebrum by the third postnatal week, remaining rather

constant thereafter. Similarly, high D-serine concentrations were determined in the human frontal cortex at gestational week 14 and remained rather constant throughout embryonic and early postnatal life.(19) D-Serine concentrations decreased later in life to half of these levels in adolescent and aged brains.(19)

In rat cerebellum, D-serine increased in the first postnatal week to its highest levels in the second week and thereafter declined dramatically to only trace levels, coinciding well with the drastic increase in DAO activity in the cerebellum during that period.(26;108;109) Furthermore, cerebellar D-serine was localized immunohistochemically to astrocytes in the granular layer and Bergmann glia that regulate granule cell migration.(26) This localization and the transient D-serine increase in the developing cerebellum, which concurs with a transient expression of NMDArs, suggest a role in cerebellar development. This is supported by the disrupted cerebellar ontogeny observed upon blockage of the glycine binding site of NMDArs during rodent development.(96;110) In addition, selective degradation of D-serine by DAO and selective inhibition of SR in 8-day old mouse cerebellar slices significantly reduced granule cell migration, whereas D-serine activated this process.(111) Granule cell migration appears to depend on an increase in intracellular calcium through NMDAr activation, as treatment with a SR inhibitor markedly diminished intracellular calcium concentrations.(111) Taken together, these results imply a role for D-serine and subsequent NMDAr activation in cerebellar granule cell migration, which might be seen as a model for neuronal migration in the entire CNS.

Chromatographic-mass spectrometric analysis of cerebrospinal fluid D-serine concentrations in healthy children, as a global marker of brain D-serine content, revealed high D-serine concentrations immediately after birth, both absolutely and relative to glycine and L-serine, declining to 15% of the initial values at infancy.(21) D-Serine concentrations were almost undetectable in untreated patients with 3-phosphoglycerate dehydrogenase deficiency, a rare inherited disorder of L-serine synthesis (Figure 1), characterized by severe CNS abnormalities, including microcephaly, intractable seizures and profound mental retardation. In one patient treated prenatally, D-serine concentrations were nearly normal at birth (21) and the clinical phenotype was normal.(112) As an NMDAr agonist, D-serine deficiency is much more likely to cause the severe CNS malformations than L-serine deficiency. The SR knock-out mouse model will help to elucidate this matter. The importance for normal CNS development of L-serine synthesis and conceivably even more so of subsequent D-serine synthesis, is further evidenced by the striking CNS abnormalities found in the first two weeks after conception in a 3-phosphoglycerate dehydrogenase deficient mouse model,(113) culminating in hypoplasia of the telencephalon, diencephalon and mesencephalon, with particularly the olfactory bulb, the ganglionic eminence and cerebellum appearing as indistinct structures. This establishes that the phosphoglycerate dehydrogenase dependent pathway of L-serine biosynthesis

(Figure 1), possibly through D-serine synthesis and NMDAr activation, is essential for embryonic CNS development, especially for brain morphogenesis.

Using the retina as a model for CNS development, the glutamate transporter excitatory amino acid transporter-1, which is the main regulator of retinal extracellular glutamate concentrations, and D-serine were both found to be present in human retinae, from 10 and 12 weeks of gestation on, respectively, which was followed by the appearance of synaptophysin, a synaptic vesicle marker.(114) Furthermore, D-serine and the glutamate transporter co-localized in radial glia-like cells that might represent Muller cells, as evidenced by the co-localization with cellular retinaldehyde-binding protein. This implies that the endogenous determinants of NMDAr activity glutamate and D-serine are present before synaptic vesicle proteins are evident and may thus play a role in shaping synaptogenesis in the developing human retina.(114) These results are consolidated by studies using the vestibular nuclei and their main connections as a model for D-serine function in synaptic plasticity. During the first three postnatal weeks, an intense period of plasticity, synaptogenesis and neuronal maturation, rat vestibular nuclei contained high D-serine concentrations in glial cells and processes, corresponding with high SR and low DAO expression. In the mature vestibular nuclei, D-serine mainly localized to neuronal cell bodies and dendrites and concentrations were low, corresponding with increasing DAO expression.(115) These strong decreases of D-serine concentrations and the glial-to-neuronal switch suggest that D-serine may have a role in neuronal plasticity, maturation and synaptogenesis during early postnatal CNS development, and possibly a distinct functional role at later developmental stages.(115)

Behavioural results of animals lacking D-serine during CNS development, such as SR knock-out mice, are currently unavailable. However, we might enhance our understanding through the behavioural results from mutant mice displaying increased NMDAr activation through elevated D-serine or glycine concentrations. One such mouse model is a naturally occurring mouse strain (ddY/DAO^{-}) lacking DAO activity and thereby displaying elevated extracellular D-serine concentrations and enhanced NMDAr function.(61) These mice showed enhanced hippocampal long-term potentiation,(61;116) improved performance in a test of spatial learning (116) and better motor coordination as evidenced by fewer foot slips when assessed on beam walking.(61) Alternative methods to increase activation of the glycine site of NMDAr involve blockage of the high affinity D-serine transporter Asc-1 and the glycine transporter GlyT1. *GlyT1*$^{-/-}$ and *Asc-1*$^{-/-}$ knock-out mice died soon after birth, *GlyT1*$^{-/-}$ with severe respiratory and motosensory deficits (117) and *Asc-1*$^{-/-}$ from tremors and seizures.(118) The GlyT-1 heterozygote (*GlyT1*$^{+/-}$) was viable and displayed enhanced NMDAr function in CA1 pyramidal cells and improved performance in a hippocampal-dependant spatial memory task.(119) Currently, there are no behavioral data of heterozygous Asc-1 mice. Taken together, these studies of mutant mice show that enhanced NMDAr activity through increased D-serine or glycine concen-

trations influences CNS function and potentially CNS development, again underscoring the importance of proper NMDAr agonist concentrations for normal brain physiology.

CLINICAL IMPLICATIONS

As described in this chapter, it has now been generally accepted that NMDArs play important roles in CNS development, with functions in neuronal proliferation, migration, maturation and synaptogenesis. Consequently, adequate NMDAr activation is essential for normal brain architecture, development and function, including long-term potentiation, the process underlying the mechanism of learning and memory. For functional NMDAr activation, proper concentrations of its endogenous agonists are required. As such, it is not surprising that an increasing body of evidence implies that adequate D-serine concentrations are necessary for the same processes in CNS development as the NMDAr.

Up to now, only one human disease, namely 3-phosphoglycerate dehydrogenase deficiency, has been associated with D-serine deficiency in cerebrospinal fluid.(21) The severe CNS abnormalities associated with this disorder could be prevented by prenatal maternal and early postnatal treatment with oral L-serine,(112) the endogenous precursor of D-serine (Figure 1). Patients starting oral L-serine treatment later in life only showed improvement of the epilepsy, with marginal effects on head circumference and psychomotor development.(120) The window of opportunity to treat the severe disease phenotype coincides with the period of extensive brain development, maximal NMDAr activity and peaking D-serine concentrations in healthy individuals, thereby strongly suggesting normalization of D-serine concentrations and consequent restoration of NMDAr activity as the mechanism of action.

The pathophysiology of many disorders associated with CNS malformations and/or disturbed CNS function are still unknown. Analysis of D-serine concentrations in cerebrospinal fluid might increase our insight in the pathogenetic mechanisms. This would not only improve our understanding of these disorders and of normal brain physiology, but might also provide novel therapeutic strategies. In disorders associated with hypofunction of the NMDAr, such as schizophrenia, strategies to increase NMDAr activity through administration of D-serine, glycine or D-cycloserine have shown mixed but encouraging results, with beneficial effects on the positive, negative and cognitive domains of schizophrenia.(64;121;122) There are issues with the administration of D-serine and glycine, in particular with the large quantities of compound that need to be dosed to increase levels in the brain. Alternative approaches include inhibition of transporters of these amino acids to block cellular uptake, for example by inhibiting the GlyT-1 and Asc-1 transporters (123) or prevention of D-serine degradation by inhibition of DAO.(124) In analogy,

modulation of NMDAr function through the glycine-binding site might also represent a novel approach for the numerous disorders associated with excitotoxicity through NMDAr overactivity. For several reasons, clinical trials with complete NMDAr blockers have been largely unsuccessful, usually because of toxicity associated with influences on either blood pressure or psychotomimetic actions.(125) Drugs that block the glycine site of NMDArs, SR inhibitors or enzymes degrading D-serine (DAO) might be beneficial in these disorders associated with excitotoxicity and cause fewer side-effects.

CONCLUSION

Although it is only 15 years ago that D-serine was first identified in the mammalian CNS, much progress has been made in unraveling its function. As an endogenous co-agonist of the NMDAr, D-serine can be expected to play a role in all the physiological and pathological conditions in which NMDArs are involved, which in fact, is consolidated in a growing body of evidence. In this chapter, we focused on the role of D-serine in the developing CNS. Similar to the NMDAr, several studies imply roles for D-serine in neuronal proliferation, migration, maturation and synaptic plasticity. This unexpected finding of D-serine as a crucial element in human brain physiology and pathology does not only increase our insight in human CNS function, but might also yield novel diagnostic and therapeutic strategies for disorders of CNS dysfunction.

REFERENCES

1. Lamzin VS, Dauter Z, Wilson KS. How nature deals with stereoisomers. Curr Opin Struct Biol 1995 December;5(6):830-6.
2. Silverman WA. The schizophrenic career of a "monster drug". Pediatrics 2002 August;110(2 Pt 1):404-6.
3. Bren L. Frances Oldham Kelsey. FDA medical reviewer leaves her mark on history. FDA Consum 2001 March;35(2):24-9.
4. Blaschke G, Kraft HP, Fickentscher K, Kohler F. [Chromatographic separation of racemic thalidomide and teratogenic activity of its enantiomers (author's transl)]. Arzneimittelforschung 1979;29(10):1640-2.
5. Corrigan JJ. D-amino acids in animals. Science 1969 April 11;164(876):142-9.
6. Auclair JL, Patton RL. On the occurrence of d-Alanine in the haemolymph of the milkweed bug, oncopeltus fasciatus. Rev Can Biol 1950 April;9(1):3-8.
7. Beatty I, Magrath D, Ennor A. Occurrence of D-serine in lombricine. Nature 1959 February 28;183(4661):591.
8. Corrigan JJ, Srinivasan NG. The occurrence of certain D-amino acids in insects. Biochemistry 1966 April;5(4):1185-90.
9. Ennor AH, Rosenberg H, Rossiter RJ, Beatty IM, Gaffney T. The isolation and characterization of D-serine ethanolamine phosphodiester from earthworms. Biochem J 1960 April;75:179-82.
10. Kreil G. Peptides containing a D-amino acid from frogs and molluscs. J Biol Chem 1994 April 15;269(15):10967-70.
11. Okuma E, Watanabe K, Abe H. Distribution of free D-amino acids in bivalve mollusks and the effects of physiological conditions on the levels of D- and L-alanine in the tissues of hard clam, Meretrix lusoria. Fish Sci 1998;64:606-11.
12. Preston RL. Occurrence of D-amino acids in higher organisms: a survey of the distribution of D-amino acids in marine vertebrates. Comp Biochem Physiol 1987;87B:55-62.
13. Seliger HH, McElroy WD, White EH, Field GF. Stereo-specificity and firefly bioluminescence, a comparison of natural and synthetic luciferins. Proc Natl Acad Sci U S A 1961 August;47:1129-34.
14. Srinivasan NG, Corrigan JJ, Meister A. D-Serine in the blood of the silkworm Bombyx mori and other lepidoptera. J Biol Chem 1962 December;237:3844-5.
15. Yasuda-Kamatani Y, Nakamura M, Minakata H, Nomoto K, Sakiyama F. A novel cDNA sequence encoding the precursor of the D-amino acid-containing neuropeptide fulicin and multiple alpha-amidated neuropeptides from Achatina fulica. J Neurochem 1995 May;64(5):2248-55.
16. Helfman PM, Bada JL, Shou MY. Considerations on the role of aspartic acid racemization in the aging process. Gerontology 1977;23(6):419-25.
17. Dunlop DS, Neidle A, McHale D, Dunlop DM, Lajtha A. The presence of free D-aspartic acid in rodents and man. Biochem Biophys Res Commun 1986 November 26;141(1):27-32.
18. Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T et al. The presence of free D-serine in rat brain. FEBS Lett 1992 January 13;296(1):33-6.
19. Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T et al. Embryonic develop-

ment and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. J Neurochem 1993 July;61(1):348-51.
20. Fuchs SA, Klomp LW, de Koning TJ. D-amino acids in the central nervous system in health and disease. Mol Genet Metab 2005 July;85(3):168-80.
21. Fuchs SA, Dorland L, de Sain-van der Velden MG, Hendriks M, Klomp LW, Berger R et al. D-serine in the developing human central nervous system. Ann Neurol 2006 October;60(4): 476-80.
22. Hashimoto A, Nishikawa T, Oka T, Takahashi K. Endogenous D-serine in rat brain: N-methyl-D-aspartate receptor-related distribution and aging. J Neurochem 1993 February;60(2):783-6.
23. Nagata Y, Horiike K, Maeda T. Distribution of free D-serine in vertebrate brains. Brain Res 1994 January 21;634(2):291-5.
24. Hashimoto A, Oka T, Nishikawa T. Extracellular concentration of endogenous free D-serine in the rat brain as revealed by in vivo microdialysis. Neuroscience 1995 June;66(3):635-43.
25. Schell MJ, Molliver ME, Snyder SH. D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release. Proc Natl Acad Sci U S A 1995 April 25; 92(9):3948-52.
26. Schell MJ, Brady RO, Jr., Molliver ME, Snyder SH. D-serine as a neuromodulator: regional and developmental localizations in rat brain glia resemble NMDA receptors. J Neurosci 1997 March 1;17(5):1604-15.
27. Wako K, Ma N, Shiroyama T, Semba R. Glial uptake of intracerebroventricularly injected D-serine in the rat brain: an immunocytochemical study. Neurosci Lett 1995 February 13;185(3):171-4.
28. Williams SM, Diaz CM, Macnab LT, Sullivan RK, Pow DV. Immunocytochemical analysis of D-serine distribution in the mammalian brain reveals novel anatomical compartmentalizations in glia and neurons. Glia 2006 March;53(4):401-11.
29. Kartvelishvily E, Shleper M, Balan L, Dumin E, Wolosker H. Neuron-derived D-serine release provides a novel means to activate N-methyl-D-aspartate receptors. J Biol Chem 2006 May 19;281(20):14151-62.
30. Yoshikawa M, Nakajima K, Takayasu N, Noda S, Sato Y, Kawaguchi M et al. Expression of the mRNA and protein of serine racemase in primary cultures of rat neurons. Eur J Pharmacol 2006 October 24;548(1-3):74-6.
31. Yasuda E, Ma N, Semba R. Immunohistochemical evidences for localization and production of D-serine in some neurons in the rat brain. Neurosci Lett 2001 February 16;299(1-2):162-4.
32. Bruckner H, Haasmann S, Friedrich A. Quantification of D-amino acids in human urine using GC-MS and HPLC. Amino Acids 1994;6(205):211.
33. Nagata Y, Masui R, Akino T. The presence of free D-serine, D-alanine and D-proline in human plasma. Experientia 1992 October 15;48(10):986-8.
34. Rotgans J, Wodarz R, Schoknecht W, Drysch K. The determination of amino-acid enantiomers in human saliva with Chirasil-Val. Arch Oral Biol 1983;28(12):1121-4.
35. Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR et al. D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. Proc Natl Acad Sci U S A 2003 May 27;100(11):6789-94.
36. De Miranda J, Panizzutti R, Foltyn VN, Wolosker H. Cofactors of serine racemase that physiologically stimulate the synthesis of the N-methyl-D-aspartate (NMDA) receptor coagonist D-serine. Proc Natl Acad Sci U S A 2002 October 29;99(22):14542-7.

37. Wolosker H, Blackshaw S, Snyder SH. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. Proc Natl Acad Sci U S A 1999 November 9;96(23):13409-14.
38. Wolosker H, Sheth KN, Takahashi M, Mothet JP, Brady RO, Jr., Ferris CD et al. Purification of serine racemase: biosynthesis of the neuromodulator D-serine. Proc Natl Acad Sci U S A 1999 January 19;96(2):721-5.
39. de Koning TJ, Klomp LW. Serine-deficiency syndromes. Curr Opin Neurol 2004 April;17(2): 197-204.
40. Wang LZ, Zhu XZ. Spatiotemporal relationships among D-serine, serine racemase, and D-amino acid oxidase during mouse postnatal development. Acta Pharmacol Sin 2003 October;24(10):965-74.
41. De Miranda J, Santoro A, Engelender S, Wolosker H. Human serine racemase: moleular cloning, genomic organization and functional analysis. Gene 2000 October 3;256(1-2):183-8.
42. Foltyn VN, Bendikov I, De Miranda J, Panizzutti R, Dumin E, Shleper M et al. Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity. J Biol Chem 2005;280 (3):1754-63.
43. Mustafa AK, Kumar M, Selvakumar B, Ho GP, Ehmsen JT, Barrow RK et al. Nitric oxide S-nitrosylates serine racemase, mediating feedback inhibition of D-serine formation. Proc Natl Acad Sci U S A 2007 February 20;104(8):2950-5.
44. Dumin E, Bendikov I, Foltyn VN, Misumi Y, Ikehara Y, Kartvelishvily E et al. Modulation of D-serine levels via ubiquitin-dependent proteasomal degradation of serine racemase. J Biol Chem 2006 July 21;281(29):20291-302.
45. Ying-Luan Z, Zhao YL, Mori H. [Roles of D-serine in the mammalian brain]. Brain Nerve 2007 July;59(7):725-30.
46. Krebs HA. Metabolism of amino acids. III. Determination of amino acids. Biochem J 1935; 29:1620-44.
47. Still J.L, Buell M.V, Knox WE, Green DE. Studies on the cyclphorase system VII. D-Aspartate oxidase. J Biol Chem 1949;179:831-7.
48. Pollegioni L, Harris CM, Molla G, Pilone MS, Ghisla S. Identification and role of ionizing functional groups at the active center of Rhodotorula gracilis D-amino acid oxidase. FEBS Lett 2001 November 2;507(3):323-6.
49. Hashimoto A, Nishikawa T, Konno R, Niwa A, Yasumura Y, Oka T et al. Free D-serine, D-aspartate and D-alanine in central nervous system and serum in mutant mice lacking D-amino acid oxidase. Neurosci Lett 1993 April 2;152(1-2):33-6.
50. Morikawa A, Hamase K, Inoue T, Konno R, Niwa A, Zaitsu K. Determination of free D-aspartic acid, D-serine and D-alanine in the brain of mutant mice lacking D-amino acid oxidase activity. J Chromatogr B Biomed Sci Appl 2001 June 5;757(1):119-25.
51. D'Aniello A, Vetere A, Petrucelli L. Further study on the specificity of D-amino acid oxidase and D-aspartate oxidase and time course for complete oxidation of D-amino acids. Comp Biochem Physiol B 1993 July;105(3-4):731-4.
52. Cline MJ, Lehrer RI. D-amino acid oxidase in leukocytes: a possible D-amino-acid-linked antimicrobial system. Proc Natl Acad Sci U S A 1969 March;62(3):756-63.
53. Tada M, Fukui K, Momoi K, Miyake Y. Cloning and expression of a cDNA encoding mouse kidney D-amino acid oxidase. Gene 1990 June 15;90(2):293-7.

54. Nagata Y, Konno R, Yasumura Y, Akino T. Involvement of D-amino acid oxidase in elimination of free D-amino acids in mice. Biochem J 1989 January 1;257(1):291-2.
55. Urai Y, Jinnouchi O, Kwak KT, Suzue A, Nagahiro S, Fukui K. Gene expression of D-amino acid oxidase in cultured rat astrocytes: regional and cell type specific expression. Neurosci Lett 2002 May 17;324(2):101-4.
56. Momoi K, Fukui K, Watanabe F, Miyake Y. Molecular cloning and sequence analysis of cDNA encoding human kidney D-amino acid oxidase. FEBS Lett 1988 September 26;238(1):180-4.
57. Molla G, Sacchi S, Bernasconi M, Pilone MS, Fukui K, Polegioni L. Characterization of human D-amino acid oxidase. FEBS Lett 2006 April 17;580(9):2358-64.
58. Kawazoe T, Tsuge H, Pilone MS, Fukui K. Crystal structure of human D-amino acid oxidase: context-dependent variability of the backbone conformation of the VAAGL hydrophobic stretch located at the si-face of the flavin ring. Protein Sci 2006 December;15(12):2708-17.
59. Pollegioni L, Piubelli L, Sacchi S, Pilone MS, Molla G. Physiological functions of D-amino acid oxidases: from yeast to humans. Cell Mol Life Sci 2007 June;64(11):1373-94.
60. Adage T, Trillat AC, Quattropani A, Perrin D, Cavarec L, Shaw J et al. In vitro and in vivo pharmacological profile of AS057278, a selective d-amino acid oxidase inhibitor with potential anti-psychotic properties. : Eur Neuropsychopharmacol 2008 Mar;18(3):200-14.
61. Almond SL, Fradley RL, Armstrong EJ, Heavens RB, Rutter AR, Newman RJ et al. Behavioral and biochemical characterization of a mutant mouse strain lacking D-amino acid oxidase activity and its implications for schizophrenia. Mol Cell Neurosci 2006 August;32(4):324-34.
62. Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C et al. Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. Proc Natl Acad Sci U S A 2003 December 9;100(25):15194-9.
63. Danysz W, Parsons AC. Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol Rev 1998 December;50(4):597-664.
64. Goff DC, Coyle JT. The emerging role of glutamate in the pathophysiology and treatment of schizophrenia. Am J Psychiatry 2001 September;158(9):1367-77.
65. Mohn AR, Gainetdinov RR, Caron MG, Koller BH. Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. Cell 1999 August 20;98(4):427-36.
66. Contreras PC. D-serine antagonized phencyclidine- and MK-801-induced stereotyped behavior and ataxia. Neuropharmacology 1990 March;29(3):291-3.
67. Takahashi T, Feldmeyer D, Suzuki N, Onodera K, Cull-Candy SG, Sakimura K et al. Functional correlation of NMDA receptor epsilon subunits expression with the properties of single-channel and synaptic currents in the developing cerebellum. J Neurosci 1996 July 15;16(14):4376-82.
68. Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. Pharmacol Rev 1999 March;51(1):7-61.
69. Johnson JW, Ascher P. Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 1987 February 5;325(6104):529-31.
70. Kleckner NW, Dingledine R. Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. Science 1988 August 12;241(4867):835-7.
71. Thomson AM. Glycine is a coagonist at the NMDA receptor/channel complex. Prog Neurobiol 1990;35(1):53-74.
72. Danysz W, Fadda E, Wroblewski JT, Costa E. [3H]D-serine labels strychnine-insensitive glycine recognition sites of rat central nervous system. Life Sci 1990;46(3):155-64.

73. Matsui T, Sekiguchi M, Hashimoto A, Tomita U, Nishikawa T, Wada K. Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration. J Neurochem 1995 July;65(1):454-8.
74. Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD et al. D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 2000 April 25;97(9):4926-31.
75. Ahmadi S, Muth-Selbach U, Lauterbach A, Lipfert P, Neuhuber WL, Zeilhofer HU. Facilitation of spinal NMDA receptor currents by spillover of synaptically released glycine. Science 2003 June 27;300(5628):2094-7.
76. Daly EC, Aprison MH. Distribution of serine hydroxymethyltransferase and glycine transaminase in several areas of the central nervous system of the rat. J Neurochem 1974 June;22(6):877-85.
77. Snyder SH, Kim PM. D-amino acids as putative neurotransmitters: focus on D-serine. Neurochem Res 2000 May;25(5):553-60.
78. Panigrahy A, Rosenberg PA, Assmann S, Foley EC, Kinney HC. Differential expression of glutamate receptor subtypes in human brainstem sites involved in perinatal hypoxia-ischemia. J Comp Neurol 2000 November 13;427(2):196-208.
79. Piggott MA, Perry EK, Perry RH, Court JA. [3H]MK-801 binding to the NMDA receptor complex, and its modulation in human frontal cortex during development and aging. Brain Res 1992 August 21;588(2):277-86.
80. Slater P, McConnell SE, D'Souza SW, Barson AJ. Postnatal changes in N-methyl-D-aspartate receptor binding and stimulation by glutamate and glycine of [3H]-MK-801 binding in human temporal cortex. Br J Pharmacol 1993 April;108(4):1143-9.
81. Chahal H, D'Souza SW, Barson AJ, Slater P. Modulation by magnesium of N-methyl-D-aspartate receptors in developing human brain. Arch Dis Child Fetal Neonatal Ed 1998 March;78(2):F116-F120.
82. Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M et al. Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. J Biol Chem 1993 February 5;268(4):2836-43.
83. Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 1994 March;12(3):529-40.
84. Akazawa C, Shigemoto R, Bessho Y, Nakanishi S, Mizuno N. Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. J Comp Neurol 1994 September 1;347(1):150-60.
85. Crair MC, Malenka RC. A critical period for long-term potentiation at thalamocortical synapses. Nature 1995 May 25;375(6529):325-8.
86. Feldman DE, Nicoll RA, Malenka RC, Isaac JT. Long-term depression at thalamocortical synapses in developing rat somatosensory cortex. Neuron 1998 August;21(2):347-57.
87. Ramoa AS, McCormick DA. Enhanced activation of NMDA receptor responses at the immature retinogeniculate synapse. J Neurosci 1994 April;14(4):2098-105.
88. Aamodt SM, Constantine-Paton M. The role of neural activity in synaptic development and its implications for adult brain function. Adv Neurol 1999;79:133-44.
89. McBain CJ, DiChiara TJ, Kauer JA. Activation of metabotropic glutamate receptors differen-

tially affects two classes of hippocampal interneurons and potentiates excitatory synaptic transmission. J Neurosci 1994 July;14(7):4433-45.
90. Scheetz AJ, Constantine-Paton M. Modulation of NMDA receptor function: implications for vertebrate neural development. FASEB J 1994 July;8(10):745-52.
91. Waters KA, Machaalani R. NMDA receptors in the developing brain and effects of noxious insults. Neurosignals 2004 July;13(4):162-74.
92. Gould E, Cameron HA, McEwen BS. Blockade of NMDA receptors increases cell death and birth in the developing rat dentate gyrus. J Comp Neurol 1994 February 22;340(4):551-65.
93. Gould E, Cameron HA. Early NMDA receptor blockade impairs defensive behavior and increases cell proliferation in the dentate gyrus of developing rats. Behav Neurosci 1997 February;111(1):49-56.
94. Cameron HA, McEwen BS, Gould E. Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. J Neurosci 1995 June;15(6):4687-92.
95. Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K et al. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 1999 January 1;283(5398):70-4.
96. Komuro H, Rakic P. Modulation of neuronal migration by NMDA receptors. Science 1993 April 2;260(5104):95-7.
97. Haberny KA, Paule MG, Scallet AC, Sistare FD, Lester DS, Hanig JP et al. Ontogeny of the N-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. Toxicol Sci 2002 July;68(1):9-17.
98. LoTurco JJ, Blanton MG, Kriegstein AR. Initial expression and endogenous activation of NMDA channels in early neocortical development. J Neurosci 1991 March;11(3):792-9.
99. Rossi DJ, Slater NT. The developmental onset of NMDA receptor-channel activity during neuronal migration. Neuropharmacology 1993 November;32(11):1239-48.
100. Behar TN, Scott CA, Greene CL, Wen X, Smith SV, Maric D et al. Glutamate acting at NMDA receptors stimulates embryonic cortical neuronal migration. J Neurosci 1999 June 1;19(11): 4449-61.
101. Hirai K, Yoshioka H, Kihara M, Hasegawa K, Sakamoto T, Sawada T et al. Inhibiting neuronal migration by blocking NMDA receptors in the embryonic rat cerebral cortex: a tissue culture study. Brain Res Dev Brain Res 1999 April 12;114(1):63-7.
102. Brooks WJ, Petit TL, Leboutillier JC, Lo R. Rapid alteration of synaptic number and postsynaptic thickening length by NMDA: an electron microscopic study in the occipital cortex of postnatal rats. Synapse 1991 May;8(1):41-8.
103. Brooks WJ, Petit TL, Leboutillier JC. Effect of chronic administration of NMDA antagonists on synaptic development. Synapse 1997 June;26(2):104-13.
104. Contestabile A. Roles of NMDA receptor activity and nitric oxide production in brain development. Brain Res Brain Res Rev 2000 April;32(2-3):476-509.
105. Brooks WJ, Weeks AC, Leboutillier JC, Petit TL. Altered NMDA sensitivity and learning following chronic developmental NMDA antagonism. Physiol Behav 1997 November;62(5): 955-62.
106. Tsien JZ, Huerta PT, Tonegawa S. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell 1996 December 27;87(7):1327-38.
107. Niewoehner B, Single FN, Hvalby O, Jensen V, Borgloh SM, Seeburg PH et al. Impaired

spatial working memory but spared spatial reference memory following functional loss of NMDA receptors in the dentate gyrus. Eur J Neurosci 2007 February;25(3):837-46.

108. Hashimoto A, Oka T, Nishikawa T. Anatomical distribution and postnatal changes in endogenous free D-aspartate and D-serine in rat brain and periphery. Eur J Neurosci 1995 August 1;7(8):1657-63.
109. Horiike K, Tojo H, Arai R, Yamano T, Nozaki M, Maeda T. Localization of D-amino acid oxidase in Bergmann glial cells and astrocytes of rat cerebellum. Brain Res Bull 1987 November;19(5): 587-96.
110. Rabacchi S, Bailly Y, Delhaye-Bouchaud N, Mariani J. Involvement of the N-methyl D-aspartate (NMDA) receptor in synapse elimination during cerebellar development. Science 1992 June 26;256(5065):1823-5.
111. Kim PM, Aizawa H, Kim PS, Huang AS, Wickramasinghe SR, Kashani AH et al. Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. Proc Natl Acad Sci U S A 2005 February 8;102(6): 2105-10.
112. de Koning TJ, Klomp LW. Prenatal and early postnatal treatment in 3-phosphoglycerate-dehydrogenase deficiency. 2004 December 18;364(9452):2221-2.
113. Yoshida K, Furuya S, Osuka S, Mitoma J, Shinoda Y, Watanabe M et al. Targeted disruption of the mouse 3-phosphoglycerate dehydrogenase gene causes severe neurodevelopmental defects and results in embryonic lethality. J Biol Chem 2004 January 30;279(5):3573-7.
114. Diaz CM, Macnab LT, Williams SM, Sullivan RK, Pow DV. EAAT1 and D-serine expression are early features of human retinal development. Exp Eye Res 2007 May;84(5):876-85.
115. Puyal J, Martineau M, Mothet JP, Nicolas MT, Raymond J. Changes in D-serine levels and localization during postnatal development of the rat vestibular nuclei. J Comp Neurol 2006 August 1;497(4):610-21.
116. Maekawa M, Watanabe M, Yamaguchi S, Konno R, Hori Y. Spatial learning and long-term potentiation of mutant mice lacking D-amino-acid oxidase. Neurosci Res 2005 September;53(1):34-8.
117. Gomeza J, Hulsmann S, Ohno K, Eulenburg V, Szoke K, Richter D et al. Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. Neuron 2003 November 13;40(4):785-96.
118. Xie X, Dumas T, Tang L, Brennan T, Reeder T, Thomas W et al. Lack of the alanine-serine-cysteine transporter 1 causes tremors, seizures, and early postnatal death in mice. Brain Res 2005 August 9;1052(2):212-21.
119. Tsai G, Ralph-Williams RJ, Martina M, Bergeron R, Berger-Sweeney J, Dunham KS et al. Gene knockout of glycine transporter 1: characterization of the behavioral phenotype. Proc Natl Acad Sci U S A 2004 June 1;101(22):8485-90.
120. de Koning TJ. Treatment with amino acids in serine deficiency disorders. J Inherit Metab Dis 2006 April;29(2-3):347-51.
121. Heresco-Levy U. Glutamatergic neurotransmission modulators as emerging new drugs for schizophrenia. Expert Opin Emerg Drugs 2005 November;10(4):827-44.
122. Tsai G, Yang P, Chung LC, Lange N, Coyle JT. D-serine added to antipsychotics for the treatment of schizophrenia. Biol Psychiatry 1998 December 1;44(11):1081-9.

123. Sur C, Kinney GG. The therapeutic potential of glycine transporter-1 inhibitors. Expert Opin Investig Drugs 2004 May;13(5):515-21.
124. Konno R, Yasumura Y. D-amino-acid oxidase and its physiological function. Int J Biochem 1992 April;24(4):519-24.
125. Kemp JA, McKernan RM. NMDA receptor pathways as drug targets. Nat Neurosci 2002 November;5 Suppl:1039-42.

Chapter 5

D-serine influences synaptogenesis in a P19 cell model

S.A. Fuchs, M.W. Roeleveld, L.W.J. Klomp, R. Berger, T.J. de Koning

Submitted

ABSTRACT

To investigate the role of NMDA receptor co-agonist D-serine in central nervous system development, we studied rat P19 cells, an established model for neuronal and glial differentiation, showing that 1. the D-serine synthesizing enzyme serine racemase was expressed upon differentiation, 2. extracellular D-serine concentrations increased upon differentiation, which was inhibited by serine racemase antagonism and 3. inhibition of D-serine synthesis or prevention of D-serine binding to the NMDA receptor increased synaptophysin expression and intercellular connections, supporting a role for NMDA receptor activation by D-serine, synthesized by serine racemase, in shaping synaptogenesis and neuronal circuitry during central nervous system development.

INTRODUCTION

Central nervous system (CNS) development is a complex process, in which N-Methyl D-Aspartate receptors (NMDArs) play an essential role; NMDAr activation is involved in neuronal migration, proliferation, maturation and survival, dendritic outgrowth, synaptic formation, brain plasticity and the onset of long-term potentiation.(1) For activation, NMDArs require simultaneous binding by glutamate to their NR2 subunit and glycine or D-serine to their NR1 subunit. D-serine appears to be the main co-agonist in most areas of human CNS,(2) where it can be synthesized from L-serine by serine racemase (SR) and metabolized by D-amino acid oxydase (DAO).

As an endogenous NMDAr agonist, D-serine is likely to be involved in CNS development. This is supported by specifically elevated D-serine concentrations in human and rodent CNS during the intense period of embryonic and early postnatal CNS development, which coincides with a transient expression and increased activity of NMDArs.(1) The severe CNS abnormalities upon failure to achieve these high D-serine concentrations, as seen in patients (3) and mutant mice (4) with 3-phosphoglycerate dehydrogenase (3-PGDH) deficiency, a rare inherited disorder in L-serine and hence D-serine synthesis, underscore the putative role of D-serine in CNS development. Degradation of D-serine by DAO and selective inhibition of SR in 8-day old mouse cerebellar slices significantly reduced granule cell migration, whereas D-serine activated this.(5) However, no evidence of disrupted neuronal migration was observed in mutant mice with a targeted disruption in exon 1 of SR, thereby lacking the ability to produce D-serine endogenously.(6) These mice displayed altered glutamatergic neurotransmission and attenuated synaptic plasticity and subtle behavioural and memory abnormalities.

To clarify the role of D-serine in CNS development, we studied rat P19 cells, an established model for neuronal and glial differentiation.(7) Neurons developing from these cells strongly resemble normal mammalian embryonic neurons, with functional glutamatergic receptors (NR1, NR2A/B, AMPA/kainate receptors and non-NMDArs (GluR1-4)). In this model, D-serine was actively synthesized by SR upon differentiation and appeared to shape synaptogenesis, potentially by preventing widespread untargeted synaptogenesis. As an NMDAr co-agonist, our results contribute to the expanding evidence indicating a role for NMDAr activation in synaptic shaping and wiring of neuronal circuitry,(8-10) and provide new evidence for a role of D-serine and SR in this process.

MATERIALS AND METHODS

Cell differentiation

Confluent P19 cells were plated 1:30 in bacterial dishes in 2 ml F12/DMEM Glutamax medium (Dulbecco), containing 10% fetal calf serum and 100µg/ml penicillin/streptomycin (Gibco Life Technologies). To induce differentiation, all-trans retinoic acid (RA, Sigma Aldrich) in DMSO was added (1µM). Control cells received DMSO without RA. Different conditions were applied by adding nothing, the SR antagonist L-serine-O-sulphate (LSOS, Sigma-Aldrich) (100µM), the competitive NR1 antagonist dichlorokynurenic acid monohydrate (DCKA, Sigma-Aldrich) (30µM) and a rescue with D-serine (Sigma-Aldrich) (10µM for LSOS, 50µM for DCKA). On day 5, cells were replated in 0.1% gelatine coated 6-well plates. Every 3-4 days, medium was changed and the different conditions were applied as before. On day 10, cytosine β-D-arabinofuranoside (ARA-C, Sigma-Aldrich) was added (20µM) to increase the proportion of neuronal cells. On day 14-17 supernatant was withdrawn for analysis, cells were photographed, harvested in Laemli sample buffer and stored at -80°C for further analyses. All experiments were performed in duplo on 7 different occasions (n=7x2).

D-serine analysis

D-serine was quantified according to the stable isotope dilution LC-MS method described previously.(11)

Western blots

Cells were lysed in Laemli sample buffer, subjected to SDS-PAGE and electrotransferred to Immobilon membranes (Millipore). Purified mouse anti-SR antibody (BD Biosciences), mouse neuron specific β-III-tubulin antibody, rabbit glial fibrillary acidic protein (GFAP) antibody and rabbit synaptophysin (neuronal presynaptic membrane protein) antibody (all from Abcam) were used to probe for the respective proteins. These were visualized by HRP-conjugated secondary antibodies and ECL (Amersham Biosciences).

RESULTS

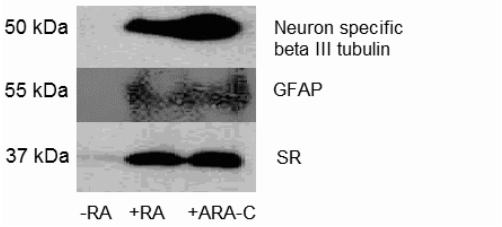
1. Undifferentiated P19 cells

Undifferentiated P19 cells did not express the neuronal marker neuron specific β-III-tubulin, the glial marker GFAP nor SR (Figure 1A) and did not excrete D-serine in supernatant (Figure 1B). In fact, D-serine concentrations in supernatant were somewhat lower than in medium (3.13μM).

2. Differentiated P19 cells

We observed dendritic outgrowth from P19 cells upon differentiation, suggesting the emergence of neurons and/or astrocytes (Figure 2). Differentiated P19 cells expressed neuron specific β-III-tubulin, GFAP and SR and expression of the neuronal marker increased when differentiation was driven towards neurons with ARA-C, (Figure 1A) as opposed to GFAP and SR. Fifteen days after inducing differentiation, extracellular D-serine concentrations increased significantly, which was prevented by LSOS (Figure 1B). Together, these results demonstrate that P19 cells were differentiated into neurons and glia expressing SR, subsequently leading to D-serine synthesis.

A.



B.

	Day 1	Day 5	Day 8	Day 11	Day 15
RA + ARA-C	2.88	3.23	2.27	1.89	7.30
+ LSOS	2.88	3.26	2.51	2.68	2.77

Figure 1. Expression of neuron specific β-III-tubulin, GFAP and functional SR upon differentiation. The upper panel (1A) represents a western blot, that shows expression of neuron specific β-III-tubulin (neuronal marker), glial fibrillary acidic protein (GFAP, glial marker) and serine racemase (SR) in undifferentiated cells (without addition of retinoic acid (-RA), lane 1), differentiated cells (after addition of RA, lane 2) and differentiated cells with additional treatment with cytosine β-D-arabinofuranoside (ARA-C, lane 3) to increase neuronal cell proportion.
The lower panel (1B) depicts D-serine concentrations in supernatant (μM), as determined by LC-MS, during differentiation with RA and ARA-C without addition of the SR inhibitor LSOS (RA+ARA-C) or after addition of LSOS to a concentration of 100 μM (+LSOS). D-serine concentration in medium (not subjected to cells) was 3.13μM.

3. Is D-serine synthesis by SR cause or consequence of differentiation?

To distinguish between these options, we attempted to create conditions without D-serine induced NMDAr activation by inhibiting D-serine synthesis (LSOS) and D-serine binding to NMDArs (DCKA). Figure 2 demonstrates that differentiation was not prevented by LSOS or DCKA. Interestingly, dendritic outgrowths appeared less concentrated and cells less interconnected upon exogenous D-serine addition, while dendritic outgrowths appeared more concentrated and cells more interconnected upon treatment with LSOS and DCKA. D-serine appeared to overcome this in the sample treated with LSOS, but not in the sample treated with DCKA. Similarly, western blots of these samples (Figure 3) showed decreased synaptophysin expression upon exogenous D-serine addition and increased expression of synaptophysin upon treatment with LSOS and DCKA, when

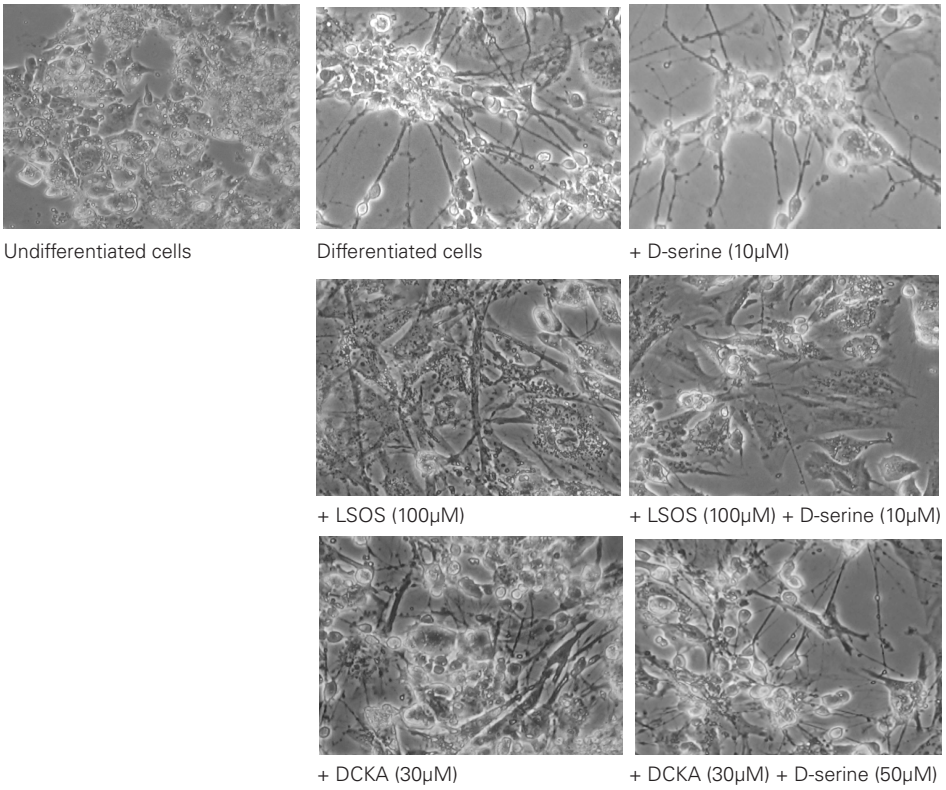


Figure 2. Differentiation in the presence and absence of D-serine induced NMDAr activation. The top panels show undifferentiated and differentiated cells (at day 15) under normal conditions and with exogenous supplementation of D-serine. In order to simulate conditions without NMDAr activation by D-serine, LSOS, a SR antagonist (middle panel) and DCKA, an antagonist of the NR1 subunit of the NMDAr (lower panel) were employed. By addition of D-serine, we attempted to rescue these conditions.

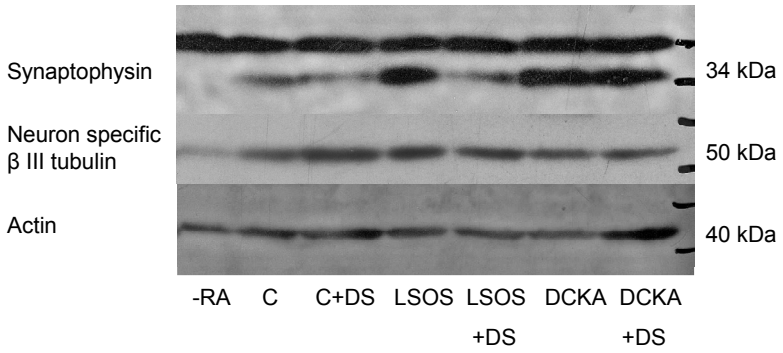


Figure 3. Expression of synaptophysin in the presence or absence of D-serine induced NMDAr activation. This western blot shows the expression of synaptophysin, neuron specific β-III-tubulin and loading control actin in undifferentiated P 19 cells (-RA, lane 1), differentiated cells (C, lane 2), differentiated cells with additional exogenous D-serine to 10μM (C+DS, lane 3), differentiated cells in the presence of the SR antagonist LSOS (LSOS, lane 4), the rescue of the former with D-serine to 10μM (LSOS+DS), differentiated cells in the presence of the NR1 antagonist DCKA (DCKA, lane 5) and the rescue of the latter with D-serine to 50μM (DCKA+DS).

compared with differentiated P19 cells with normal NMDAr activation. D-serine rescue normalized synaptophysin expression in LSOS treated cells, but not in DCKA treated cells.

DISCUSSION

In this study, we induced and visualized P19 cell differentiation into neurons and glia and evidenced for the first time D-serine synthesis by SR upon differentiation. Similar to GFAP, SR expression was not evidently increased by ARA-C, concurring with the predominant glial localization of D-serine and SR.(12) Inhibition of D-serine synthesis or D-serine binding to NMDArs increased synaptophysin expression and the former was overcome by D-serine supplementation. This might signify that suppression of NMDAr activation by decreased D-serine synthesis or NR1 antagonism leads to increased synaptic formation. This concurs with the finding that NMDAr activation is critical in the regression of functional synapses in the developing rat cerebellum.(8) Similarly, partial deletion of NR1 subunits of NMDArs in organotypic hippocampal cells profoundly increased numbers of functional synapses between neurons and strength of unitary connections *in vitro* and *in vivo*.(10) Conversely, reintroduction of NMDArs in NR1-deficient neurons reduced the number of functional inputs. Based on these findings, the authors proposed a new model for maturation of excitatory synapses in which ongoing activation of NMDArs prevents premature synaptic maturation by ensuring that only punctuated bursts of activity lead to induction of functional synapses for the activity-dependent wiring of neuronal circuitry.

(10) Our results imply that endogenous D-serine might be a crucial factor in activating NMDArs, thereby preventing premature synaptic maturation.

Evaluation of CNS development in SR knock-out mice is interesting in this respect. These mice display attenuated synaptic plasticity, a spatial memory deficit and subtle behavioural abnormalities, including mild hyperactivity and increased anxiety.(6) According to our results, this might be attributable to differences in synaptic shaping induced by decreased D-serine concentrations. In fact, NMDAr antagonists influenced rat hippocampal mossy fiber synaptogenesis and inhibited spatial learning.(13) Similarly, NMDArs seem to be involved in synaptic plasticity in the amygdala, which appears to play a role in anxiety.(14) Since no structural CNS abnormalities were described in the SR knock-out mice, a vast migration defect induced by D-serine depletion seems less likely, but compensations in the constitutive knockout and residual D-serine concentrations (10% of the wild type (6)) may occlude the effects of D-serine depletion.

Schizophrenia has been strongly associated with both altered synaptic shaping (15) and decreased D-serine concentrations, alterations in D-serine synthesizing and metabolizing enzymes and genes encoding for these enzymes.(16) Our results putatively link altered synaptic shaping and decreased D-serine concentrations in schizophrenia. Similarly, in bipolar disorder, an increase in synaptophysin and synaptosomal-associated protein-25 was observed in post-mortem brains, when compared to control brains,(17) which, considering our results, might be due to decreased D-serine concentrations, caused by altered D-serine metabolism, because genes coding for enzymes associated with D-serine metabolism have been implicated in bipolar disorder.(16) Likewise, D-serine showed some anxiolytic properties in patients with post-traumatic stress disorder,(18) suggesting an absolute or relative D-serine deficiency, potentially leading to altered synaptic plasticity, which has been associated with anxiety.(14)

Support for our finding of D-serine in a regulatory role in tissue development comes from chondrogenesis, where SR negatively regulated maturation in chondrocytes.(19) D-Serine suppressed several chondrocytic maturation markers in rat chondrocytes and delayed chondral mineralization in mouse metatarsals.(20) D-Serine, synthesized by SR, may thus negatively regulate chondrocyte differentiation, similar to the negative regulatory activity during neuronal differentiation in our results.

In conclusion, our P19 cell studies showed that 1. SR was expressed upon differentiation, 2. extracellular D-serine concentrations increased upon differentiation, which was inhibited by SR antagonism, 3. inhibition of D-serine synthesis or prevention of D-serine binding to NMDArs appeared to lead to altered synaptogenesis, supporting a role for NMDAr activation by D-serine, synthesized by SR, in shaping synaptogenesis and neuronal circuitry.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from The Netherlands Organisation for Health Research and Development (grant 920-03-345). We thank O. van Beekum for directing our attention to and supplying us with rat P19 cells.

REFERENCES

1. Fuchs SA, Klomp LWJ, de Koning TJ. D-serine in the developing human central nervous system: Clinical implications. In: Heresco-Levy U, Javitt DC, editors. Glutamate in Neuropsychiatric Disorders.Kerala, India: Research Signpost; 2008. p. 75-97.
2. Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD et al. D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 2000 April 25;97(9):4926-31.
3. de Koning TJ, Klomp LW, van Oppen AC, Beemer FA, Dorland L, van dB, I et al. Prenatal and early postnatal treatment in 3-phosphoglycerate-dehydrogenase deficiency. Lancet 2004 December 18;364(9452):2221-2.
4. Yoshida K, Furuya S, Osuka S, Mitoma J, Shinoda Y, Watanabe M et al. Targeted disruption of the mouse 3-phosphoglycerate dehydrogenase gene causes severe neurodevelopmental defects and results in embryonic lethality. J Biol Chem 2004 January 30;279(5):3573-7.
5. Kim PM, Aizawa H, Kim PS, Huang AS, Wickramasinghe SR, Kashani AH et al. Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. Proc Natl Acad Sci U S A 2005 February 8;102(6):2105-10.
6. Basu AC, Tsai GE, Ma CL, Ehmsen JT, Mustafa AK, Han L et al. Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. Mol Psychiatry 2009 July;14(7):719-27.
7. Bain G, Ray WJ, Yao M, Gottlieb DI. From embryonal carcinoma cells to neurons: the P19 pathway. Bioessays 1994 May;16(5):343-8.
8. Rabacchi S, Bailly Y, Delhaye-Bouchaud N, Mariani J. Involvement of the N-methyl D-aspartate (NMDA) receptor in synapse elimination during cerebellar development. Science 1992 June 26;256(5065):1823-5.
9. Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C et al. Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. Proc Natl Acad Sci U S A 2003 December 9;100(25):15194-9.
10. Adesnik H, Li G, During MJ, Pleasure SJ, Nicoll RA. NMDA receptors inhibit synapse unsilencing during brain development. Proc Natl Acad Sci U S A 2008 April 8;105(14):5597-602.
11. Fuchs SA, de Sain-van der Velden MG, de Barse MM, Roeleveld MW, Hendriks M, Dorland L et al. Two mass-spectrometric techniques for quantifying serine enantiomers and glycine in cerebrospinal fluid: potential confounders and age-dependent ranges. Clin Chem 2008 September;54(9):1443-50.
12. Schell MJ, Molliver ME, Snyder SH. D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release. Proc Natl Acad Sci U S A 1995 April 25; 92(9):3948-52.
13. Ramirez-Amaya V, Balderas I, Sandoval J, Escobar ML, Bermudez-Rattoni F. Spatial long-term memory is related to mossy fiber synaptogenesis. J Neurosci 2001 September 15;21(18): 7340-8.
14. Pape HC, Pare D. Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol Rev 2010 April;90(2):419-63.
15. Eastwood SL. The synaptic pathology of schizophrenia: is aberrant neurodevelopment and plasticity to blame? Int Rev Neurobiol 2004;59:47-72.

16. Detera-Wadleigh SD, McMahon FJ. G72/G30 in schizophrenia and bipolar disorder: review and meta-analysis. Biol Psychiatry 2006 July 15;60(2):106-14.
17. Scarr E, Gray L, Keriakous D, Robinson PJ, Dean B. Increased levels of SNAP-25 and synaptophysin in the dorsolateral prefrontal cortex in bipolar I disorder. Bipolar Disord 2006 April;8(2):133-43.
18. Heresco-Levy U, Vass A, Bloch B, Wolosker H, Dumin E, Balan L et al. Pilot controlled trial of D-serine for the treatment of post-traumatic stress disorder. Int J Neuropsychopharmacol 2009 October;12(9):1275-82.
19. Takarada T, Hinoi E, Takahata Y, Yoneda Y. Serine racemase suppresses chondrogenic differentiation in cartilage in a Sox9-dependent manner. J Cell Physiol 2008 May;215(2):320-8.
20. Takarada T, Takahata Y, Iemata M, Hinoi E, Uno K, Hirai T et al. Interference with cellular differentiation by D-serine through antagonism at N-methyl-D-aspartate receptors composed of NR1 and NR3A subunits in chondrocytes. J Cell Physiol 2009 September;220(3):756-64.

D-serine in human pathology

Chapter 6

Increased concentrations of both NMDA receptor co-agonists D-serine and glycine in global ischemia: a potential novel treatment target for perinatal asphyxia

S.A. Fuchs, C.M.P.C.D. Peeters-Scholte, M.M.J. de Barse, M.W. Roeleveld, L.W.J. Klomp, R. Berger, T.J. de Koning

Submitted

ABSTRACT

Worldwide, perinatal asphyxia is an important cause of morbidity and mortality among term-born children. Overactivation of the N-Methyl-D-Aspartate receptor (NMDAr) plays a central role in the pathogenesis of cerebral hypoxia-ischemia, but the role of both endogenous NMDAr co-agonists D-serine and glycine remains largely elusive. *Methods:* We investigated D-serine and glycine concentration changes in 1. Rat glioma cells, subjected to oxygen and glucose deprivation (OGD), 2. cerebrospinal fluid (CSF) from human newborns affected by perinatal asphyxia 3. CSF from piglets exposed to hypoxia-ischemia by occlusion of both carotid arteries and hypoxia. *Results:* Extracellular concentrations of glycine and D-serine were markedly increased in rat glioma cells exposed to OGD, presumably through increased synthesis from L-serine. Upon reperfusion glycine concentrations normalized and D-serine concentrations were significantly lowered. The *in vivo* studies corroborated the finding of initially elevated and then normalizing concentrations of glycine and decreased D-serine concentrations upon reperfusion. *Conclusion*: Global cerebral ischemia leads to significant increases of both endogenous NMDAr co-agonists, which, in combination with elevated glutamate concentrations, are bound to lead to massive NMDAr activation, excitotoxicity and neuronal damage. Influencing these NMDAr co-agonist concentrations provides an interesting treatment target for this common, devastating and currently untreatable condition.

INTRODUCTION

Perinatal asphyxia is the consequence of disturbed gas exchange between mother and fetus, for example after placental pathology or umbilical cord accidents. The impairment in blood supply leads to insufficient delivery of oxygen, glucose and other blood-borne fuels to fetal organs, including the brain. The short-term clinical consequences include persistent low Apgar scores, multi-organ failure and neurological sequelae. In the long-term, this might result in cerebral palsy, mental retardation, visual and acoustic impairment and epilepsy.(1, 2)

Perinatal asphyxia is one of the most important causes of morbidity and mortality among term-born children and in spite of improvements in perinatal care, the incidence and outcome remain relatively unchanged. Of the yearly estimated 4 million neonatal deaths worldwide, approximately 1 million is caused by perinatal asphyxia.(3) Of infants with moderate encephalopathy, ten percent die, and thirty percent of those who survive, have disabilities. Among infants with severe encephalopathy, sixty percent die, and many, if not all survivors are handicapped.(1, 2) Treatment is currently aimed at supportive intensive care, recently combined with hypothermia.(4)

It has been firmly established that excessive excitation of the N-methyl-D-aspartate receptor (NMDAr) plays a central role in the pathogenesis of hypoxic-ischemic damage, especially in the developing brain.(5) The NMDAr requires simultaneous binding by glutamate and a co-agonist for activation. It had long been assumed that glycine was the endogenous co-agonist. However, recent studies have shown that D-serine is the main endogenous co-agonist for most NMDAr subtypes in most brain areas.(6) Since NMDArs appear not to be saturated during physiological conditions,(7) elevations in glycine or D-serine concentrations may lead to increased toxicity during pathological conditions, such as hypoxia-ischemia. The synthesizing (8) and metabolizing (9) enzymes of D-serine and glycine have recently been characterized (Figure 1). By pharmacological manipulation, this might yield new potential candidates for pharmacological intervention after perinatal asphyxia.

Despite this exciting hypothesis, most research has focused on glutamate, and the contribution of D-serine and glycine to the pathogenesis of perinatal asphyxia remains largely elusive. Therefore, the aim of this study was to investigate D-serine and glycine concentration changes in three different models for global ischemia.

1. Cell cultures: we subjected rat glioma cells to oxygen-glucose deprivation (OGD), a commonly used *in-vitro* model for global ischemia, although duration and depth of oxygen and glucose deprivation vary between studies.(10) Although neurons are far more susceptible to ischemic brain damage than neighboring astrocytes, glioma cells were studied, because of emerging evidence that astrocytes contribute substantially to neuronal fate in cerebral hypoxia-ischemia,(11) for instance by releasing neurotransmitters such as glutamate and serving as lactate shuttles for neurons.(12)

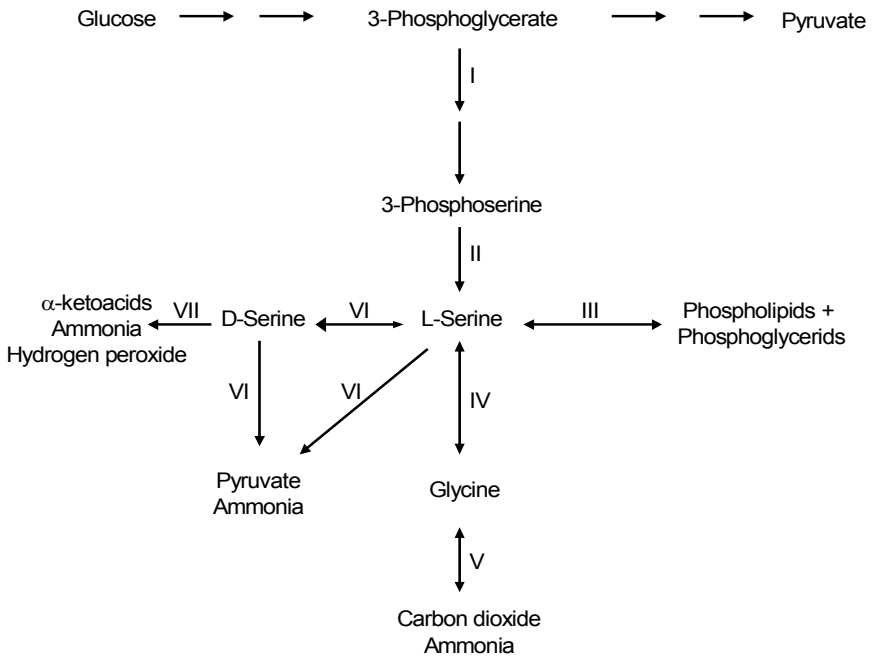


Figure 1. Pathways involved in L-serine and D-serine biosynthesis, catabolism and exogenous sources. I= 3-phosphoglycerate dehydrogenase, II= 3-phosphoserine phosphatase, III= synthesis of phospholipids and phosphoglycerides from cytidine diphosphodiacylglycerol and palmitoyl-coenzyme A, IV = serine hydroxymethyltransferase, V = glycine cleavage system, VI = serine racemase, VII= D-amino acid oxidase.

2. Human newborns: analysis of cerebrospinal fluid (CSF) from newborns with and without perinatal asphyxia. Despite being a global marker for concentration differences in brain regions, CSF is the most accessible central nervous system marker in these critically ill newborns.

3. Piglets: analysis of CSF from piglets exposed to hypoxia-ischemia by occlusion of both common carotid arteries, combined with hypoxia. Piglet brains correspond well with those of term-born neonates,(13) and this animal model is commonly used to study global ischemia.(14, 15)

Studies in our *in vitro* and *in vivo* models demonstrated time dependent changes in D-serine and glycine concentrations upon hypoxia-ischemia and reperfusion. The initial increases in concentrations of both NMDAr co-agonists, together with the known increases in extracellular glutamate concentrations upon hypoxia-ischemia,(16-19) are bound to lead to massive NMDAr activation, resulting in excitotoxicity and neuronal damage and thus provide an interesting therapeutic target for this common, devastating and currently untreatable condition.

METHODS AND MATERIALS

Cell cultures

Materials

Rat Glioma C6 cells were cultured in F-12 Kaighn's medium supplemented with 10% fetal calf serum and 100µg/ml penicillin / streptomycin (all from Gibco Life Technologies, Invitrogen). Medium with and without glucose (RPMI 1640 BE-12 702F and BE-12 752F, respectively, Bio Whittaker) were used to create normoglycemic and hypoglycemic conditions, respectively. Hypoxia was applied by culturing samples in an in VIVO$_2$ Hypoxia workstation 1000 (Biotrace International), equipped with a Ruskinn gas mixer module V$_2$ (Biotrace International). Oxygen level was set at 1%, CO_2 at 5% and H_2 at 0%.

Experimental design

The experiment was started by washing 6 x 6 well plates (C2-C4 and S2-S4 started on day 1; C1 and S1 started 24 hours (h) later) of near confluent glioma C6 cells twice with phosphate buffered saline and applying the experimental conditions (6 different samples per condition) as depicted in Figure 2. L-serine-O-sulphate (LSOS), an inhibitor of serine racemase (SR) and thus of D-serine synthesis,(20) was added to two experimental conditions (C3 and S3). Supernatant medium was collected at the end of each condition and stored at -20ºC until analysis. This experiment (8 conditions; n=6 for each condition) was repeated on 4 different occasions.

Human newborns

CSF samples were obtained from full-term newborn infants (n=4; gestational age>37 weeks), who had undergone a lumbar puncture at our hospital within 48h of life to rule out meningitis, but bacterial culture and cell count negated infection. The diagnosis of asphyxia was based upon the following criteria:

1. Intrapartum distress as indicated by the cardiotochographic pattern (late decelerations ≥ 1 hour, or severe abnormalities as absent variability or persistent bradycardia ≥ 30 minutes prior to birth), early passage of thick meconium or scalp-pH<7.2 immediately before birth.
2. Need for neonatal resuscitation with positive pressure ventilation for >3 minutes, Apgar score≤5, or umbilical /first postnatal pH<7.1 and/or base deficit>10 mmol/l.

Subjects with central nervous system pathology or malformations and hemorrhagic CSF samples were excluded from analysis. Samples were kept at 4ºC for 1 week, before being stored at -80ºC until analysis. Use of the samples for our study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands.

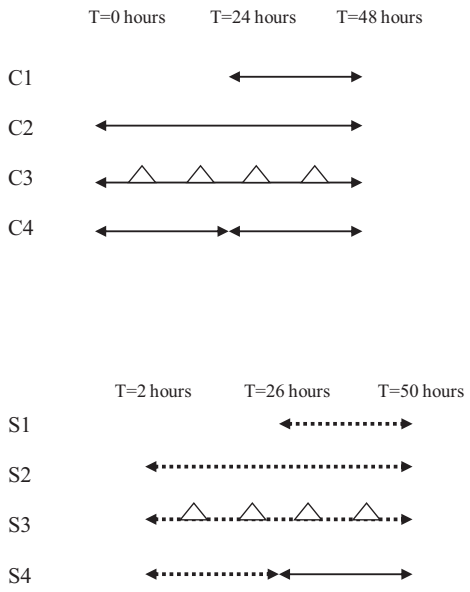


Figure 2. Experimental design of the Glioma C6 studies.
◄——► normoxia and normoglycemia (21% O_2; RPMI 12-702 medium)
◄----------► oxygen and glucose deprivation (1% O_2; RPMI 12-752 medium)
C1-C4=control 1-4; S1-S4 =sample 1-4.
The competitive serine racemase antagonist L-serine O-sulphate (LSOS △) was added to a medium concentration of 100 µM to C3 and S3.

Piglets

7 Newborn Dutch store piglets with a postnatal age range from 1-3 days were exposed to hypoxia-ischemia by occlusion of both common carotid arteries and reduction the fraction of inspired oxygen for 1h as described previously.(15) CSF was withdrawn at 3h (n=2) or 24h (n=5) after the start of 1h hypoxia-ischemia. CSF was also withdrawn in 3 control piglets, which underwent the same surgical procedure but were not exposed to hypoxia-ischemia.

Quantitative amino acid determination

D-Serine, L-serine and glycine concentrations were measured by stable isotope dilution gas and liquid chromatographic-mass spectrometric methods as described before.(21)

Statistical analyses

Amino acid concentrations in supernatant of glioma C6 cells were compared between experimental and control conditions using the unpaired Student's T-test. Similarly, amino acid concentrations in CSF from patients and piglets after perinatal asphyxia were compared with control CSF, using the unpaired Student's T-test. The level of significance was set at $p<0.05$.

RESULTS

Rat glioma C6 cells

Cells were incubated according to our experimental protocol (see methods and materials section). In control conditions, D-serine concentrations in supernatant medium increased after 24h to +0.42μM (Figure 3) and after 48h to 0.64μM ($p<0.05$ when comparing 24h with 48h). LSOS did not affect the increase in D-serine (0.64μM after 48h versus 0.78μM after 48h + LSOS, non-significant). Glycine and L-serine concentrations decreased during 24-48h of normal conditions, suggesting uptake by glioma cells.

Application of OGD for 24h or 48h resulted in increases in D-serine concentrations from +0.42μM (control condition) to +0.69μM at 24h and from 0.64μM (control condition) to 0.91μM at 48h ($p<0.05$ in both cases). After addition of the SR antagonist LSOS, the difference in D-serine concentrations between control and OGD conditions disappeared (+0.78μM (48h control conditions + LSOS) versus +0.89μM (48h OGD + LSOS), non-significant). After OGD, glycine concentrations increased from -78μM (control condition) to +109μM at 24h and from -151μM (control condition) to +175μM at 48h ($p<< 0.01$ in both cases; $p<0.05$ when comparing 24 to 48h OGD). This significant increase was not affected by addition of LSOS.

Interestingly, when simulating reperfusion by applying normoxia/normoglycemia (NO/NG) after 24h OGD, D-serine concentrations were significantly lowered when compared to control conditions (+0.66μM (control condition) versus +0.34μM (24h OGD + 24h NO/NG), $p<0.01$). Concentrations of glycine normalized, by decreasing to a lesser extent than during control conditions (-116μM (control condition) versus -52μM (24h OGD + 24h NO/NG).

Concentrations of L-serine, the common precursor of D-serine and glycine, and other amino acids (including aspartate, another excitatory amino acid not involved in NMDAr activity, data not shown) decreased after OGD and in the reperfusion experiment, but less than during control conditions, suggesting that the rise in NMDAr agonists is caused by specific synthesis and not mere efflux due to for instance increased cell wall permeability.

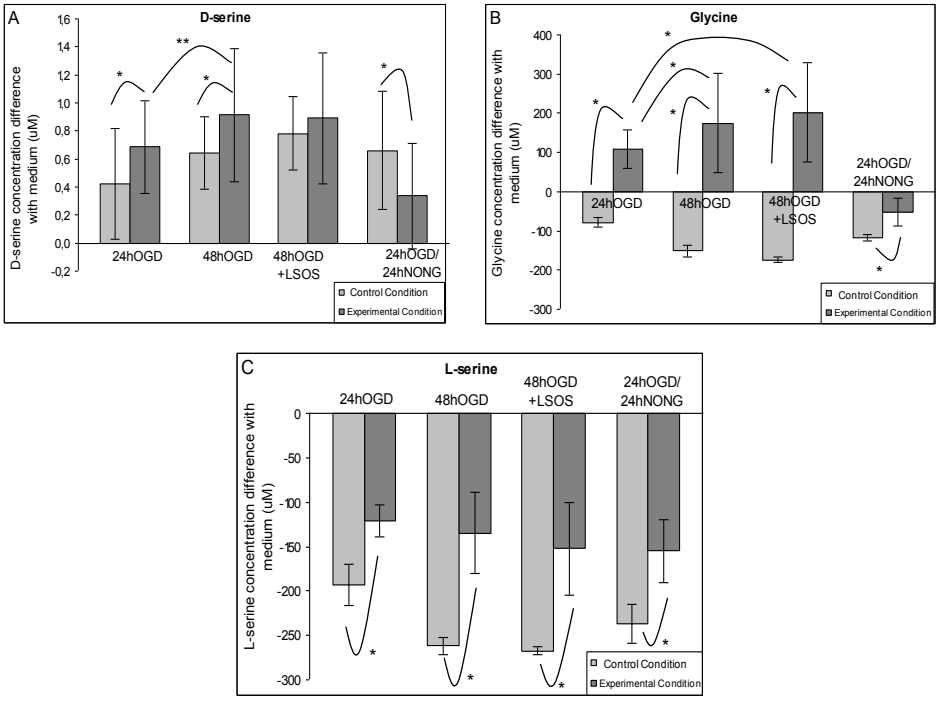


Figure 3. Medium concentration differences of the NMDAr agonists D-serine, glycine and their common precursor L-serine after oxygen and glucose deprivation (OGD). The figures represent the mean ± standard deviation of the concentration differences of D-serine (A), glycine (B) and L-serine (C) between fresh medium and supernatant after 24h OGD, 48h OGD, 48h OGD with the serine racemase antagonist LSOS, and 24h OGD followed by 24h normoxia/normoglycemia (NO/NG). These graphs represent the mean of 6 different samples and 6 different controls during 4 different experiments. * represents a significant change with a p-value<0.05 and ** 0.05<p<0.1.

In order to gain insight in the mechanism responsible for changes in D-serine and glycine concentrations, a fifth condition was added (similar to S1 and C1, Figure 2) by addition of 3mg of the labelled stable isotope $^{13}C_3{}^{15}N$ L-serine (Cambridge Isotope Laboratories, Inc.) to 100ml medium (to a concentration of 285 µM), to which NO/NG, OGD, normoxia/hypoglycemia and hypoxia/normoglycemia was applied during 48h, because the effects were most evident after 48h (Figure 3). By labelling L-serine, the common endogenous precursor of D-serine and glycine, we investigated D-serine and glycine synthesis from L-serine by quantifying arising labelled D-serine and glycine (Table 1). In this experiment, D-serine concentrations remained unaffected by 48h OGD when compared to NO/NG (0.04µM and 0.03µM respectively, non-significant), but these concentrations were very low, nearing the limit of detection of the analytical method. Glycine concentrations increased significantly and markedly after 48h OGD (57.0µM), when compared to control conditions (34.3µM, p<0.01). Synthesis of labeled glycine after 48h of control conditions is interesting when considering the decrease in total glycine concentrations in medium

Table 1. Changes in D-serine and glycine synthesis from L-serine after hypoxia, hypoglycemia and oxygen-glucose deprivation.

	$^{13}C_3$ L-serine use (μM)	$^{13}C_3$ D-serine synthesis (μM)	$^{13}C_2$ glycine synthesis (μM)	$^{13}C_3$ D-serine synthesis/ $^{13}C_3$ L-serine use (%)	$^{13}C_2$ glycine synthesis/ $^{13}C_3$ L-serine use (%)
NO/NG	191.3 (SD 3.8)	0.03 (SD 0.04)	34.3 (SD 1.8)	0.02 (SD 0.02)	18.0 (SD 1.0)
HO	162.8 (SD 6.7)	0.12 (SD 0.07)	45.8 (SD 4.4)	0.08 (SD 0.05, p=0.05)	28.3 (SD 4.0, p<0.01)
HG	231.7 (SD 4.6)	0.12 (SD 0.07)	10.7 (SD 1.3)	0.05 (SD 0.03, p=0.09)	4.6 (SD 0.6, p<0.01)
OGD	111.7 (SD 10.8)	0.04 (SD 0.06)	57.0 (SD 3.6)	0.04 (SD 0.06, p=0.51)	51.5 (SD 7.4, p<0.01)

D-serine and glycine synthesis from L-serine was studied by analysis of arising $^{13}C_3$ D-serine and $^{13}C_2$ glycine concentrations in the medium upon addition of $^{13}C_3$ L-serine. The table depicts medium concentration changes in $^{13}C_3$ L-serine (added to a medium concentration of 285 μM), $^{13}C_3$ D-serine and $^{13}C_2$ glycine after rat glioma C6 cells were exposed to 48 hours of NO/NG, hypoxia (HO), hypoglycemia (HG) or OGD (n=6 for each condition). The last two columns represent the percentage of $^{13}C_3$ D-serine and $^{13}C_2$ glycine synthesis, respectively, of total $^{13}C_3$ L-serine use and the p-value (Student's T-test) after comparison with the NO/NG condition (N=6 for all conditions).

after 48 hours (Figure 3), implying simultaneous synthesis and use of glycine under normal conditions. Under NO/NG conditions, D-serine synthesis accounted for 0.02% and glycine synthesis for 18% of L-serine use. D-Serine synthesis from L-serine was not significantly increased by hypoxia, hypoglycemia or the combination ($0.05<p<0.1$ for OGD), but again, concentrations of labeled D-serine were very low for quantification with our analytical method. Glycine synthesis from L-serine almost doubled to 28% ($p<0.01$) upon hypoxia, decreased to 4.6% ($p<0.01$) upon hypoglycemia and almost tripled to 51.5% ($p<0.01$) upon OGD.

Human newborns

Despite the limited number of subjects (n=4), we found a significant and marked increase in glycine concentrations (+50%, $p<0.05$) and percentage glycine of the sum of L-serine and glycine (an indication for the amount of glycine synthesis from L-serine; +100%, $p<0.01$) in CSF of patients with perinatal asphyxia when compared with controls (Table 2). D-Serine concentrations showed a trend to be lower (-25%) in CSF withdrawn 24-48h after ischemia asphyxia and so did the percentage D-serine of the sum of D- and L-serine (an indication for the quantity of D-serine synthesis from L-serine; -50%), when compared with control CSF.

Piglets

Three hours after starting hypoxia-ischemia, piglets showed an almost 8-fold increase in glycine concentrations in CSF when compared to control piglets (Figure 4). Glycine concentrations remained markedly increased (4-fold $p<0.05$) in CSF 24h after starting hypoxia-ischemia when compared to control piglets, but less than 3h after hypoxia-

ischemia (2-fold reduction). D-Serine concentrations were not significantly increased 3h after starting hypoxia-ischemia, but they were reduced at 24h when compared with both

Table 2. Amino acid concentrations and their ratios in cerebrospinal fluid from neonates with and without perinatal asphyxia.

	N	D-serine (μM)	L-serine (μM)	Glycine (μM)	D-ser/tot ser (%)	Gly/L-ser+ gly (%)	D-ser/gly (%)
Controls	2	12.1 (0.2)	48.0 (0.1)	9.4 (1.3)	20.1 (0.2)	16.4 (1.9)	1.3 (0.2)
Asphyxia	2	9.4 (3.2)	34.6 (0.4)	14.8 (0.2)	21.1 (5.5)	29.9 (0.1)	0.6 (0.2)
p-value		0.35	<0.01*	0.03*	0.82	<0.01*	0.08

CSF amino acid concentrations determined by LC-MS and their ratios in neonates with and without perinatal asphyxia. All samples were taken within the first 48 hours of life. The table represents mean values (standard deviation). Asterixes represent statistically significant values.

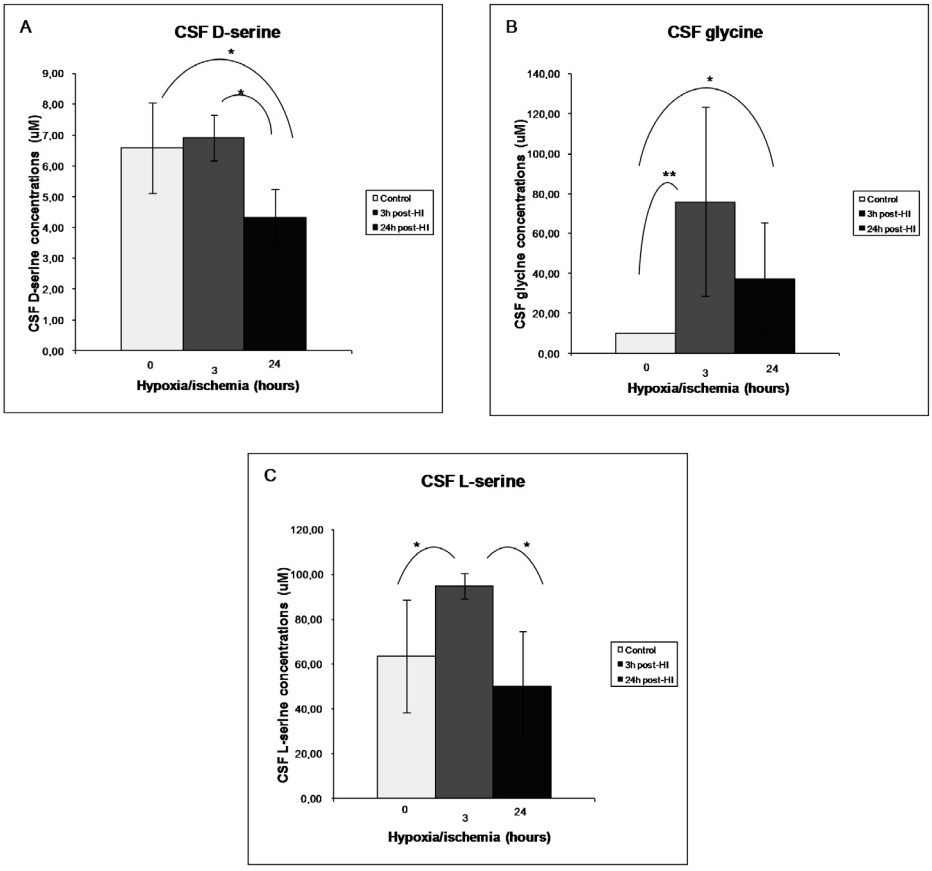


Figure 4. D-serine, glycine and L-serine concentrations in CSF from control piglets and 3 and 24h after application of cerebral hypoxia-ischemia. CSF concentrations of D-serine (A), glycine (B), and L-serine (C) as determined by GC-MS in control piglets (n=3), 3h after start of hypoxia-ischemia (HI) (n=2) and 24h after start of hypoxia-ischemia (n=5) *p<0.05; **p<0.1 (0.068).

control piglets and 3h after starting hypoxia-ischemia (40% reduction, $p<0.05$ in both cases). L-Serine concentrations increased significantly in CSF 3h after hypoxia-ischemia (50%, $p<0.05$) and returned to control values 24h after hypoxia-ischemia.

DISCUSSION

Since perinatal asphyxia is an important contributor to neonatal mortality and morbidity without curative treatment options, this study was aimed to elucidate the mechanism of NMDAr associated excitotoxicity in hypoxia-ischemia in order to develop future treatment strategies. The necessity for NMDArs to be activated not only by glutamate but also by glycine or D-serine may be regarded as a safety mechanism to prevent excitotoxicity. Our results imply that this safety mechanism fails in global cerebral ischemia, since we found that concentrations of both glycine and D-serine significantly increased during hypoxic-ischemic conditions, followed by increased but normalizing glycine concentrations and decreased D-serine concentrations upon reperfusion.

Both D-serine and glycine concentrations appear to rise through increased synthesis from L-serine. For D-serine, this is evidenced by the observation that after antagonism of D-serine synthesis, the rise in D-serine concentrations upon OGD was prevented, when compared to control conditions. This concurred with our isotope studies, showing a trend for increased synthesis of labelled D-serine from labelled L-serine upon hypoxia, hypoglycemia and OGD. These results did not reach significance, presumably because of the small sample size (n=6) and the very low concentrations of labelled D-serine, reaching the limit of detection of our analytical method. The decreases in neurotoxicity after ischemia in SR knock-out mice (22) further consolidate a central role for D-serine synthesis from L-serine by SR during OGD and subsequent neuronal damage. Similarly, glycine synthesis of L-serine, estimated by determining the percentage glycine from the total of L-serine and glycine, almost doubled in CSF from asphyxiated neonates, when compared with non-asphyxiated neonates. In our isotope studies, glycine synthesis from L-serine almost tripled from 18% under control conditions to 51.5% after OGD. Since use of labelled L-serine decreased after OGD, this might represent a specific shift of L-serine use towards D-serine and glycine synthesis.

The relative contribution of D-serine and glycine to NMDAr induced neuronal death following ischemia remains unclear. Addition of D-amino acid oxidase (DAO), the enzyme that degrades D-serine, led to diminished cell death upon application of NMDA or OGD, which was reversed by addition of either D-serine or glycine.(23) Another study showed that NMDA-elicited neurotoxicity in rat hippocampal slices was virtually abolished by complete removal of D-serine.(24) The effect of endogenous glycine could be observed only after simultaneous removal of endogenous D-serine and blockage of the glycine

transporter GlyT1. Thus, although levels of glycine were 10-fold higher than D-serine in this study, endogenous D-serine appeared to be the dominant co-agonist for NMDA-elicited neurotoxicity.(24) Similarly, mutant mice with a targeted deletion of SR showed dramatically diminished infarct volume in several brain regions following middle cerebral artery occlusion,(22) and brain cultures from SR knock-out mice exhibited markedly diminished nitric oxide formation and neurotoxicity. Concluding, most recent studies identify D-serine as the main excitotoxic co-agonist after cerebral ischemia, despite robust increases in glycine concentrations.(25) Moreover, simultaneous elevation of both NMDAr co-agonists as seen in our cell culture experiments, in combination with the known increases in extracellular glutamate concentrations, (16-19) is bound to lead to NMDAr associated excitotoxicity.

During the reperfusion phase, we observed increased but normalizing glycine concentrations *in vitro* and *in vivo*. This concurs with the few reports describing increased glycine concentrations in mammalian CSF or microdialysates after global ischemia.(26-28) To our knowledge, we are the first to have investigated extracellular concentrations of both D-serine and glycine after OGD. Only one paper describing focal ischemia demonstrated large elevations of glutamate, glycine and D-serine concentrations in microdialysis samples from rabbit cortex during ischemia and also during reperfusion.(29) In contrast to these results, we observed significantly reduced D-serine concentrations during the reperfusion phase, both *in vitro* and *in vivo*. This concurs with the finding of delayed transient decreases of SR (mRNA and protein) and D-serine in the ipsilateral temporo-parietal mouse cortex after permanent focal ischemia.(30) In accordance with the model of a safety mechanism, this reduction in D-serine concentrations during reperfusion might represent an attempt to compensate for increased NMDAr activity. Potentially, elevated glycine concentrations contribute to the decrease in D-serine concentrations, because glycine inhibits SR activity at these concentrations.(31) In the piglet studies, we observed a remarkable rise in L-serine concentrations 3h after hypoxia-ischemia, which might concur with decreased L-serine use, which was observed in the glioma cell studies and confirmed upon L-serine isotope analysis. This rise in L-serine concentrations was not seen in human newborns with perinatal asphyxia, which might be due to the fact that these samples were taken later than 3h after the asphyxiating incident, explaining the resemblance between their CSF pattern and piglet CSF 24h after hypoxia-ischemia. L-Serine has recently been found to exert a neuroprotective effect against ischemia,(32) but speculating on this L-serine rise seems precarious, since there were only two piglets studied 3h after hypoxia-ischemia.

Our *in vitro* and *in vivo* results and their concurrence have several more implications. First, it underscores the usability of rat glioma C6 cells and this study design as a model to study glia in global ischemia. Second, it provides further evidence that glia are relevant signalling partners and not merely supporting cells for neurons, as was long

believed.(33-36) Third, despite the relatively small sample size, we found alterations in the concentrations of the NMDAr co-agonists in human and piglet CSF, which represents just a global marker for changes in the central nervous system. This emphasizes the magnitude of concentration changes of the NMDAr co-agonists in specific brain areas.
In conclusion, our results encourage the development of effective and safe drugs preventing NMDAr overactivation after global ischemia. Therapeutic agents for hypoxia-ischemia, including competitive and non-competitive NMDAr inhibitors, as well as NR1 antagonists have been largely disappointing,(37) partly due to significant side effects. (38, 39) New strategies, such as manipulating co-agonist concentrations, might form a more subtle alternative. The protective effect of hypothermia, a currently promising method to decrease neuronal damage in asphyxiated infants with severe encephalopathy, might at least in part rely on reducing glutamate (26, 40) and glycine (26, 41) concentrations, as observed in rabbit hippocampus microdialysates and in superfusates from rat cerebral cortical slices. Although unfortunately D-serine concentrations have not been investigated in hypothermia as of yet, these results show that reduction of the concentrations of the NMDAr (co)-agonists might be beneficial in global ischemia. Targeting the glycine and particularly the D-serine synthetic pathway may provide an attractive novel treatment option for asphyxiated neonates.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from The Netherlands Organization for Health Research and Development (to S.A.F: 920-03-345). We thank Marc Vooijs of the department of Pathology of the University Medical Center Utrecht for using the hypoxia working station obtained with financial support from the Maurits and Anna de Kock Foundation. We thank Bart van de Sluis and Leyla Tabataie for their expert assistance and helpful discussions.

REFERENCES

1. Robertson CM, Finer NN, Grace MG 1989 School performance of survivors of neonatal encephalopathy associated with birth asphyxia at term. J Pediatr 114:753-760
2. Shankaran S, Woldt E, Koepke T, Bedard MP, Nandyal R 1991 Acute neonatal morbidity and long-term central nervous system sequelae of perinatal asphyxia in term infants. Early Hum Dev 25:135-148
3. Lawn JE, Cousens S, Zupan J 2005 4 million neonatal deaths: when? Where? Why? Lancet 365:891-900
4. Selway LD 2010 State of the science: hypoxic ischemic encephalopathy and hypothermic intervention for neonates. Adv Neonatal Care 10:60-66
5. Sattler R, Tymianski M 2001 Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. Mol Neurobiol 24:107-129
6. Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD, Rogawski MA, Snyder SH 2000 D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 97:4926-4931
7. Bergeron R, Meyer TM, Coyle JT, Greene RW 1998 Modulation of N-methyl-D-aspartate receptor function by glycine transport. Proc Natl Acad Sci U S A 95:15730-15734
8. Wolosker H, Blackshaw S, Snyder SH 1999 Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. Proc Natl Acad Sci U S A 96:13409-13414
9. Molla G, Sacchi S, Bernasconi M, Pilone MS, Fukui K, Pollegioni L 2006 Characterization of human D-amino acid oxidase. FEBS Lett 580:2358-2364
10. Jones PA, May GR, McLuckie JA, Iwashita A, Sharkey J 2004 Apoptosis is not an invariable component of in vitro models of cortical cerebral ischaemia. Cell Res 14:241-250
11. Rossi DJ, Brady JD, Mohr C 2007 Astrocyte metabolism and signaling during brain ischemia. Nat Neurosci 10:1377-1386
12. Mangia S, Simpson IA, Vannucci SJ, Carruthers A 2009 The in vivo neuron-to-astrocyte lactate shuttle in human brain: evidence from modeling of measured lactate levels during visual stimulation. J Neurochem 109 Suppl 1:55-62
13. Dobbing J, Sands J 1979 Comparative aspects of the brain growth spurt. Early Hum Dev 3: 79-83
14. Fritz KI, Groenendaal F, Andersen C, Ohnishi ST, Mishra OP, Delivoria-Papadopoulos M 1999 Deleterious brain cell membrane effects after NMDA receptor antagonist administration to newborn piglets. Brain Res 816:438-445
15. Peeters-Scholte C, Koster J, Veldhuis W, van den Tweel E, Zhu C, Kops N, Blomgren K, Bar D, van Buul-Offers S, Hagberg H, Nicolay K, van BF, Groenendaal F 2002 Neuroprotection by selective nitric oxide synthase inhibition at 24 hours after perinatal hypoxia-ischemia. Stroke 33:2304-2310
16. Benveniste H, Drejer J, Schousboe A, Diemer NH 1984 Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 43:1369-1374
17. Gucuyener K, Atalay Y, Aral YZ, Hasanoglu A, Turkyilmaz C, Biberoglu G 1999 Excitatory

amino acids and taurine levels in cerebrospinal fluid of hypoxic ischemic encephalopathy in newborn. Clin Neurol Neurosurg 101:171-174

18. Puka-Sundvall M, Gilland E, Bona E, Lehmann A, Sandberg M, Hagberg H 1996 Development of brain damage after neonatal hypoxia-ischemia: excitatory amino acids and cysteine. Metab Brain Dis 11:109-123
19. Ueda Y, Obrenovitch TP, Lok SY, Sarna GS, Symon L 1992 Changes in extracellular glutamate concentration produced in the rat striatum by repeated ischemia. Stroke 23:1125-1130
20. Panizzutti R, De MJ, Ribeiro CS, Engelender S, Wolosker H 2001 A new strategy to decrease N-methyl-D-aspartate (NMDA) receptor coactivation: inhibition of D-serine synthesis by converting serine racemase into an eliminase. Proc Natl Acad Sci U S A 98:5294-5299
21. Fuchs SA, de Sain-van der Velden MG, de Barse MM, Roeleveld MW, Hendriks M, Dorland L, Klomp LW, Berger R, de Koning TJ 2008 Two mass-spectrometric techniques for quantifying serine enantiomers and glycine in cerebrospinal fluid: potential confounders and age-dependent ranges. Clin Chem 54:1443-1450
22. Mustafa AK, Ahmad AS, Zeynalov E, Gazi SK, Sikka G, Ehmsen JT, Barrow RK, Coyle JT, Snyder SH, Dore S 2010 Serine racemase deletion protects against cerebral ischemia and excitotoxicity. J Neurosci 30:1413-1416
23. Katsuki H, Nonaka M, Shirakawa H, Kume T, Akaike A 2004 Endogenous D-serine is involved in induction of neuronal death by N-methyl-D-aspartate and simulated ischemia in rat cerebrocortical slices. J Pharmacol Exp Ther 311:836-844
24. Shleper M, Kartvelishvily E, Wolosker H 2005 D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices. J Neurosci 25: 9413-9417
25. Katsuki H, Watanabe Y, Fujimoto S, Kume T, Akaike A 2007 Contribution of endogenous glycine and d-serine to excitotoxic and ischemic cell death in rat cerebrocortical slice cultures. Life Sci 81:740-749
26. Baker AJ, Zornow MH, Grafe MR, Scheller MS, Skilling SR, Smullin DH, Larson AA 1991 Hypothermia prevents ischemia-induced increases in hippocampal glycine concentrations in rabbits. Stroke 22:666-673
27. Phillis JW, Walter GA 1989 Effect of a brief hypoxic/hypotensive episode on the in vivo release of cerebral cortical gamma-aminobutyric acid and glycine. Brain Res 504:121-123
28. Roldan A, Figueras-Aloy J, Deulofeu R, Jimenez R 1999 Glycine and other neurotransmitter amino acids in cerebrospinal fluid in perinatal asphyxia and neonatal hypoxic-ischaemic encephalopathy. Acta Paediatr 88:1137-1141
29. Lo EH, Pierce AR, Matsumoto K, Kano T, Evans CJ, Newcomb R 1998 Alterations in K+ evoked profiles of neurotransmitter and neuromodulator amino acids after focal ischemia-reperfusion. Neuroscience 83:449-458
30. Wang LZ, Zhu XZ 2004 Serine racemase expression in mouse cerebral cortex after permanent focal cerebral ischemia. Acta Pharmacol Sin 25:436-441
31. Strisovsky K, Jiraskova J, Mikulova A, Rulisek L, Konvalinka J 2005 Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxylate substrate and inhibitors and analysis of the beta-eliminase activity. Biochemistry 44:13091-13100

32. Wang GH, Jiang ZL, Chen ZQ, Li X, Peng LL 2010 Neuroprotective effect of L-serine against temporary cerebral ischemia in rats. J Neurosci Res 88:2035-2045
33. Billard JM 2008 D-serine signalling as a prominent determinant of neuronal-glial dialogue in the healthy and diseased brain. J Cell Mol Med 12:1872-1884
34. Halassa MM, Fellin T, Haydon PG 2007 The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol Med 13:54-63
35. Oliet SH, Mothet JP 2009 Regulation of N-methyl-D-aspartate receptors by astrocytic D-serine. Neuroscience 158:275-283
36. Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA, Oliet SH 2006 Glia-derived D-serine controls NMDA receptor activity and synaptic memory. Cell 125:775-784
37. Madden K 2002 NMDA receptor antagonists and glycine site NMDA antagonists. Curr Med Res Opin 18 Suppl 2:s27-s31
38. Groenendaal F, Rademaker CM, Toet MC, de Vries LS 2002 Effects of magnesium sulphate on amplitude-integrated continuous EEG in asphyxiated term neonates. Acta Paediatr 91: 1073-1077
39. Levene M, Blennow M, Whitelaw A, Hanko E, Fellman V, Hartley R 1995 Acute effects of two different doses of magnesium sulphate in infants with birth asphyxia. Arch Dis Child Fetal Neonatal Ed 73:F174-F177
40. Illievich UM, Zornow MH, Choi KT, Scheller MS, Strnat MA 1994 Effects of hypothermic metabolic suppression on hippocampal glutamate concentrations after transient global cerebral ischemia. Anesth Analg 78:905-911
41. Simpson RE, Walter GA, Phillis JW 1991 The effects of hypothermia on amino acid neurotransmitter release from the cerebral cortex. Neurosci Lett 124:83-86

Chapter 7

Cerebrospinal fluid D-serine and glycine concentrations are unaltered and unaffected by olanzapine therapy in male schizophrenic patients

S.A. Fuchs, M.M.J. De Barse, F.E. Scheepers, W. Cahn, L. Dorland, M.G. de Sain-van der Velden, L.W.J. Klomp, R. Berger, R.S. Kahn, T.J. de Koning

Eur Neuropsychopharmacol 2008;18:333-8

ABSTRACT

N-Methyl D-aspartate (NMDA)-receptor hypofunction has been implicated in the pathophysiology of schizophrenia and D-serine and glycine add-on therapy to antipsychotics has shown beneficial effects in schizophrenic patients. Nevertheless, previous studies have not shown consistently altered D-serine concentrations in cerebrospinal fluid (CSF) of schizophrenic patients. To confirm and extend these results, CSF concentrations of both endogenous NMDA-receptor co-agonists D-serine and glycine and their common precursor L-serine were analyzed simultaneously in 17 healthy controls and 19 schizophrenic patients before and 6 weeks after daily olanzapine (10mg) treatment. CSF D-serine, L-serine and glycine concentrations and their relative ratios were similar between schizophrenic patients and controls and no differences were observed before and after olanzapine therapy. Thus, the NMDA-receptor hypofunction hypothesis in schizophrenia is not explained by olanzapine therapy-dependent absolute or relative decreases in CSF D-serine and glycine concentrations in this series of male patients, thereby not providing convenient markers for the disorder.

INTRODUCTION

Schizophrenia is a serious psychiatric condition, affecting 0.5-1% of the general population (Goldner et al., 2002). It is characterized by positive symptoms (including hallucinations and delusions), negative symptoms (including blunted affect and emotional withdrawal) and cognitive symptoms. The pathophysiology remains elusive. Abnormal glutamatergic neurotransmission through N-methyl D-aspartate (NMDA)-receptors might be involved, as NMDA-receptor antagonists induce schizophrenia-like psychoses in healthy individuals and exacerbate psychotic symptoms in schizophrenics (Lahti et al., 2001). In addition, some effective antipsychotic drugs, such as clozapine, potentiate NMDA-receptor mediated neurotransmission (Arvanov et al., 1997) and increase glutamate concentrations in serum (Goff et al., 1996;Evins et al., 1997) and specific brain areas (Daly and Moghaddam, 1993;Yamamoto and Cooperman, 1994). Both clozapine (Olney and Farber, 1994) and olanzapine (Farber et al., 1996) block NMDA-receptor antagonist-induced neurotoxicity, suggesting that these atypical antipsychotics share glutamatergic properties (Heresco-Levy, 2003). Furthermore, mutant mice with absent or reduced expression of NMDA-receptor subunits, display behavioral abnormalities similar to those observed in pharmacologically-induced animal models of schizophrenia (Miyamoto et al., 2001;Mohn et al., 1999). Finally, a number of genetic schizophrenia susceptibility loci comprise genes with roles in NMDA-receptor function or glutamatergic neurotransmission (Harrison and Owen, 2003). Together, all these studies strongly imply contribution of NMDA-receptor dysfunction to the pathophysiology of schizophrenia, collectively referred to as the NMDA-receptor hypofunction hypothesis (Goff and Coyle, 2001).

NMDA-receptors require simultaneous binding by glutamate and one of the endogenous co-agonists D-serine or glycine for activation. D-Serine and glycine add-on treatment to antipsychotics induces beneficial effects especially on negative symptoms of schizophrenia (Tuominen et al., 2006). Therefore, it was hypothesized that altered concentrations of these obligatory NMDA-receptor co-agonists contribute to NMDA-receptor dysfunction in schizophrenia (Hashimoto et al., 2003;Kumashiro et al., 1995) and several studies have investigated cerebrospinal fluid (CSF) D-serine concentrations (Bendikov et al., 2007;Hashimoto et al., 2005). Despite reporting lowered D-serine to total serine ratio (Hashimoto et al., 2005) or decreased D-serine in only the female schizophrenic patients (Bendikov et al., 2007), none showed unequivocally decreased CSF D-serine or glycine concentrations in schizophrenia. In this study, we hoped to confirm and extend these results with the simultaneous analysis of CSF D-serine and glycine. Furthermore, since some atypical antipsychotics possess glutamatergic properties (Heresco-Levy, 2003) that might be effected by binding to NMDA-receptors or influencing endogenous NMDA-receptor agonist concentrations, we hypothesized that alterations in D-serine

and glycine concentrations might constitute a therapeutic target for olanzapine. If in fact, olanzapine acts by increasing decreased D-serine or glycine concentrations, this would provide a helpful diagnostic marker and a clinical predictor of drug efficacy. Consequently, we analyzed absolute and relative CSF concentrations of D-serine, glycine, and their common endogenous precursor L-serine (Fu et al., 2001;Herbig et al., 2002;Wolosker et al., 1999) in healthy individuals and patients with schizophrenia before and after olanzapine therapy.

EXPERIMENTAL PROCEDURES

Subjects

Nineteen male schizophrenic patients participated in this study. Subject characteristics are summarized in Table 1. Design of the olanzapine study has been published elsewhere (Scheepers et al., 2002). Briefly, patients were evaluated using the Comprehensive Assessment for Symptoms and History (CASH) interview (Andreasen et al., 1992) and met DSM-IV criteria for schizophrenia, paranoid type (n=15) or disorganized type (n=4). Upon giving written informed consent, patients underwent a lumbar puncture after being free of antipsychotic medication for at least 2 weeks (4 weeks for clozapine; exclusion of patients with depot medication ≤two months prior to this trial) and after 6 weeks of daily treatment with 10mg olanzapine. Since 3 subjects failed to complete the study (1 receiving increased olanzapine dosage upon aggressive behavior, 1 failure of the second lumbar puncture and 1 lost to follow-up), and 3 CSF samples were not usable (1 hemorrhagic; 2 not enough material), olanzapine effects were evaluated in 13 patients. Samples were kept at -80°C until analysis.

Control CSF was obtained from samples, submitted for exclusion of meningitis from July to December 2004. Exclusion criteria were contamination with erythrocytes (>100/ml), leukocytes (>10/ml) and protein (>0.5 mg/ml), age <10 years or >55 years, intracranial pathology, epilepsy and psychiatric disorders. Both male and female subjects were included, since CSF D-serine, L-serine and glycine concentrations were not sex-dependent in healthy controls (99 healthy subjects, 48 males and 51 females, data not shown). Not to interfere with the normal routine in the laboratory of microbiology, samples were kept at 4°C for 1 week, before being stored at -80°C (we demonstrated that this procedure did not affect D-serine, L-serine or glycine concentrations, Fuchs et al., submitted). The study was approved by the medical ethics committee of the University Medical Center Utrecht.

Table 1. Subject characteristics of the schizophrenic patients

Subject	Age [a]	D [b]	DOI [c]	Drugs [d]	PANSS [e]			CGI [f]		
	(years)		(years)		1	2	8	1	2	8
1	21.0	2	4	2a	35	44	41	4	4	4
2	30.2	2	4	3	47	46	28	5	5	3
3	22.0	1	0.1	3	43	39	28	5	4	3
4	20.7	1	1.3	1	35	35	31	4	4	3
5	26.1	1	3	2	40	30	32	4	4	3
6	27.2	2	2.5	0	52	40	37	6	6	4
7	32.1	1	2.5	1+3	31	39	32	4	4	3
8	40.8	1	6	2a	43	40	25	4	5	3
9	30.3	1	3	1	37	46	31	5	5	4
10	41.4	1	0.5	1+3	39	31	22	3	3	2
11	32.0	1	10	2b+4	64	64	48	6	6	5
12	37.6	1	6	0	58	43	43	6	6	5
13	24.1	1	0.5	2a	48	45	41	5	5	5
14	35.8	1	7	0	44	[h]	[h]	5	5	[h]
15	32.5	1	15.5	3	47	43	35	5	5	4
16	34.6	1	2.5	2a+4	44	38	25	5	5	3
17	39.6	2	15	1	50	57	35	6	6	4
18	49.5	1	13	0	46	46	37	5	5	4
19	28.6	1	3.5	0	56	48	[h]	6	6	[h]
Mean (SD) 1-13 [g]	29.7 (7.1)		3.3 (2.8)		44.0 (9.6)	41.7 (8.5)	33.8 (7.7)	4.7 (0.9)	4.7 (0.9)	3.6 (1.0)
Mean (SD) 1-19 [g]	31.9 (7.7)		5.3 (4.8)		45.2 (8.4)	43.0 (8.2)	33.6 (7.1)	4.9 (0.9)	4.9 (0.9)	3.6 (0.9)

[a] Age in years; [b] D=diagnosis: 1=schizophrenia, paranoid type; 2=schizophrenia, disorganized type. No patient had any form of personality disorder; [c] DOI=Duration of illness in years; [d] Drugs=Drugs before study. 0=none, 1=typical antipsychotics, 2=atypical antipsychotics (2a=risperidone, 2b=clozapine), 3=benzodiazepines, 4=SSRIs. The protocol required a wash-out period of 4 weeks for clozapine and 2 weeks for all other medication; [e] PANSS=Positive and Negative Symptom Scale as evaluated by an experienced psychiatrist at visit 1, 2 and 8. Visit 1= day of inclusion in the study (on former medication). Visit 2=start of olanzapine therapy (after wash-out of former medication). Visit 8= end of the 6 weeks olanzapine trial; [f] CGI= Clinical global impression, scored by an experienced psychiatrist at visit 1, 2 and 8. 1=normal, not at all ill, 2=borderline ill, 3=mildly ill, 4=moderately ill, 5=markedly ill, 6=severely ill, 7=among the most extremely ill patients; [g] Patient 1-13 were included for olanzapine studies. Since there were no suitable CSF samples after 6 weeks of olanzapine treatment, patient 14-19 were analyzed only for comparison with healthy subjects; [h] No evaluation was performed due to patient absence or altered drug dosage.

Analysis of CSF D-serine, L-serine and glycine

D-Serine, L-serine and glycine concentrations were simultaneously measured by a stable isotope dilution method based on the method of Bruckner (Bruckner and Hausch, 1994). A detailed description of this validated method will be published elsewhere (Fuchs et al., submitted).

Statistical analyses

CSF of schizophrenic patients was analyzed in duplicate and mean amino acid concentrations and ratios were compared to control amino acid concentrations using two-tailed Student's t-tests. The effect of olanzapine was analyzed with two-tailed paired Student's t-tests. The level of significance was set at p=0.05.

RESULTS

19 Male schizophrenic patients (mean age:31.9 years (SD=7.7)), mean duration of illness:5.3 years (SD=4.8)) and 17 healthy individuals (6 males, 11 females, mean age:38.3 years (SD=11.5)) participated in this study. Age did not differ significantly (p=0.07;Table 1+2). Patient characteristics are presented in Table 1.

Table 2. D-Serine, L-serine and glycine in cerebrospinal fluid of healthy subjects and schizophrenic patients

	N	Age in years	D-serine (µmol/l)	L-serine (µmol/l)	Glycine (µmol/l)	D-serine/ total serine (%)	Glycine/ glycine+ L-serine (%)	D-serine/ glycine
Controls	17	38.3 (11.5)	3.3 (1.9)	34.6 (5.8)	8.4 (2.9)	8.4 (4.1)	19.6 (6.2)	0.4 (0.2)
Patients with schizophrenia	19	31.9 (7.7)	3.1 (1.0)	32.9 (5.0)	7.1 (1.7)	8.5 (2.0)	18.0 (4.4)	0.5 (0.2)
p-value t-test		0.07	0.75	0.36	0.12	0.95	0.37	0.67

D-Serine, L-serine and glycine concentrations (in µmol/l) and the percentage D-serine of total serine (%), percentage glycine of the sum of glycine and L-serine (%) and the ratio of D-serine to glycine were determined in cerebrospinal fluid from 17 healthy control subjects and 19 patients with schizophrenia. The table represents the mean values (standard deviations) and p-values of the two-tailed Student's *t*-tests (level of significance: $p<0.05$). N = number of subjects.

Table 3. D-Serine, L-serine and glycine in cerebrospinal fluid of patients with schizophrenia before and after olanzapine therapy.

	Before treatment	After treatment	p-value
D-serine (µmol/l)	3.1 (0.8)	3.2 (0.9)	0.78
L-serine (µmol/l)	34.5 (4.1)	35.8 (3.3)	0.22
glycine (µmol/l)	7.0 (1.2)	6.9 (1.1)	0.46
D-serine/total serine (%)	8.3 (1.8)	8.3 (2.2)	0.95
glycine/(glycine+L-serine) (%)	16.9 (3.4)	16.2 (3.1)	0.18
D-serine/glycine	0.5 (0.1)	0.5 (0.1)	0.59

In cerebrospinal fluid of 13 male schizophrenic patients, D-serine, L-serine and glycine concentrations (in µmol/l) and the percentage D-serine of total serine (%), percentage glycine of the sum of glycine and L-serine (%) and ratio of D-serine to glycine were determined before and after 6 weeks of daily treatment with 10mg olanzapine. The table represents the mean values (standard deviations) and p-values of the two-tailed paired Student's *t*-tests (level of significance: $p<0.05$).

CSF D-serine, L-serine and glycine concentrations were non-significantly different between schizophrenic patients and healthy individuals (p>0.1;Table 2). Since both D-serine and glycine are synthesized from L-serine, we expressed these data as percentage D-serine of total serine and percentage glycine of the sum of glycine and L-serine. The ratio of D-serine to glycine represents the relative contribution of each NMDA-receptor co-agonist. None of these ratios differed significantly between healthy individuals and schizophrenic patients (p>0.3;Table 2). Similarly, D-serine, L-serine and glycine concentrations and the ratios all remained unaffected by olanzapine therapy (p>0.1;Table 3).

DISCUSSION

Despite accumulating evidence that NMDA-receptor dysfunction contributes to the pathogenesis of schizophrenia, the underlying mechanism remains unclear. A deficiency of NMDA-receptor agonists would provide a plausible pathophysiological mechanism (Boks et al., 2007), and restitution of this deficiency would then account for (part of) the effectiveness of some antipsychotic medication. However, CSF concentrations of D-serine, L-serine, glycine and their ratios were not significantly different between healthy individuals and male schizophrenic patients before and after olanzapine therapy.

Our results partly concur with previous studies on CSF D-serine concentrations in schizophrenic patients. Similar to our results, one study reported unaltered D-serine concentrations compared to healthy individuals, but the percentage D-serine of total serine was significantly decreased (Hashimoto et al., 2005). However, we would rather attribute this to the observed increased L-serine concentrations (21.5μM versus 18.6μM, p=0.069) than to decreased D-serine concentrations (1.3μM versus 1.4μM, p=0.778). Another recent study reported 25% decreased CSF D-serine concentrations in schizophrenic patients, compared with healthy controls (Bendikov et al., 2007). Interestingly, protein levels of D-serine synthesizing and metabolizing enzymes were also altered in schizophrenic brains. However, if we compare male schizophrenic patients with male controls, CSF D-serine concentrations were non-significantly different. When combining the results from these three studies, there is no consistent CSF D-serine decrease in schizophrenia, at least not in males. In these patients, CSF D-serine can not serve as a reliable diagnostic marker for the disorder. Since all three study populations were small, these results should be confirmed in larger populations, which is hampered by the obvious difficulty to obtain CSF from large groups of schizophrenic patients and healthy controls.

An important extension in the present study is the simultaneous determination of D-serine and glycine. Concentrations of both NMDA-receptor co-agonists influence each other, not only by interconversion through L-serine (Fu et al., 2001;Herbig et al., 2002;Wo-

losker et al., 1999), but also through competitive inhibition of the D-serine synthesizing enzyme serine racemase by glycine (Dunlop and Neidle, 2005;Strisovsky et al., 2005). Furthermore, alterations in the proper homeostatic control of the two NMDA-receptor co-agonists may have relevant functional consequences (Fuchs et al., 2006). However, patient CSF amino acid concentrations and ratios in the present study did not differ from healthy controls nor from a healthy younger population we reported previously (Fuchs et al., 2006). Moreover, the normal CSF glycine concentrations in schizophrenic patients are important in the light of the beneficial effect of glycine transporter type-I inhibitors in patients with schizophrenia (Tsai et al., 2004), and the possible consequential hypothesis of altered glycine transporter function in schizophrenia (Tsai et al., 2006).

To our knowledge, we are the first to evaluate the effect of an atypical antipsychotic on CSF D-serine, L-serine and glycine concentrations. Olanzapine did not affect any of these concentrations. Similarly, previous analyses in this same patient group failed to show an effect of olanzapine on CSF glutamate concentrations (Scheepers et al., 2002). Thus, despite evidence suggestive of glutamatergic properties of atypical antipsychotics (Heresco-Levy, 2003), CSF concentrations of none of the endogenous NMDA-receptor agonists were affected by olanzapine therapy.

There are several explanations for the unaltered NMDA-receptor agonist concentrations in our study. First, D-serine, glycine and/or glutamate concentrations are altered in the schizophrenic brain, but these alterations are not reflected in our results. D-serine and glycine were implied in the pathophysiology of schizophrenia because negative symptoms ameliorated upon D-serine and glycine addition to antipsychotic treatment (Tuominen et al., 2006). Additionally, schizophrenia was genetically associated with *G72*, whose product activates the D-serine degrading enzyme D-amino acid oxidase (DAO) (Chumakov et al., 2002;Detera-Wadleigh and McMahon, 2006). Similarly, both a single-nucleotide polymorphism in the *Serine Racemase* gene (Morita et al., 2007) and the *Protein interacting with C-kinase* (PICK) gene, which interacts with serine racemase (Fujii et al., 2006), were associated with schizophrenia. All these studies imply a role for D-serine and glycine in the pathophysiology of schizophrenia. Conceivably, we did not detect significant olanzapine dependent CSF alterations in schizophrenic patients, because these occur only in specific brain areas and are not discernable in global bio-fluids as CSF (Tsai et al., 1998). In analogy, altered serine racemase expression was detected only in the hippocampus (Steffek et al., 2006) and frontal cortex (Bendikov et al., 2007), but not in other areas of schizophrenic brains, thereby potentially not affecting CSF amino acid concentrations. Alternatively, we possibly failed to detect significant differences, because the study population was small and so is the expected difference in CSF concentrations between patients and controls (Bendikov et al., 2007;Hashimoto et al., 2005). Finally, the distinctive heterogeneity in schizophrenic patients might result in subgroups with and without amino acid deficiencies.

The second explanation for our results supposes another pathophysiological mechanism than olanzapine dependent D-serine, glycine or glutamate deficiency in schizophrenia. In that case, the NMDA-receptor hypofunction hypothesis must be explained by some other defect in NMDA-receptor neurotransmission, like fewer glycine/D-serine binding-sites, altered NMDA-receptor subunit composition, or decreased sensitivity of the subunits for their agonists. In accordance, mice with genetically engineered mutations affecting the glycine/D-serine binding-site show persistent hyperactivity and stereotypic behaviors, indicating that dysfunction of the glycine/D-serine binding-site itself can cause behavioral abnormalities similar to those seen in schizophrenia (Ballard et al., 2002).

In conclusion, we detected normal CSF D-serine and glycine concentrations in male schizophrenic patients, before and after olanzapine therapy. We conclude from our and previous CSF studies that D-serine and glycine cannot serve as reliable biomarkers for this disorder.

REFERENCES

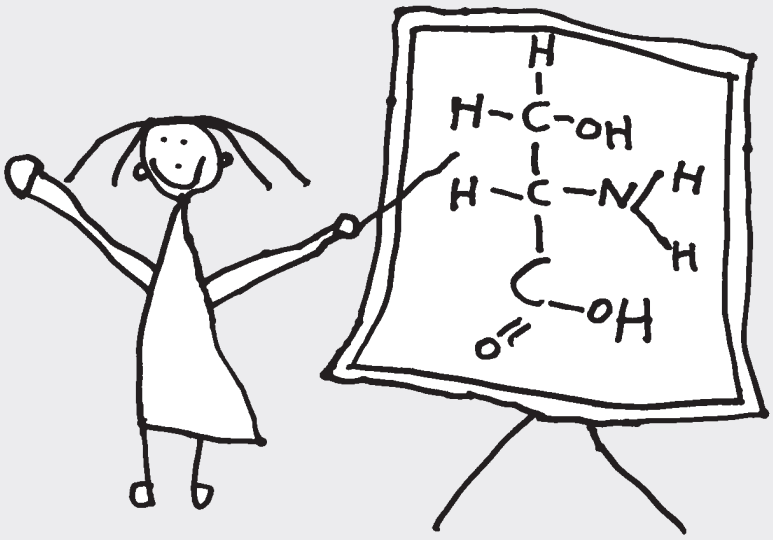
1. Andreasen, N.C., Flaum, M., Arndt, S. (1992) The Comprehensive Assessment of Symptoms and History (CASH). An instrument for assessing diagnosis and psychopathology. Arch. Gen. Psychiatry. 49, 615-623.
2. Arvanov, V.L., Liang, X., Schwartz, J., Grossman, S., Wang, R.Y. (1997) Clozapine and haloperidol modulate N-methyl-D-aspartate- and non-N-methyl-D-aspartate receptor-mediated neurotransmission in rat prefrontal cortical neurons in vitro. J. Pharmacol. Exp. Ther. 283, 226-234.
3. Ballard, T.M., Pauly-Evers, M., Higgins, G.A., Ouagazzal, A.M., Mutel, V., Borroni, E., Kemp, J.A., Bluethmann, H., Kew, J.N. (2002) Severe impairment of NMDA receptor function in mice carrying targeted point mutations in the glycine binding site results in drug-resistant nonhabituating hyperactivity. J. Neurosci. 22, 6713-6723.
4. Bendikov, I., Nadri, C., Amar, S., Panizzutti, R., De, M.J., Wolosker, H., Agam, G. (2007) A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. Schizophr. Res. 90, 41-51.
5. Boks, M.P., Rietkerk, T., van de Beek, M.H., Sommer, I.E., De Koning, T.J., Kahn, R.S. (2007) Reviewing the role of the genes G72 and DAAO in glutamate neurotransmission in schizophrenia. Eur. Neuropsychopharmacol. 17, 567-572.
6. Bruckner, H. and Hausch, M. (1994) Gas chromatographic characterization of free D-amino acids in the blood serum of patients with renal disorders and of healthy volunteers. J. Chromatogr. 614, 7-17.
7. Chumakov, I., Blumenfeld, M., Guerassimenko, O., Cavarec, L., Palicio, M., Abderrahim, H., Bougueleret, L., Barry, C., Tanaka, H., La Rosa, P., Puech, A., Tahri, N., Cohen-Akenine, A., Delabrosse, S., Lissarrague, S., Picard, F.P., Maurice, K., Essioux, L., Millasseau, P., Grel, P., Debailleul, V., Simon, A.M., Caterina, D., Dufaure, I., Malekzadeh, K., Belova, M., Luan, J.J., Bouillot, M., Sambucy, J.L., Primas, G., Saumier, M., Boubkiri, N., Martin-Saumier, S., Nasroune, M., Peixoto, H., Delaye, A., Pinchot, V., Bastucci, M., Guillou, S., Chevillon, M., Sainz-Fuertes, R., Meguenni, S., Aurich-Costa, J., Cherif, D., Gimalac, A., Van Duijn, C., Gauvreau, D., Ouellette, G., Fortier, I., Raelson, J., Sherbatich, T., Riazanskaia, N., Rogaev, E., Raeymaekers, P., Aerssens, J., Konings, F., Luyten, W., Macciardi, F., Sham, P.C., Straub, R.E., Weinberger, D.R., Cohen, N., Cohen, D. (2002) Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. Proc. Natl. Acad. Sci. U. S. A. 99, 13675-13680.
8. Daly, D.A. and Moghaddam, B. (1993) Actions of clozapine and haloperidol on the extracellular levels of excitatory amino acids in the prefrontal cortex and striatum of conscious rats. Neurosci. Lett. 152, 61-64.
9. Detera-Wadleigh, S.D. and McMahon, F.J. (2006) G72/G30 in Schizophrenia and Bipolar Disorder: Review and Meta-analysis. Biol. Psychiatry. 60, 106-114.
10. Dunlop, D.S. and Neidle, A. (2005) Regulation of serine racemase activity by amino acids. Brain Res. Mol. Brain Res. 133, 208-214.
11. Evins, A.E., Amico, E.T., Shih, V., Goff, D.C. (1997) Clozapine treatment increases serum glutamate and aspartate compared to conventional neuroleptics. J. Neural Transm. 104, 761-766.

12. Farber, N.B., Foster, J., Duhan, N.L., Olney, J.W. (1996) Olanzapine and fluperlapine mimic clozapine in preventing MK-801 neurotoxicity. Schizophr. Res. 21, 33-37.
13. Fu, T.F., Rife, J.P., Schirch, V. (2001) The role of serine hydroxymethyltransferase isozymes in one-carbon metabolism in MCF-7 cells as determined by (13)C NMR. Arch. Biochem. Biophys. 393, 42-50.
14. Fuchs, S.A., Dorland, L., De Sain-Van Der Velden, M.G., Hendriks, M., Klomp, L.W., Berger, R., De Koning, T.J. (2006) D-serine in the developing human central nervous system. Ann. Neurol. 60, 476-480.
15. Fujii, K., Maeda, K., Hikida, T., Mustafa, A.K., Balkissoon, R., Xia, J., Yamada, T., Ozeki, Y., Kawahara, R., Okawa, M., Huganir, R.L., Ujike, H., Snyder, S.H., Sawa, A. (2006) Serine racemase binds to PICK1: potential relevance to schizophrenia. Mol. Psychiatry. 11, 150-157.
16. Goff, D.C. and Coyle, J.T. (2001) The emerging role of glutamate in the pathophysiology and treatment of schizophrenia. Am. J. Psychiatry. 158, 1367-1377.
17. Goff, D.C., Tsai, G., Manoach, D.S., Flood, J., Darby, D.G., Coyle, J.T. (1996) D-cycloserine added to clozapine for patients with schizophrenia. Am. J. Psychiatry. 153, 1628-1630.
18. Goldner, E.M., Hsu, L., Waraich, P., Somers, J.M. (2002) Prevalence and incidence studies of schizophrenic disorders: a systematic review of the literature. Can. J. Psychiatry. 47, 833-843.
19. Harrison, P.J. and Owen, M.J. (2003) Genes for schizophrenia? Recent findings and their pathophysiological implications. Lancet. 361, 417-419.
20. Hashimoto, K., Engberg, G., Shimizu, E., Nordin, C., Lindstrom, L.H., Iyo, M. (2005) Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naive schizophrenic patients. Prog. Neuropsychopharmacol. Biol. Psychiatry. 29, 767-769.
21. Hashimoto, K., Fukushima, T., Shimizu, E., Komatsu, N., Watanabe, H., Shinoda, N., Nakazato, M., Kumakiri, C., Okada, S., Hasegawa, H., Imai, K., Iyo, M. (2003) Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. Arch. Gen. Psychiatry. 60, 572-576.
22. Herbig, K., Chiang, E.P., Lee, L.R., Hills, J., Shane, B., Stover, P.J. (2002) Cytoplasmic serine hydroxymethyltransferase mediates competition between folate-dependent deoxyribonucleotide and S-adenosylmethionine biosyntheses. J. Biol. Chem. 277, 38381-38389.
23. Heresco-Levy, U. (2003) Glutamatergic neurotransmission modulation and the mechanisms of antipsychotic atypicality. Prog. Neuropsychopharmacol. Biol. Psychiatry. 27, 1113-1123.
24. Kumashiro, S., Hashimoto, A., Nishikawa, T. (1995) Free D-serine in post-mortem brains and spinal cords of individuals with and without neuropsychiatric diseases. Brain Res. 681, 117-125.
25. Lahti, A.C., Weiler, M.A., Tamara Michaelidis, B.A., Parwani, A., Tamminga, C.A. (2001) Effects of ketamine in normal and schizophrenic volunteers. Neuropsychopharmacology. 25, 455-467.
26. Miyamoto, Y., Yamada, K., Noda, Y., Mori, H., Mishina, M., Nabeshima, T. (2001) Hyperfunction of dopaminergic and serotonergic neuronal systems in mice lacking the NMDA receptor epsilon1 subunit. J. Neurosci. 21, 750-757.
27. Mohn, A.R., Gainetdinov, R.R., Caron, M.G., Koller, B.H. (1999) Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. Cell. 98, 427-436.
28. Morita, Y., Ujike, H., Tanaka, Y., Otani, K., Kishimoto, M., Morio, A., Kotaka, T., Okahisa, Y.,

Matsushita, M., Morikawa, A., Hamase, K., Zaitsu, K., Kuroda, S. (2007) A genetic variant of the serine racemase gene is associated with schizophrenia. Biol. Psychiatry. 61, 1200-1203.

29. Olney, J.W. and Farber, N.B. (1994) Efficacy of clozapine compared with other antipsychotics in preventing NMDA-antagonist neurotoxicity. J. Clin. Psychiatry. 55 Suppl B, 43-46.
30. Scheepers, F.E., Gispen-De Wied, C.C., Westenberg, H.G., Kahn, R.S. (2002) Effect of olanzapine on glutamate levels in cerebrospinal fluid of patients with schizophrenia. J. Clin. Psychopharmacol. 22, 333-334.
31. Steffek, A.E., Haroutunian, V., Meador-Woodruff, J.H. (2006) Serine racemase protein expression in cortex and hippocampus in schizophrenia. Neuroreport. 17, 1181-1185.
32. Strisovsky, K., Jiraskova, J., Mikulova, A., Rulisek, L., Konvalinka, J. (2005) Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxylate substrate and inhibitors and analysis of the beta-eliminase activity. Biochemistry. 44, 13091-13100.
33. Tsai, G., Lane, H.Y., Yang, P., Chong, M.Y., Lange, N. (2004) Glycine transporter I inhibitor, N-methylglycine (sarcosine), added to antipsychotics for the treatment of schizophrenia. Biol. Psychiatry. 55, 452-456.
34. Tsai, G., van Kammen, D.P., Chen, S., Kelley, M.E., Grier, A., Coyle, J.T. (1998) Glutamatergic neurotransmission involves structural and clinical deficits of schizophrenia. Biol. Psychiatry. 44, 667-674.
35. Tsai, S.J., Cheng, C.Y., Hong, C.J., Liao, D.L., Hou, S.J., Yen, F.C., Liou, Y.J. (2006) Association study of polymorphisms in glycine transporter with schizophrenia. J. Neural Transm. 113, 1545-1549.
36. Tuominen, H.J., Tiihonen, J., Wahlbeck, K. (2006) Glutamatergic drugs for schizophrenia. Cochrane. Database. Syst. Rev. CD003730.
37. Wolosker, H., Blackshaw, S., Snyder, S.H. (1999) Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. Proc. Natl. Acad. Sci. U. S. A. 96, 13409-13414.
38. Yamamoto, B.K. and Cooperman, M.A. (1994) Differential effects of chronic antipsychotic drug treatment on extracellular glutamate and dopamine concentrations. J. Neurosci. 14, 4159-4166.

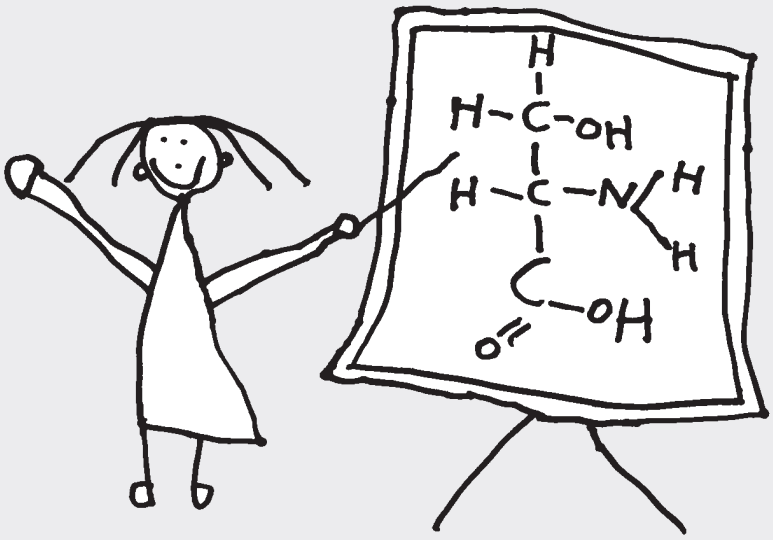
Discussion



Chapter 8

General discussion

Submitted in adapted form



D-SERINE: THE RIGHT OR WRONG ISOFORM?

Only a few decades ago, D-amino acids were considered to be restricted to some bacteria and insects. In the last few years, evidence is accumulating that D-amino acids occur in mammals (**chapter 1**) and we are only starting to unravel their biological function. Most research has focused on D-serine, which was found to occur in high concentrations in the mammalian brain.(1) D-Serine can be synthesized and metabolized endogenously by the enzymes serine racemase (SR) (2) and D-amino acid oxidase (DAO),(3) respectively. In mammalian central nervous system (CNS), D-serine was found to function as a neurotransmitter by binding to the N-methyl-D-aspartate receptor (NMDAr). NMDArs are widely expressed in the CNS, where they play important roles in physiological and pathological processes,(4) including synaptic plasticity and neurotoxicity. NMDArs are tetrameric glutamate-gated ion channels, occurring as multiple subtypes which differ in their subunit composition, which mostly comprises an NR1 subunit, combined with at least one of four NR2 (A-D) subunits. Depending on the subunit composition, the biophysical and pharmacological properties of NMDArs vary.(5) One of the unique features of NMDArs is that they are only activated upon simultaneous binding by glutamate to their NR2 subunit and by a co-agonist to their NR1 subunit. Similar to glycine, which was long thought to be the endogenous NMDAr co-agonist, D-serine can bind to the NR1 subunit. Binding to the co-agonist site is not only essential for NMDAr activation, but also exerts a neuromodulatory role, by increasing the affinity of the NMDAr for glutamate,(6) decreasing its desensitization (7) and promoting NMDAr turnover by internalization.(8) This regulation of NMDAr activity by a second agonist might be regarded as a safety mechanism to protect against the deleterious effects of overstimulation of the NMDAr (excitotoxicity). Had this safety mechanism not existed, then the brain would potentially have been more at risk for excitotoxicity and subsequent neurotoxicity, with the abundance of glutamate in the CNS.

To investigate this putatively important role of D-serine in human CNS, we developed chiral chromatographic separation techniques, combined with mass spectrometric detection to determine concentrations of D-serine, glycine and their common precursor L-serine simultaneously in biological fluids (**chapter 2**). These stable isotope dilution analysis techniques represent valuable tools to address scientific research questions on the role of D-serine (or other D-amino acids) in mammalian physiology and pathology, with the ultimate goal of developing novel diagnostic, therapeutic and prognostic strategies for human diseases. In this respect, our generation of age-dependent reference values in human cerebrospinal fluid (CSF) (**chapters 2-3**) is pivotal in enabling diagnosis and follow-up of patients with disorders associated with alterations in CNS D-serine, L-serine or glycine concentrations. The studies presented in this thesis - all using these

analytical techniques - contribute to the emergent awareness that D-serine plays an important role as an endogenous NMDAr co-agonist in human physiology and pathology, as underscored by following lines of evidence:

1. D-SERINE IN MAMMALIAN PHYSIOLOGY

One of the main reasons to suspect D-serine of being a mammalian endogenous NMDAr co-agonist, was the close anatomical co-localization between D-serine and NMDArs.(9;10) D-Serine and NMDAr immunoreactivity was high in cranial areas of rat brain (especially in the frontal cortex) and lower in caudal areas,(9;10) where the D-serine degrading enzyme DAO was present.(11) In contrast, glycine immunoreactivity predominated in caudal areas of the brain, where the density of NMDArs was lower, with lowest glycine immunoreactivity in the telencephalon.(9) In addition, extracellular D-serine concentrations in the brain, as determined by microdialysis, exceeded the concentrations of many L-amino acids, and were similar to glycine concentrations in the cerebral cortex.(12) Moreover, in cloned NMDArs, D-serine affinity for the NR1 subunit was high,(13) indeed threefold higher than glycine. This might be explained by the crystal structure of the NR1 subunit of NMDArs, where D-serine displaces a water molecule from the binding pocket and makes three additional hydrogen bonds, when compared to glycine.(14)

Evidence for a contribution of endogenous D-serine to physiological NMDAr co-activation came from the decrease in spontaneous NMDAr activity (up to 60%) in immature rat cerebellar slices, rat hippocampus slices and primary hippocampal cell cultures upon removal of extracellular D-serine by DAO, the effect of which was fully reversed by application of exogenous D-serine.(15) Similarly, using enzymatic degradation of D-serine, endogenous D-serine was found to be required for NMDAr mediated light-evoked responses in the vertebrate retina.(16;17) In fact, upon enzymatic degradation of D-serine, light-evoked NMDAr contribution was similar to that observed after blockage of NMDArs with antagonists.(16) Additionally, electrophysiological experiments performed in the supra-optic nucleus of the hypothalamus demonstrated that endogenous D-serine degradation by recombinant DAO blocked NMDAr responses in hypothalamic slices, as opposed to endogenous glycine degradation by a glycine oxidase enzyme.(18) Finally, SR knock-out mice displaying 90% decreased D-serine concentrations showed severely altered glutamatergic neurotransmission,(19) with an overall decrease in NMDAr mediated neurotransmission, as demonstrated by lower S-nitrosylation of intracellular proteins. Together, these studies point to an important role for D-serine as a determinant of NMDAr mediated neurotransmission in mammalian physiology.

2. D-SERINE IN SPECIFIC PHYSIOLOGICAL PROCESSES

CNS development

The first clue for a specific role for D-serine in CNS development came from the finding of specifically and markedly elevated D-serine concentrations in human and rodent CNS (10;20;21) during the intense period of embryonic and early postnatal CNS development, coinciding with a transient expression (22-25) and increased activity (26-28) of NMDArs. Similarly, we found peaking D-serine concentrations in human CSF during the early postnatal (and presumably embryonic) period (**chapter 3**, (29)). The severe CNS abnormalities upon failure to achieve these specific and marked increases in D-serine concentrations, as seen in patients (30) and mutant mice (31) with 3-phosphoglycerate dehydrogenase deficiency (3-PGDH), a rare inherited disorder in L-serine and hence D-serine synthesis, suggest a role for D-serine in CNS development (**chapter 3**).
More specifically, degradation of D-serine by DAO and selective inhibition of SR and hence of D-serine synthesis in 8-day old mouse cerebellar slices significantly reduced granule cell migration, whereas D-serine activated this process,(32) implying a specific role for D-serine in neuronal migration, putatively through NMDAr activation.(33) This is supported by the unambiguous mass spectrometric identification of SR in the perireticular nucleus, a transient structure of the developing brain in humans, suggested to be mainly involved in neuronal migration.(34) Moreover, our P19 studies (**chapter 5**) imply a role for D-serine, synthesized by SR, in shaping synaptogenesis, as specific inhibition of SR and NR1 antagonism increased synaptophysin expression and interconnections between differentiated neuronal and glial cells, which was overcome by D-serine supplementation in the case of SR inhibition. Similarly, partial deletion of NR1 subunits of NMDArs in organotypic hippocampal cells profoundly increased numbers of functional synapses between neurons and strength of unitary connections *in vitro* and *in vivo*, which was overcome by reintroduction of NMDArs.(35) Conceivably, endogenous D-serine is a crucial factor in activating NMDArs, thereby preventing premature synaptic maturation by ensuring that only punctuated bursts of activity lead to induction of functional synapses for the activity-dependent wiring of neuronal circuitry.(35;36) This specific role for D-serine in synaptogenesis is further consolidated by observations in SR knock-out mice.(19) The subtle behavioural abnormalities these mice display have been associated with altered synaptic shaping, including mild hyperactivity and increased anxiety (37) and a spatial memory deficit.(38) In contrast, the naturally occurring mouse strain (ddY/DAO$^-$), lacking DAO activity and thereby displaying elevated extracellular D-serine concentrations and enhanced NMDAr function,(39) show improved spatial learning (40) and better motor coordination,(39) which might be associated with altered synaptic shaping due to increased D-serine concentrations.

Learning and memory

Long-term potentiation (LTP) of synaptic transmission in the hippocampus refers to an enhancement in signal transmission between neurons upon synchronic stimulation and is one of the processes underlying synaptic plasticity. It is widely considered one of the major cellular mechanisms underlying learning and memory in vertebrates.(41) The best understood form of LTP is induced by NMDAr activation, and D-serine released from astrocytes appears to play a role in this process. In fact, LTP could be induced in neurons grown on a layer of astrocytes, as opposed to neurons cultured in glial conditioned medium, which was overcome by D-serine supplementation.(42) LTP induction was suppressed by NMDAr antagonists and enzymatic D-serine degradation. Similarly, in adult rat CA1 pyramidal hippocampus cells, LTP depended on calcium dependent release of D-serine from astrocytes.(43) LTP could be blocked by decreasing NR1 occupancy by D-serine depletion (through depletion of stored D-serine and SR inhibition) or disruption of exocytosis, which was reversible by exogenous D-serine or glycine application. Further support comes from SR knock-out mice displaying a 90% reduction in D-serine concentrations, impaired NMDAr transmission and attenuated LTP,(19) while ddY/DAO$^-$ mice display elevated extracellular D-serine concentrations, enhanced NMDAr function and enhanced hippocampal LTP.(39;40) Additional evidence for a role of D-serine in LTP comes from studies evaluating learning and memory deterioration occurring with aging. SR Expression, D-serine concentrations, NMDAr mediated synaptic potentials and LTP were all significantly decreased in CA1 hippocampal slices from aged rats when compared with young rats, which was rescued by exogenous D-serine supplementation.(44;45) Similarly, when comparing two rat strains which model healthy aging versus accelerated aging with cognitive impairments, the latter model showed decreased hippocampal SR mRNA and protein and decreased D-serine concentrations, combined with normal DAO expression, and marked reductions in NMDAr mediated synaptic potentials and LTP, which were restored by exogenous D-serine addition.(46) Correspondingly, hippocampal slices from a senescence-accelerated mouse model showed significant and exaggerated LTP suppression with age, when compared to normal mice, which was overcome by D-serine supplementation.(47) Addition of DAO also decreased LTP, which was rescued by exogenous application of D-serine. D-serine effects were inhibited by antagonists of the NMDAr co-agonist site. Together, these results all robustly demonstrate the importance of D-serine, synthesized by SR, for NMDAr activation and subsequent LTP induction, which underlies learning and memory.

3. D-SERINE IN MAMMALIAN PATHOLOGY

Excitotoxicity

Glutamate toxicity, or excitotoxicity, occurs mainly through excessive NMDAr activation and subsequent massive calcium influx in the cell.(4) This appears to be the major reason for neurotoxicity and cell death upon hypoxia/ischemia and in neurodegenerative conditions.(48) D-Serine appears to be an important endogenous co-agonist in this process, since removal of endogenous D-serine with DAO (49) or D-serine deaminase,(50) a D-serine degrading enzyme with higher affinity and specificity to D-serine than commercially available DAO, virtually abolished NMDA-elicited neurotoxicity in rat cerebrocortical and organotypic hippocampal slices. The effect of endogenous glycine could be observed only after simultaneous removal of endogenous D-serine and blockage of the glycine transporter GlyT1.(50) Moreover, SR knock-out mice displaying a 90% reduction in extracellular D-serine concentrations demonstrated reduced neurotoxicity induced by NMDA injections in their forebrains.(51)

Perinatal asphyxia

Perinatal asphyxia refers to hypoxic/ischemic pathology occurring around birth, and represents one of the main causes of morbidity and mortality among term-born children without curative treatment options.(52) Excitotoxicity plays an essential role in the pathogenesis.(48) The role of D-serine in this process was implicated by the reduction of cell death after addition of DAO to rat cerebrocortical slices exposed to oxygen-glucose deprivation (OGD), which was reversed by addition of either D-serine or glycine.(49) Similarly, mutant mice with a targeted deletion of SR showed dramatically diminished infarct volume in several brain regions following middle cerebral artery occlusion,(53) and brain cultures from SR knock-out mice exhibited markedly diminished nitric oxide formation and neurotoxicity. Our OGD studies (**chapter 6**) showed marked increases in glycine and D-serine concentrations, putatively through increased synthesis from L-serine, which together with robustly increased glutamate concentrations,(54) are bound to lead to massive NMDAr activation and neurotoxicity. During our reperfusion experiments, glycine concentrations normalized but remained increased, while D-serine concentrations were significantly reduced, both *in vitro* and *in vivo*. This concurs with the finding of delayed transient decreases of D-serine concentrations and SR mRNA and protein in the ipsilateral temporoparietal mouse cortex after permanent focal ischemia.(55) These reduced D-serine concentrations during reperfusion might be due to SR inhibition by elevated glycine concentrations.(56) This might represent an attempt to

compensate for increased NMDAr activity, providing additional evidence for a role for D-serine in regulating NMDAr activity.
Collectively, D-serine synthesized by SR appears to play a neurotoxic role in hypoxia-ischemia. Manipulation of CNS D-serine concentrations might provide an interesting treatment target for patients with this devastating condition, especially since results from treatment with NMDAr antagonists, targeting both NR1 and NR2 subunits, have been largely disappointing.(57)

Schizophrenia

Schizophrenia is a severely debilitating psychiatric condition, characterized by psychotic features (including hallucinations), negative symptoms (including social withdrawal) and cognitive defects (including impairment in attention, learning and memory). Schizophrenia affects 1% of the population worldwide. As described in **chapter 7**, there is vast support for NMDAr hypofunction as a pathophysiological mechanism in schizophrenia. Some ambiguous evidence for a contribution of D-serine to the NMDAr hypofunction, comes from the finding of reduced D-serine concentrations (58) and a reduced D-serine to total serine ratio in CSF (58;59) and serum (60) of patients with schizophrenia. However, differences with controls were small and inconsistent between sexes, and we were unable to replicate these findings (**chapter 7**). Potentially, CSF or serum as a marker for extracellular concentration differences might be too global to detect subtle differences in specific brain areas.(61) Moreover, no reductions in D-serine concentrations were detected in the parietal or prefrontal cortex from patients with schizophrenia.(58;62)
More convincing results for a role of D-serine in the pathology of schizophrenia comes from genetic association studies, showing an association between schizophrenia with the gene *G72*,(63-71) whose product was thought to activate the D-serine degrading enzyme DAO, although recent studies have not been able to replicate this activating interaction with DAO.(72) In addition, several meta-analyses of genetic association studies in schizophrenia provide some support for an association between the *DAO* gene and schizophrenia.(73-75) More robust is the finding of increased DAO expression (76-78) and activity (76) in cerebellum, parietal cortex (79) and hippocampus (80) of patients with schizophrenia, when compared with controls. In addition, duration of illness correlated with DAO expression and activity in the hippocampus (58) and cerebellum.(76) Presumably, this increase in DAO expression and activity causes decreased D-serine concentrations in specific brain areas and thereby contributes to NMDAr hypofunction in schizophrenia.
Single-nucleotide polymorphisms in SR (81) and SR genetic variants in humans showed an association with schizophrenia.(82) Similarly, the protein interacting with C-kinase (PICK), which interacts with SR,(83) has been implicated as a susceptibility gene for schizophrenia.(83;84) Results from studies on SR expression and immunoreactivity are

ambiguous and SR activity has not been assessed in schizophrenia as of yet. No change in SR expression was observed in the parietal cortex,(77) cerebellum or prefrontal cortex, when comparing schizophrenic patients with controls.(78) SR protein levels and ratio of SR/DAO were reduced in postmortem prefrontal cortex and hippocampus from patients with schizophrenia,(58) while in other patient groups SR immunoreactivity was increased in the hippocampus (85) and the prefrontal cortex,(78) and unchanged in other brain areas when compared to controls.(78;85) Heterogeneity of patients, the disease entity and the different brain areas might contribute to these conflicting results. SR exon 1 knock-out mice displaying 90% D-serine reduction exhibited relatively subtle behavioural abnormalities, reflecting hyperactivity, impaired spatial memory and elevated anxiety, which are all relevant to schizophrenia.(19) Prepulse inhibition was not reduced, while this is a consistent finding in schizophrenia.(86) Mice with an ENU-induced mutation resulting in complete loss of SR activity and 95% reduced D-serine concentrations displayed similar behavioural deficits relevant to schizophrenia, including impairment in spatial discrimination and sociability, but no alteration in anxiety.(82) Conversely, these mice showed impaired prepulse inhibition. The behavioural symptoms of these mice were aggravated by NMDAr antagonists and ameliorated by D-serine. Expression profiling revealed that the SR mutation influenced several genes, linked to schizophrenia and cognitive ability, and altered transcript levels were normalized by D-serine. The signs of subtle negative symptoms and cognitive impairment associated with schizophrenia observed in both SR knock-out mice models might explain the beneficial effect of D-serine or glycine addition to conventional antipsychotic treatment on negative symptoms, with a trend effect on cognitive symptoms.(87) Failure to reproduce the complete schizophrenia phenotype in the SR knock-out mice might be due to compensatory mechanisms, as evidenced for NMDAr kinetics and molecular composition.(19) Moreover, given the current belief that schizophrenia represents a disorder of complex genetics with a strong environmental component, alterations in one genetic pathway implicated in the disorder would not be expected to replicate the entire phenotype.

Some of the difficulties in unveiling the underlying molecular mechanisms in schizophrenia, specifically for the role of gene *G72*, are associated with the lack of appropriate animal models. It has been hypothesized that genes responsible for higher cognitive functions have developed very recently on the evolutionary scale, especially those unique to humans, and thus relevant to psychiatric conditions such as schizophrenia.(88) This might explain the poor genetic conservation of rodent and human gene sequences of genes implicated in schizophrenia, including catechol-*O*-methyltransferase (COMT) and disrupted-in-schizophrenia-1 (DISC1).(89) Similarly, no *G72* ortholog was found in mice.(90) This poor genetic homology might be crucial in interpreting mouse behavioural phenotypes as a model for human symptoms of schizophrenia and might hamper the use of rodent models in schizophrenia research.

Summarizing, there is strong evidence for NMDAr dysfunction in schizophrenia, and numerous reports on altered extracellular D-serine concentrations or their regulation converge to a role for D-serine in the NMDAr hypofunction theory. The cognitive symptoms in schizophrenia, including impairment in learning and memory concur with the role of D-serine in LTP generation. Hopefully, future studies, potentially using primate animal models, will unveil the exact role of D-serine and the underlying mechanisms in schizophrenia and thereby contribute to improved treatment and quality of life for patients with this incapacitating condition.

Amyotrophic Lateral Sclerosis (ALS)

ALS is the most common adult-onset motor neuron disease. It is a currently incurable rapidly progressive disorder, characterized by muscle weakness, atrophy, paralysis and death from respiratory failure. Pathologically, it is characterized by massive motoneuronal loss, inclusion bodies in remaining neurons and astrocytes and gliosis around dying neurons in the ventral horns of spinal cords.(91) The mechanism of selective motoneuronal death is still unclear, but excitotoxicity mediated by ionotropic glutamate receptors has been proposed to play a principal role.(91-93) While approximately 90% of ALS patients are sporadic, 10% are inherited and 20% of those have mutations in the gene encoding superoxide dismutase 1 (SOD1). Transgenic mice with a glycine to alanine switch at codon 93 in SOD1 have become the benchmark pre-clinical model for screening ALS therapies.(94) In spinal cords from these mice, SR and D-serine concentrations were increasingly elevated with disease progression,(95) while elevated glutamate concentrations remained unchanged with disease progression.(92) In addition, primary spinal cord neurons from these mice were more vulnerable to NMDA-elicited toxicity than those from control mice in a D-serine dependent manner and removal of endogenous D-serine protected motoneurons against NMDAr mediated cell death.(95) Exogenous addition of glycine to these cells significantly reduced NMDA-induced toxicity, putatively through competitive inhibition of SR.(56) *In vitro*, SR expression was induced by an extracellular proinflammatory factor and also by transiently expressed mutant SOD1 in microglial cells.(95) In spinal cords of patients with familial and sporadic forms of ALS, the levels of SR and D-serine were also greatly increased.(95)

Based on these findings of increased D-serine concentrations in spinal cords of ALS patients, we determined concentrations of D-serine, glycine and their common precursor L-serine in CSF from patients with ALS, both sporadic and with SOD1 mutations (Table 1). Unfortunately, we did not identify any significant D-serine, glycine or L-serine concentration or ratio difference between CSF from controls and patients with ALS. Potentially, concentration differences in the spinal cord are too subtle to be detected in CSF with our analytical method. Furthermore, DAO has recently been identified im-

Table 1. D-serine, L-serine and glycine concentrations in cerebrospinal fluid from patients with ALS and controls.

	N	Age (years)	D-serine (μM)	L-serine (μM)	Glycine (μM)	D-serine / total serine (%)	Glycine / glycine + L-serine (%)	D-serine / glycine
Controls	19	63 (15)	1.8 (0.3)	26.2 (4.8)	7.0 (1.9)	6.5 (1.7)	21.3 (6.2)	0.27 (0.1)
ALS total	36	59 (13)	1.8 (0.4)	27.5 (6.2)	8.0 (4.8)	6.4 (1.7)	21.6 (6.1)	0.27 (0.1)
	p-value		0.73	0.42	0.38	0.78	0.84	0.86
ALS SOD1	15	56 (15)	1.9 (0.5)	27.0 (5.1)	7.2 (2.9)	6.6 (1.7)	20.5 (5.0)	0.29 (0.1)
	p-value		0.44	0.65	0.82	0.86	0.70	0.48
ALS sporadic	21	60 (12)	1.8 (0.3)	27.9 (6.9)	8.5 (5.7)	6.2 (1.8)	22.4 (6.7)	0.25 (0.1)
	p-value		0.91	0.38	0.26	0.59	0.59	0.37

D-serine, L-serine, glycine concentrations and their ratios in CSF from controls and patients with ALS, both sporadic or with SOD1 mutation. The p-value for the difference with controls was calculated with an unpaired Student's *t*-test.

munohistochemically in the human and rat choroid plexus, potentially thereby regulating CSF D-serine levels (96) and as such occluding concentration differences in specific areas in the CNS. Our results signify that CSF amino acid determination with our method does not provide a valuable diagnostic tool for ALS. Other methods to directly investigate D-serine concentrations in the ventral horns of spinal cords, such as spinal cord (NMR) spectroscopy (97) might form an interesting alternative in this regard.

Additional evidence for a role of D-serine in the pathophysiology of ALS comes from a recent genome screen in families with confirmed ALS without known familial ALS mutations, identifying a locus on chromosome 12 in a single family with three generations of affected members.(98) Upon screening of candidate genes within this region, a mutation in DAO was found, which, upon expression in neuronal cell lines, revealed a mutated protein with almost total loss of activity, impairing cell viability and promoting ubiquitinated aggregate formation when compared with the wild type protein. Lentiviral mediated transduction of this DAO mutation in primary motor neuron cultures or in astrocytes co-cultured with motor neurons increased apoptosis in neuronal cells. In addition, ddY/DAO$^-$ mice, lacking DAO activity and displaying elevated levels of D-serine, also display enhanced NMDAr mediated excitatory postsynaptic currents in spinal cord dorsal horn neurons.(99) Finally, enzymes involved in L-serine and consequently in D-serine biosynthesis, including phosphoserine phosphatase and 3-PGDH are among the earliest transcriptional changes detected in motor neurons in the SOD1 mouse model. (100)

In conclusion, the enhancement of glutamate toxicity by increased D-serine concentrations in the spinal cord, through alterations in D-serine biosynthesis and metabolism, might represent an underlying mechanism for ALS motoneuronal death. Further studies on modulation of these elevated D-serine concentrations in the spinal cord may help to develop neuroprotective strategies against this currently untreatable fatal condition.

4. CONTRIBUTION OF D-SERINE VERSUS GLYCINE IN MAMMALIAN NMDAR NEUROTRANSMISSION

It is still a matter of debate whether D-serine or glycine serves as the main determinant of NMDAr activity in different brain regions and in different conditions, although most recent reports appear to favour D-serine.(18;50;101) Both amino acids are present in CNS and display relatively comparable affinities to the NR1 subunit of NMDArs. The relative contribution of both co-agonists might depend on their availability at synaptic sites. Specific D-serine transporters have not yet been discovered. The affinity of neutral amino acid transporters for D-serine is low to moderate,(102-104) and encompass the higher affinity Na+-independent alanine-serine-cysteine 1 transporter (asc-1 transporter, IC_{50} 20μM) (105-108) and the low affinity Na+ and/or K+-dependent transporters,(104;108;109) including the alanine-serine-cysteine transporter 2 (ASCT2, IC_{50} 1mM). The ASCT2 has been suggested to serve as an important release rather than uptake mechanism.(109) In contrast, high-affinity glycine transport systems tightly regulate glycine access to NMDAr sites.(110-112) Antagonists of the high affinity glycine transporter type 1 (GlyT1) potentiated NMDAr activity in hippocampal pyramidal neurons (111) and in prefrontal cortex slices and prefrontal neurons in anesthetized rats. (113) Similarly, blocking GlyT1 led to full occupancy of NMDAr co-agonist sites in the salamander retina, while blocking the glycine transporter type 2 (GlyT2) had no effect on the light-evoked NMDAr currents or on their sensitivity to exogenous D-serine.(101) In the retina from heterozygous mice deficient in GlyT1, NMDAr co-agonist sites were more saturated and less enhanced by D-serine when compared with wild type controls. (114) Ultrastructural studies in the brain revealed the adjacency of GlyT1 transporters to NMDArs.(115-116) All these studies converge to the hypothesis that GlyT1 activity keeps glycine concentrations near NMDArs sufficiently low to allow D-serine to be the major regulator of NMDAr activity. In support, exogenous D-serine was more than 30 times as effective in potentiating NMDAr currents when compared with glycine in retinal ganglion cells of the tiger salamander(101) and in brain stem slice preparations,(110) requiring low physiological D-serine concentrations (micromolar range), as opposed to supraphysiological glycine concentrations (≥100μM). Similarly, when observing NMDA-elicited neurotoxicity in organotypic hippocampal slices, the effect of endogenous glycine could only be observed after simultaneous removal of endogenous D-serine and blockage of GlyT1,(50) despite ten-fold higher endogenous glycine than D-serine concentrations.

The glycine cleavage system may also play a role in maintaining subsaturating glycine concentrations near NMDArs. In astrocytes, the glycine cleavage system degrades glycine efficiently, thereby generating the cytosol / extracellular concentration gradient,(117-119) which enables glycine transporters to transfer glycine from the synaptic

space into astrocytes. Conversely, the role of DAO remains controversial in regulating D-serine concentrations near NMDArs, because D-serine and NMDArs are predominantly present in the forebrain, where DAO activity is virtually undetectable.(11) This correlates with the long half-life of D-serine in the brain (~16h).(18) When observing the *in vivo* effects of administration of L-serine, D-serine and glycine to the infant rat, intraperitoneal injection of L-serine or glycine induced elevated L-serine concentrations in the brain and a delayed and prolonged increase in D-serine concentrations. Systemic administration of D-serine resulted in increased neocortical L-serine and D-serine but not glycine content.(120) Potentially, this illustrates that extracellular glycine concentrations are more tightly regulated and that D-serine is more prone to accumulate in CNS when its production is excessive or its metabolism reduced. Thereby, D-serine presumably diffuses more easily to extrasynaptic locations and activates NMDArs in the proximity but also further away from the release sites.(18) This will provide a more general activation of NMDArs, which may play a crucial role in the massive activation that occurs in excitotoxicity.(50)

5. IMPLICATIONS AND FUTURE DIRECTIONS

Culminating evidence for a key role for endogenous D-serine in regulating NMDAr activity in physiological and pathological conditions has some interesting implications. Our development of high-throughput analytical techniques to determine D-serine concentrations in biological fluids and generation of age-dependent ranges in apparently healthy subjects (**chapter 2**) will provide us with new diagnostic and prognostic strategies for CNS disorders associated with NMDAr pathology. In addition, influencing D-serine concentrations might represent an interesting therapeutic target, which appears feasible as endogenous D-serine concentrations might be manipulated by administration of L-serine,(30;120) and by manipulation of its synthesizing and metabolizing enzymes, which both have been identified and characterized.(2;3;121)
However, a number of issues remain to be addressed in order to increase our understanding of the role of D-serine and other D-amino acids in physiological and pathological conditions for translational purposes:

1. Regulation of synaptic D-serine concentrations

As a key endogenous NMDAr regulator and thus potentially as an important contributor to the pathophysiology of the variety of conditions associated with NMDAr dysregulation, it is eminent to elucidate the mechanisms responsible for controlling synaptic D-serine concentrations, with the ultimate potential to influence these processes for therapeutic purposes.

As described before, there are several low- and moderate affinity transporters for cellular uptake of D-serine. Evidence from gene knock-out studies implicates Asc-1 as the primary transporter mediating D-serine re-uptake in neurons,(107) while the lower affinity transporter ASCT2 might be the dominant transporter involved in removal of synaptic D-serine into glia (108) (Figure 1). The contribution of these transporters to the

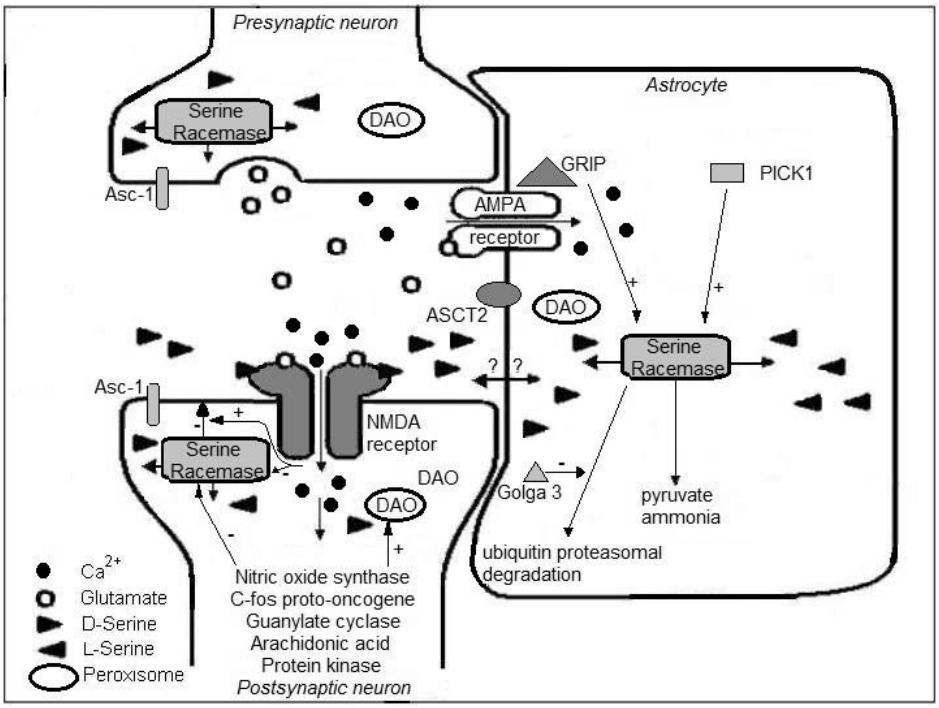


Figure 1. Regulation of synaptic D-serine concentrations. Regulation of synaptic D-serine concentrations has not been fully elucidated. Potentially, several low affinity (ASCT2) transporters and higher affinity (Asc-1) transporters are involved in neuronal (Asc-1) and glial (ASCT2) D-serine uptake. Upon uptake in neurons and astrocytes, D-serine might be degraded by peroxisomal and potentially also by non-peroxisomal DAO. However, DAO activity is virtually absent in the forebrain. Alternatively, SR might regulate D-serine concentrations in both astrocytes and neurons by enabling D-serine synthesis from L-serine and the reverse and by catalyzing degradation of both D- and L-serine. Regulation of SR activity appears to involve nitrosylation, which inhibits SR activity. Nitrosylation is increased by NMDAr activity, since this activates neuronal nitric-oxide synthase. This effect on D-serine concentrations is enhanced by increased DAO activity by nitrosylation. SR activity is dramatically decreased by translocation of cytosolic SR to the plasma membrane, which is promoted by NMDAr activation. Similarly, increased NMDAr activity through increased glycine concentrations might also constitute a feedback loop, since glycine competitively inhibits SR. Moreover, NMDAr antagonism has been found to upregulate SR mRNA and protein expression. SR is also activated by several proteins that have been found to interact with SR, including glutamate receptor interacting protein (GRIP) and protein interacting with C kinase 1 (PICK1). Golgin subfamily A member 3 (Golga3) is another protein interacting with SR, leading to decreased ubiquitin-proteasomal degradation of SR, thereby increasing SR and subsequently D-serine concentrations.

regulation of synaptic D-serine concentrations in mammalian physiology requires further investigation. Upon uptake in neurons and astrocytes, D-serine might be degraded by peroxisomal (122) and potentially also by non-peroxisomal (72;123) DAO. However, since DAO activity is virtually absent in the forebrain,(11) its role in regulating synaptic D-serine concentrations might be limited. Investigation of patients with peroxisomal disorders, and thus putatively lacking peroxisomal DAO might be helpful in clarifying the role of DAO in D-serine metabolism. Another potential regulator of synaptic D-serine concentrations is SR, which has been found to localize not only to astrocytes,(2) but possibly preferentially to neurons,(124;125) concurring with the preferential neuronal localization of Asc-1.(107) SR is responsible for D-serine synthesis from L-serine,(121) and the reverse, albeit with lower affinity. In addition, SR catalyzes degradation of both D- and L-serine by elimination of water to yield pyruvate and ammonia.(126;127) The *V*max/*K*m ratio for racemization of D- into L-serine is threefold higher than for elimination to pyruvate,(128) and the elimination reaction appears to compete with the isomerisation reaction mechanism for regulating intracellular D-serine levels, especially in forebrain areas, which lack DAO activity.(126)

Regulation of SR activity has not been fully elucidated. Nitrosylation was found to physiologically inhibit SR activity, and NMDAr stimulation activating neuronal nitric-oxide synthase enhances SR nitrosylation, supporting a feedback mechanism of NMDAr activity on D-serine concentrations.(129) This feedback mechanism might be enhanced by increased DAO activity by nitrosylation.(130) Recently, NMDAr activation has also been found to promote translocation of cytosolic SR to the plasma membrane and dendrites in primary neuronal cultures of rat brain, leading to dramatically decreased SR activity (131) and as such contribute to a feedback mechanism on D-serine concentrations and NMDAr activity. Similarly, increased NMDAr activity through increased glycine concentrations might also constitute a feedback loop, since glycine competitively inhibits SR (reported *K*i vary from 0.15 (56) -1.63mM (128) for mouse SR and these values are thought to be 2- to 5-fold lower for human than for mouse SR,(132) with estimated glycine astrocytic concentrations of 3-6mM). Moreover, NMDAr antagonism has been found to upregulate SR mRNA and protein expression.(133) All these feedback mechanisms between NMDAr activity and SR activity and expression might serve to regulate D-serine concentrations and thereby control NMDAr activity.

SR is also activated by several proteins that have been found to interact with SR, including glutamate receptor interacting protein (GRIP).(32) Similarly, protein interacting with C kinase 1 (PICK1) was found to increase D-serine concentrations upon transfection with SR in HEK293 cells,(134) which was inhibited by decreasing PICK1 expression or using SR or PICK1 variants that could not interact. Concurrently, D-serine concentrations were reduced in the forebrain of PICK1 knock-out mice. Golgin subfamily A member 3 (Golga3) is another protein interacting with SR, leading to decreased ubiquitin-proteasomal deg-

radation of SR, thereby increasing SR and subsequently D-serine concentrations (135) (Figure 1).
Further elucidation of the regulation of synaptic D-serine concentrations might contribute to the development of novel treatment approaches, including targeting D-serine transporters and D-serine synthesizing and metabolizing enzymes, either directly or through their interacting proteins.

2. Role of D-serine in pathology

Since D-serine appears to be an essential regulator of NMDAr activity, D-serine might be involved in all conditions associated with altered NMDAr activity. Some of these disorders, such as schizophrenia, have been extensively studied. To a lesser extent, the same accounts for ALS, memory defects with ageing, perinatal asphyxia and stroke. However, the exact pathophysiological mechanism and the contribution of D-serine to the pathophysiology are still largely unknown for all these disorders and further investigation is required. In addition, it would be interesting to expand this body of research with other poorly treatable disorders that have been associated with NMDAr pathology, but in which D-serine has not been comprehensively investigated, including neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, epilepsy) or other psychiatric disorders (post traumatic stress disorder, bipolar disorder, pain and anxiety disorders). Differentiating tissues constitute another interesting field to study, since SR and D-serine play important roles in CNS development (**chapter 3-5**) and a similar role has been observed in chondrogenesis.(136) The high-throughput analytical methods to determine D-serine and glycine concentrations we developed (**chapter 2**) and the age-dependent reference ranges are currently employed in the routine diagnostic laboratory of our hospital and might help in the evaluation of disorders associated with CNS pathology and/or NMDAr dysfunction and potentially yield other physiological and pathological conditions in which D-serine plays a role.

3. Function of other D-amino acids in physiology and pathology

It is highly probable that not only D-serine, but also other mammalian D-amino acids serve specific functions in mammalian physiology and pathology (**chapter 1**). The two other most abundant D-amino acids in mammalian brain are D-aspartate and D-alanine. Mammalian aspartate racemase, converting L- to D-aspartate, has recently been identified and cloned.(137) Aspartate racemase co-localizes with D-aspartate in neuroendocrine tissues and the brain, concurring with the putative neuroendocrine role of D-aspartate and its role in regulating neuronal development, potentially as another endogenous ligand of NMDArs.(137) D-Alanine can also act as an NMDAr co-agonist

(138;139) and human DAO has a higher affinity for D-alanine than for D-serine.(3) An endogenous synthetic enzyme has not been identified as of yet. The first suggestion for a role in human pathology comes from significant improvement of positive and cognitive symptoms upon addition of D-alanine to antipsychotic treatment in patients with schizophrenia.(140) Subsequently, D-alanine concentrations were determined in plasma of patients with schizophrenia during the acute phase and during remission.(141) There was no difference in D-alanine plasma levels between controls and drug-naive patients with schizophrenia. However, in patients with schizophrenia, lower plasma D-alanine levels correlated with more severe schizophrenic symptoms (especially positive symptoms). With resolution of symptoms after the acute phase, D-alanine concentrations increased to significantly higher levels, when compared to controls. Consequently, plasma D-alanine levels might constitute a therapeutic marker for schizophrenia. As a potential NMDAr co-agonist, this contributes to the NMDAr hypofunction theory in schizophrenia. Obviously, further investigation in the field of D-amino acids is required. This might be facilitated by our generation of a high-throughput chromatographic-mass spectrometric analytical method to determine numerous D-amino acids simultaneously in different biological fluids, including blood and CSF. This will enable us to study the role of D-amino acids in mammalian physiology and pathology. In addition to generating exciting insight in mammalian physiology and pathology, hopefully, this will contribute to the development of novel diagnostic, prognostic and treatment modalities.

REFERENCES

1. Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T et al. The presence of free D-serine in rat brain. FEBS Lett 1992 January 13;296(1):33-6.
2. Wolosker H, Blackshaw S, Snyder SH. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. Proc Natl Acad Sci U S A 1999 November 9;96(23):13409-14.
3. Molla G, Sacchi S, Bernasconi M, Pilone MS, Fukui K, Pollegioni L. Characterization of human D-amino acid oxidase. FEBS Lett 2006;580:2358-64.
4. Danysz W, Parsons CG. Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol Rev 1998 December;50(4):597-664.
5. Paoletti P, Neyton J. NMDA receptor subunits: function and pharmacology. Curr Opin Pharmacol 2007 February;7(1):39-47.
6. Fadda E, Danysz W, Wroblewski JT, Costa E. Glycine and D-serine increase the affinity of N-methyl-D-aspartate sensitive glutamate binding sites in rat brain synaptic membranes. Neuropharmacology 1988 November;27(11):1183-5.
7. Lerma J, Zukin RS, Bennett MV. Glycine decreases desensitization of N-methyl-D-aspartate (NMDA) receptors expressed in Xenopus oocytes and is required for NMDA responses. Proc Natl Acad Sci U S A 1990 March;87(6):2354-8.
8. Nong Y, Huang YQ, Ju W, Kalia LV, Ahmadian G, Wang YT et al. Glycine binding primes NMDA receptor internalization. Nature 2003 March 20;422(6929):302-7.
9. Hashimoto A, Nishikawa T, Oka T, Takahashi K. Endogenous D-serine in rat brain: N-methyl-D-aspartate receptor-related distribution and aging. J Neurochem 1993 February;60(2):783-6.
10. Schell MJ, Brady RO, Jr., Molliver ME, Snyder SH. D-serine as a neuromodulator: regional and developmental localizations in rat brain glia resemble NMDA receptors. J Neurosci 1997 March 1;17(5):1604-15.
11. Horiike K, Tojo H, Arai R, Nozaki M, Maeda T. D-amino-acid oxidase is confined to the lower brain stem and cerebellum in rat brain: regional differentiation of astrocytes. Brain Res 1994 August 1;652(2):297-303.
12. Hashimoto A, Oka T, Nishikawa T. Extracellular concentration of endogenous free D-serine in the rat brain as revealed by in vivo microdialysis. Neuroscience 1995 June;66(3):635-43.
13. Matsui T, Sekiguchi M, Hashimoto A, Tomita U, Nishikawa T, Wada K. Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration. J Neurochem 1995 July;65(1):454-8.
14. Furukawa H, Gouaux E. Mechanisms of activation, inhibition and specificity: crystal structures of the NMDA receptor NR1 ligand-binding core. EMBO J 2003 June 16;22(12):2873-85.
15. Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD et al. D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 2000 April 25;97(9):4926-31.
16. Gustafson EC, Stevens ER, Wolosker H, Miller RF. Endogenous D-serine contributes to NMDA-receptor-mediated light-evoked responses in the vertebrate retina. J Neurophysiol 2007 July;98(1):122-30.
17. Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR et al. D-serine and

serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. Proc Natl Acad Sci U S A 2003 May 27;100(11):6789-94.

18. Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA et al. Glia-derived D-serine controls NMDA receptor activity and synaptic memory. Cell 2006 May 19;125(4): 775-84.
19. Basu AC, Tsai GE, Ma CL, Ehmsen JT, Mustafa AK, Han L et al. Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. Mol Psychiatry 2009 July;14(7):719-27.
20. Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T et al. Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. J Neurochem 1993 July;61(1):348-51.
21. Hashimoto A, Oka T, Nishikawa T. Anatomical distribution and postnatal changes in endogenous free D-aspartate and D-serine in rat brain and periphery. Eur J Neurosci 1995 August 1;7(8):1657-63.
22. Chahal H, D'Souza SW, Barson AJ, Slater P. Modulation by magnesium of N-methyl-D-aspartate receptors in developing human brain. Arch Dis Child Fetal Neonatal Ed 1998 March;78(2):F116-F120.
23. Panigrahy A, Rosenberg PA, Assmann S, Foley EC, Kinney HC. Differential expression of glutamate receptor subtypes in human brainstem sites involved in perinatal hypoxia-ischemia. J Comp Neurol 2000 November 13;427(2):196-208.
24. Piggott MA, Perry EK, Perry RH, Court JA. [3H]MK-801 binding to the NMDA receptor complex, and its modulation in human frontal cortex during development and aging. Brain Res 1992 August 21;588(2):277-86.
25. Slater P, McConnell SE, D'Souza SW, Barson AJ. Postnatal changes in N-methyl-D-aspartate receptor binding and stimulation by glutamate and glycine of [3H]-MK-801 binding in human temporal cortex. Br J Pharmacol 1993 April;108(4):1143-9.
26. Crair MC, Malenka RC. A critical period for long-term potentiation at thalamocortical synapses. Nature 1995 May 25;375(6529):325-8.
27. Feldman DE, Nicoll RA, Malenka RC, Isaac JT. Long-term depression at thalamocortical synapses in developing rat somatosensory cortex. Neuron 1998 August;21(2):347-57.
28. Ramoa AS, McCormick DA. Enhanced activation of NMDA receptor responses at the immature retinogeniculate synapse. J Neurosci 1994 April;14(4):2098-105.
29. Fuchs SA, Dorland L, de Sain-van der Velden MG, Hendriks M, Klomp LW, Berger R et al. D-serine in the developing human central nervous system. Ann Neurol 2006 October;60(4): 476-80.
30. de Koning TJ, Klomp LW, van Oppen AC, Beemer FA, Dorland L, van dB, I et al. Prenatal and early postnatal treatment in 3-phosphoglycerate-dehydrogenase deficiency. Lancet 2004 December 18;364(9452):2221-2.
31. Yoshida K, Furuya S, Osuka S, Mitoma J, Shinoda Y, Watanabe M et al. Targeted disruption of the mouse 3-phosphoglycerate dehydrogenase gene causes severe neurodevelopmental defects and results in embryonic lethality. J Biol Chem 2004 January 30;279(5):3573-7.
32. Kim PM, Aizawa H, Kim PS, Huang AS, Wickramasinghe SR, Kashani AH et al. Serine race-

mase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. Proc Natl Acad Sci U S A 2005 February 8;102(6):2105-10.
33. Komuro H, Rakic P. Modulation of neuronal migration by NMDA receptors. Science 1993 April 2;260(5104):95-7.
34. Hepner F, Pollak A, Ulfig N, Yae-Kyung M, Lubec G. Mass spectrometrical analysis of human serine racemase in foetal brain. J Neural Transm 2005 June;112(6):805-11.
35. Adesnik H, Li G, During MJ, Pleasure SJ, Nicoll RA. NMDA receptors inhibit synapse unsilencing during brain development. Proc Natl Acad Sci U S A 2008 April 8;105(14):5597-602.
36. Rabacchi S, Bailly Y, Delhaye-Bouchaud N, Mariani J. Involvement of the N-methyl D-aspartate (NMDA) receptor in synapse elimination during cerebellar development. Science 1992 June 26;256(5065):1823-5.
37. Pape HC, Pare D. Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol Rev 2010 April;90(2):419-63.
38. Ramirez-Amaya V, Balderas I, Sandoval J, Escobar ML, Bermudez-Rattoni F. Spatial long-term memory is related to mossy fiber synaptogenesis. J Neurosci 2001 September 15;21(18): 7340-8.
39. Almond SL, Fradley RL, Armstrong EJ, Heavens RB, Rutter AR, Newman RJ et al. Behavioral and biochemical characterization of a mutant mouse strain lacking D-amino acid oxidase activity and its implications for schizophrenia. Mol Cell Neurosci 2006 August;32(4):324-34.
40. Maekawa M, Watanabe M, Yamaguchi S, Konno R, Hori Y. Spatial learning and long-term potentiation of mutant mice lacking D-amino-acid oxidase. Neurosci Res 2005 September;53(1):34-8.
41. Bliss TV, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 1993 January 7;361(6407):31-9.
42. Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C et al. Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. Proc Natl Acad Sci U S A 2003 December 9;100(25):15194-9.
43. Henneberger C, Papouin T, Oliet SH, Rusakov DA. Long-term potentiation depends on release of D-serine from astrocytes. Nature 2010 January 14;463(7278):232-6.
44. Junjaud G, Rouaud E, Turpin F, Mothet JP, Billard JM. Age-related effects of the neuromodulator D-serine on neurotransmission and synaptic potentiation in the CA1 hippocampal area of the rat. J Neurochem 2006 August;98(4):1159-66.
45. Mothet JP, Rouaud E, Sinet PM, Potier B, Jouvenceau A, Dutar P et al. A critical role for the glial-derived neuromodulator D-serine in the age-related deficits of cellular mechanisms of learning and memory. Aging Cell 2006 June;5(3):267-74.
46. Turpin FR, Potier B, Dulong JR, Sinet PM, Alliot J, Oliet SH et al. Reduced serine racemase expression contributes to age-related deficits in hippocampal cognitive function. Neurobiol Aging 2009 October 1.
47. Yang S, Qiao H, Wen L, Zhou W, Zhang Y. D-serine enhances impaired long-term potentiation in CA1 subfield of hippocampal slices from aged senescence-accelerated mouse prone/8. Neurosci Lett 2005 April 29;379(1):7-12.
48. Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci 1990;13:171-82.
49. Katsuki H, Nonaka M, Shirakawa H, Kume T, Akaike A. Endogenous D-serine is involved in

induction of neuronal death by N-methyl-D-aspartate and simulated ischemia in rat cerebrocortical slices. J Pharmacol Exp Ther 2004 November;311(2):836-44.

50. Shleper M, Kartvelishvily E, Wolosker H. D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices. J Neurosci 2005 October 12;25(41):9413-7.
51. Inoue R, Hashimoto K, Harai T, Mori H. NMDA- and beta-Amyloid1-42-induced neurotoxicity is attenuated in serine racemase knock-out mice. J Neurosci 2008 December 31;28(53): 14486-91.
52. Lawn JE, Cousens S, Zupan J. 4 million neonatal deaths: when? Where? Why? Lancet 2005 March 5;365(9462):891-900.
53. Mustafa AK, Ahmad AS, Zeynalov E, Gazi SK, Sikka G, Ehmsen JT et al. Serine racemase deletion protects against cerebral ischemia and excitotoxicity. J Neurosci 2010 January 27; 30(4):1413-6.
54. Benveniste H, Drejer J, Schousboe A, Diemer NH. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 1984 November;43(5):1369-74.
55. Wang LZ, Zhu XZ. Serine racemase expression in mouse cerebral cortex after permanent focal cerebral ischemia. Acta Pharmacol Sin 2004 April;25(4):436-41.
56. Dunlop DS, Neidle A. Regulation of serine racemase activity by amino acids. Brain Res Mol Brain Res 2005 February 18;133(2):208-14.
57. Madden K. NMDA receptor antagonists and glycine site NMDA antagonists. Curr Med Res Opin 2002;18 Suppl 2:s27-s31.
58. Bendikov I, Nadri C, Amar S, Panizzutti R, De MJ, Wolosker H et al. A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. Schizophr Res 2007 February;90(1-3):41-51.
59. Hashimoto K, Engberg G, Shimizu E, Nordin C, Lindstrom LH, Iyo M. Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naive schizophrenic patients. Prog Neuropsychopharmacol Biol Psychiatry 2005 June;29(5):767-9.
60. Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N et al. Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. Arch Gen Psychiatry 2003 June;60(6):572-6.
61. Tsai G, van Kammen DP, Chen S, Kelley ME, Grier A, Coyle JT. Glutamatergic neurotransmission involves structural and clinical deficits of schizophrenia. Biol Psychiatry 1998 October 15;44(8):667-74.
62. Kumashiro S, Hashimoto A, Nishikawa T. Free D-serine in post-mortem brains and spinal cords of individuals with and without neuropsychiatric diseases. Brain Res 1995 May 29; 681(1-2):117-25.
63. Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H et al. Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. Proc Natl Acad Sci U S A 2002 October 15;99(21):13675-80.
64. Detera-Wadleigh SD, McMahon FJ. G72/G30 in Schizophrenia and Bipolar Disorder: Review and Meta-analysis. Biol Psychiatry 2006 July 15;60(2):106-14.
65. Goldberg TE, Straub RE, Callicott JH, Hariri A, Mattay VS, Bigelow L et al. The G72/G30 gene

complex and cognitive abnormalities in schizophrenia. Neuropsychopharmacology 2006 September;31(9):2022-32.

66. Korostishevsky M, Kaganovich M, Cholostoy A, Ashkenazi M, Ratner Y, Dahary D et al. Is the G72/G30 locus associated with schizophrenia? single nucleotide polymorphisms, haplotypes, and gene expression analysis. Biol Psychiatry 2004 August 1;56(3):169-76.
67. Korostishevsky M, Kremer I, Kaganovich M, Cholostoy A, Murad I, Muhaheed M et al. Transmission disequilibrium and haplotype analyses of the G72/G30 locus: suggestive linkage to schizophrenia in Palestinian Arabs living in the North of Israel. Am J Med Genet B Neuropsychiatr Genet 2006 January 5;141B(1):91-5.
68. Ma J, Qin W, Wang XY, Guo TW, Bian L, Duan SW et al. Further evidence for the association between G72/G30 genes and schizophrenia in two ethnically distinct populations. Mol Psychiatry 2006 May;11(5):479-87.
69. Schumacher J, Jamra RA, Freudenberg J, Becker T, Ohlraun S, Otte AC et al. Examination of G72 and D-amino-acid oxidase as genetic risk factors for schizophrenia and bipolar affective disorder. Mol Psychiatry 2004 February;9(2):203-7.
70. Wang X, He G, Gu N, Yang J, Tang J, Chen Q et al. Association of G72/G30 with schizophrenia in the Chinese population. Biochem Biophys Res Commun 2004 July 9;319(4):1281-6.
71. Zou F, Li C, Duan S, Zheng Y, Gu N, Feng G et al. A family-based study of the association between the G72/G30 genes and schizophrenia in the Chinese population. Schizophr Res 2005 March 1;73(2-3):257-61.
72. Sacchi S, Bernasconi M, Martineau M, Mothet JP, Ruzzene M, Pilone MS et al. pLG72 modulates intracellular D-serine levels through its interaction with D-amino acid oxidase: effect on schizophrenia susceptibility. J Biol Chem 2008 August 8;283(32):22244-56.
73. Allen NC, Bagade S, McQueen MB, Ioannidis JP, Kavvoura FK, Khoury MJ et al. Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. Nat Genet 2008 July;40(7):827-34.
74. Shi J, Gershon ES, Liu C. Genetic associations with schizophrenia: meta-analyses of 12 candidate genes. Schizophr Res 2008 September;104(1-3):96-107.
75. Sun J, Kuo PH, Riley BP, Kendler KS, Zhao Z. Candidate genes for schizophrenia: a survey of association studies and gene ranking. Am J Med Genet B Neuropsychiatr Genet 2008 October 5;147B(7):1173-81.
76. Burnet PW, Eastwood SL, Bristow GC, Godlewska BR, Sikka P, Walker M et al. D-amino acid oxidase activity and expression are increased in schizophrenia. Mol Psychiatry 2008 July;13(7):658-60.
77. Kapoor R, Lim KS, Cheng A, Garrick T, Kapoor V. Preliminary evidence for a link between schizophrenia and NMDA-glycine site receptor ligand metabolic enzymes, d-amino acid oxidase (DAAO) and kynurenine aminotransferase-1 (KAT-1). Brain Res 2006 August 23;1106(1):205-10.
78. Verrall L, Walker M, Rawlings N, Benzel I, Kew JN, Harrison PJ et al. d-Amino acid oxidase and serine racemase in human brain: normal distribution and altered expression in schizophrenia. Eur J Neurosci 2007 September;26(6):1657-69.
79. Madeira C, Freitas ME, Vargas-Lopes C, Wolosker H, Panizzutti R. Increased brain D-amino acid oxidase (DAAO) activity in schizophrenia. Schizophr Res 2008 April;101(1-3):76-83.
80. Habl G, Zink M, Petroianu G, Bauer M, Schneider-Axmann T, von WM et al. Increased D-

amino acid oxidase expression in the bilateral hippocampal CA4 of schizophrenic patients: a post-mortem study. J Neural Transm 2009 December;116(12):1657-65.

81. Morita Y, Ujike H, Tanaka Y, Otani K, Kishimoto M, Morio A et al. A genetic variant of the serine racemase gene is associated with schizophrenia. Biol Psychiatry 2007 May 15;61(10): 1200-3.
82. Labrie V, Fukumura R, Rastogi A, Fick LJ, Wang W, Boutros PC et al. Serine racemase is associated with schizophrenia susceptibility in humans and in a mouse model. Hum Mol Genet 2009 September 1;18(17):3227-43.
83. Fujii K, Maeda K, Hikida T, Mustafa AK, Balkissoon R, Xia J et al. Serine racemase binds to PICK1: potential relevance to schizophrenia. Mol Psychiatry 2006 February;11(2):150-7.
84. Dev KK, Henley JM. The schizophrenic faces of PICK1. Trends Pharmacol Sci 2006 November;27(11):574-9.
85. Steffek AE, Haroutunian V, Meador-Woodruff JH. Serine racemase protein expression in cortex and hippocampus in schizophrenia. Neuroreport 2006 July 31;17(11):1181-5.
86. Braff DL, Grillon C, Geyer MA. Gating and habituation of the startle reflex in schizophrenic patients. Arch Gen Psychiatry 1992 March;49(3):206-15.
87. Tuominen HJ, Tiihonen J, Wahlbeck K. Glutamatergic drugs for schizophrenia: a systematic review and meta-analysis. Schizophr Res 2005 January 1;72(2-3):225-34.
88. Clark AG, Glanowski S, Nielsen R, Thomas PD, Kejariwal A, Todd MA et al. Inferring nonneutral evolution from human-chimp-mouse orthologous gene trios. Science 2003 December 12;302(5652):1960-3.
89. Bord L, Wheeler J, Paek M, Saleh M, Lyons-Warren A, Ross CA et al. Primate disrupted-in-schizophrenia-1 (DISC1): high divergence of a gene for major mental illnesses in recent evolutionary history. Neurosci Res 2006 November;56(3):286-93.
90. Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H et al. Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. Proc Natl Acad Sci U S A 2002 October 15;99(21):13675-80.
91. Bruijn LI, Miller TM, Cleveland DW. Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annu Rev Neurosci 2004;27:723-49.
92. Rothstein JD, Tsai G, Kuncl RW, Clawson L, Cornblath DR, Drachman DB et al. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. Ann Neurol 1990 July;28(1): 18-25.
93. Spreux-Varoquaux O, Bensimon G, Lacomblez L, Salachas F, Pradat PF, Le FN et al. Glutamate levels in cerebrospinal fluid in amyotrophic lateral sclerosis: a reappraisal using a new HPLC method with coulometric detection in a large cohort of patients. J Neurol Sci 2002 January 15;193(2):73-8.
94. Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 1994 June 17;264(5166):1772-5.
95. Sasabe J, Chiba T, Yamada M, Okamoto K, Nishimoto I, Matsuoka M et al. D-serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. EMBO J 2007 September 19;26(18):4149-59.
96. Ono K, Shishido Y, Park HK, Kawazoe T, Iwana S, Chung SP et al. Potential pathophysiological role of D-amino acid oxidase in schizophrenia: immunohistochemical and in situ hybridization

study of the expression in human and rat brain. J Neural Transm 2009 October;116(10): 1335-47.

97. Ciccarelli O, Wheeler-Kingshott CA, McLean MA, Cercignani M, Wimpey K, Miller DH et al. Spinal cord spectroscopy and diffusion-based tractography to assess acute disability in multiple sclerosis. Brain 2007 August;130(Pt 8):2220-31.
98. Mitchell J, Paul P, Chen HJ, Morris A, Payling M, Falchi M et al. Familial amyotrophic lateral sclerosis is associated with a mutation in D-amino acid oxidase. Proc Natl Acad Sci U S A 2010 April 20;107(16):7556-61.
99. Wake K, Yamazaki H, Hanzawa S, Konno R, Sakio H, Niwa A et al. Exaggerated responses to chronic nociceptive stimuli and enhancement of N-methyl-D-aspartate receptor-mediated synaptic transmission in mutant mice lacking D-amino-acid oxidase. Neurosci Lett 2001 January 5;297(1):25-8.
100. Lobsiger CS, Boillee S, Cleveland DW. Toxicity from different SOD1 mutants dysregulates the complement system and the neuronal regenerative response in ALS motor neurons. Proc Natl Acad Sci U S A 2007 May 1;104(18):7319-26.
101. Stevens ER, Gustafson EC, Miller RF. Glycine transport accounts for the differential role of glycine vs. D-serine at NMDA receptor coagonist sites in the salamander retina. Eur J Neurosci 2010 March;31(5):808-16.
102. Hayashi F, Takahashi K, Nishikawa T. Uptake of D- and L-serine in C6 glioma cells. Neurosci Lett 1997 December 19;239(2-3):85-8.
103. Nakauchi J, Matsuo H, Kim DK, Goto A, Chairoungdua A, Cha SH et al. Cloning and characterization of a human brain Na(+)-independent transporter for small neutral amino acids that transports D-serine with high affinity. Neurosci Lett 2000 June 30;287(3):231-5.
104. Ribeiro CS, Reis M, Panizzutti R, De MJ, Wolosker H. Glial transport of the neuromodulator D-serine. Brain Res 2002 March 8;929(2):202-9.
105. Helboe L, Egebjerg J, Moller M, Thomsen C. Distribution and pharmacology of alanine-serine-cysteine transporter 1 (asc-1) in rodent brain. Eur J Neurosci 2003 October;18(8): 2227-38.
106. Matsuo H, Kanai Y, Tokunaga M, Nakata T, Chairoungdua A, Ishimine H et al. High affinity D- and L-serine transporter Asc-1: cloning and dendritic localization in the rat cerebral and cerebellar cortices. Neurosci Lett 2004 March 25;358(2):123-6.
107. Rutter AR, Fradley RL, Garrett EM, Chapman KL, Lawrence JM, Rosahl TW et al. Evidence from gene knockout studies implicates Asc-1 as the primary transporter mediating d-serine reuptake in the mouse CNS. Eur J Neurosci 2007 March;25(6):1757-66.
108. Shao Z, Kamboj A, Anderson CM. Functional and immunocytochemical characterization of D-serine transporters in cortical neuron and astrocyte cultures. J Neurosci Res 2009 August 15;87(11):2520-30.
109. Dun Y, Mysona B, Itagaki S, Martin-Studdard A, Ganapathy V, Smith SB. Functional and molecular analysis of D-serine transport in retinal Muller cells. Exp Eye Res 2007 January;84(1): 191-9.
110. Berger AJ, Dieudonne S, Ascher P. Glycine uptake governs glycine site occupancy at NMDA receptors of excitatory synapses. J Neurophysiol 1998 December;80(6):3336-40.
111. Bergeron R, Meyer TM, Coyle JT, Greene RW. Modulation of N-methyl-D-aspartate receptor function by glycine transport. Proc Natl Acad Sci U S A 1998 December 22;95(26):15730-4.

112. Supplisson S, Bergman C. Control of NMDA receptor activation by a glycine transporter co-expressed in Xenopus oocytes. J Neurosci 1997 June 15;17(12):4580-90.
113. Chen L, Muhlhauser M, Yang CR. Glycine tranporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons in vitro and in vivo. J Neurophysiol 2003 February;89(2):691-703.
114. Reed BT, Sullivan SJ, Tsai G, Coyle JT, Esguerra M, Miller RF. The glycine transporter GlyT1 controls N-methyl-D-aspartic acid receptor coagonist occupancy in the mouse retina. Eur J Neurosci 2009 December;30(12):2308-17.
115. Cubelos B, Gimenez C, Zafra F. Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. Cereb Cortex 2005 April;15(4):448-59.
116. Smith KE, Borden LA, Hartig PR, Branchek T, Weinshank RL. Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. Neuron 1992 May;8(5):927-35.
117. Sakata Y, Owada Y, Sato K, Kojima K, Hisanaga K, Shinka T et al. Structure and expression of the glycine cleavage system in rat central nervous system. Brain Res Mol Brain Res 2001 October 19;94(1-2):119-30.
118. Sato K, Yoshida S, Fujiwara K, Tada K, Tohyama M. Glycine cleavage system in astrocytes. Brain Res 1991 December 13;567(1):64-70.
119. Zafra F, Aragon C, Gimenez C. Molecular biology of glycinergic neurotransmission. Mol Neurobiol 1997 June;14(3):117-42.
120. Takahashi K, Hayashi F, Nishikawa T. In vivo evidence for the link between L- and D-serine metabolism in rat cerebral cortex. J Neurochem 1997 September;69(3):1286-90.
121. Wolosker H, Sheth KN, Takahashi M, Mothet JP, Brady RO, Jr., Ferris CD et al. Purification of serine racemase: biosynthesis of the neuromodulator D-serine. Proc Natl Acad Sci U S A 1999 January 19;96(2):721-5.
122. Moreno S, Nardacci R, Cimini A, Ceru MP. Immunocytochemical localization of D-amino acid oxidase in rat brain. J Neurocytol 1999 March;28(3):169-85.
123. Verrall L, Burnet PW, Betts JF, Harrison PJ. The neurobiology of D-amino acid oxidase and its involvement in schizophrenia. Mol Psychiatry 2010 February;15(2):122-37.
124. Miya K, Inoue R, Takata Y, Abe M, Natsume R, Sakimura K et al. Serine racemase is predominantly localized in neurons in mouse brain. J Comp Neurol 2008 October 20;510(6):641-54.
125. Yoshikawa M, Takayasu N, Hashimoto A, Sato Y, Tamaki R, Tsukamoto H et al. The serine racemase mRNA is predominantly expressed in rat brain neurons. Arch Histol Cytol 2007 July;70(2):127-34.
126. Foltyn VN, Bendikov I, De Miranda J, Panizzutti R, Dumin E, Shleper M et al. Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity. J Biol Chem 2005 January 21;280(3):1754-63.
127. Strisovsky K, Jiraskova J, Barinka C, Majer P, Rojas C, Slusher BS et al. Mouse brain serine racemase catalyzes specific elimination of L-serine to pyruvate. FEBS Lett 2003 January 30; 535(1-3):44-8.
128. Strisovsky K, Jiraskova J, Mikulova A, Rulisek L, Konvalinka J. Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxylate substrate and inhibitors and analysis of the beta-eliminase activity. Biochemistry 2005 October 4; 44(39):13091-100.
129. Mustafa AK, Kumar M, Selvakumar B, Ho GP, Ehmsen JT, Barrow RK et al. Nitric oxide

S-nitrosylates serine racemase, mediating feedback inhibition of D-serine formation. Proc Natl Acad Sci U S A 2007 February 20;104(8):2950-5.

130. Shoji K, Mariotto S, Ciampa AR, Suzuki H. Mutual regulation between serine and nitric oxide metabolism in human glioblastoma cells. Neurosci Lett 2006 February 20;394(3):163-7.
131. Balan L, Foltyn VN, Zehl M, Dumin E, Dikopoltsev E, Knoh D et al. Feedback inactivation of D-serine synthesis by NMDA receptor-elicited translocation of serine racemase to the membrane. Proc Natl Acad Sci U S A 2009 May 5;106(18):7589-94.
132. Hoffman HE, Jiraskova J, Ingr M, Zvelebil M, Konvalinka J. Recombinant human serine racemase: enzymologic characterization and comparison with its mouse ortholog. Protein Expr Purif 2009 January;63(1):62-7.
133. Hashimoto A, Yoshikawa M, Andoh H, Yano H, Matsumoto H, Kawaguchi M et al. Effects of MK-801 on the expression of serine racemase and d-amino acid oxidase mRNAs and on the D-serine levels in rat brain. Eur J Pharmacol 2007 January 19;555(1):17-22.
134. Hikida T, Mustafa AK, Maeda K, Fujii K, Barrow RK, Saleh M et al. Modulation of D-serine levels in brains of mice lacking PICK1. Biol Psychiatry 2008 May 15;63(10):997-1000.
135. Dumin E, Bendikov I, Foltyn VN, Misumi Y, Ikehara Y, Kartvelishvily E et al. Modulation of D-serine levels via ubiquitin-dependent proteasomal degradation of serine racemase. J Biol Chem 2006 July 21;281(29):20291-302.
136. Takarada T, Hinoi E, Takahata Y, Yoneda Y. Serine racemase suppresses chondrogenic differentiation in cartilage in a Sox9-dependent manner. J Cell Physiol 2008 May;215(2):320-8.
137. Kim PM, Duan X, Huang AS, Liu CY, Ming GL, Song H et al. Aspartate racemase, generating neuronal D-aspartate, regulates adult neurogenesis. Proc Natl Acad Sci U S A 2010 February 16;107(7):3175-9.
138. Sakata K, Fukushima T, Minje L, Ogurusu T, Taira H, Mishina M et al. Modulation by L- and D-isoforms of amino acids of the L-glutamate response of N-methyl-D-aspartate receptors. Biochemistry 1999 August 3;38(31):10099-106.
139. Tanii Y, Nishikawa T, Hashimoto A, Takahashi K. Stereoselective antagonism by enantiomers of alanine and serine of phencyclidine-induced hyperactivity, stereotypy and ataxia in the rat. J Pharmacol Exp Ther 1994 June;269(3):1040-8.
140. Tsai GE, Yang P, Chang YC, Chong MY. D-alanine added to antipsychotics for the treatment of schizophrenia. Biol Psychiatry 2006 February 1;59(3):230-4.
141. Hatano T, Ohnuma T, Sakai Y, Shibata N, Maeshima H, Hanzawa R et al. Plasma alanine levels increase in patients with schizophrenia as their clinical symptoms improve-Results from the Juntendo University Schizophrenia Projects (JUSP). Psychiatry Res 2010 May 15;177(1-2): 27-31.

To conclude

Summary

Amino acids are among the most important molecules for living beings, since they are used to build peptides and proteins. Depending on their spatial positioning, amino acids can occur as D- or L-amino acids. This spatial configuration determines the structural interactions and is essential for the function of peptides and proteins in complex chiral environments such as the human body. It was long thought that in nature all amino acids occurred in the L-form. In this respect, identification of D-amino acids in different organisms, including mammals, was revolutionary. In **chapter 1**, an overview is given of the different D-amino acids occurring in mammals and their putative function, focusing on D-serine. D-Serine was found to be synthesized and metabolized in the human central nervous system (CNS). Moreover, D-serine appeared to function as a neurotransmitter by binding to the N-methyl-D-aspartate receptor (NMDAr). It had long been known that the NMDAr is an exceptional receptor, requiring binding by two different agonists for its activation. In addition to glutamate, glycine had long been believed to be the necessary co-agonist. However, recent studies have demonstrated that D-serine is another endogenous co-agonist of the NMDAr in the human CNS. The NMDAr is broadly distributed throughout the mammalian CNS and has been implicated in physiological processes such as CNS development, memory and learning and in pathological processes including neurodegenerative conditions (such as amyotrophic lateral sclerosis (ALS), polyneuropathies, Parkinson's, Alzheimer's and Huntington's disease), but also in stroke, chronic pain, epilepsy and psychiatric disorders such as schizophrenia. Since by now, it is generally appreciated that D-serine is an important endogenous obligatory co-agonist in most regions of the brain, D-serine might well be involved in all processes associated with NMDAr (dys-)function.

The first part of the thesis (**D-serine and L-serine analysis**) describes how we developed two different chiral chromatographic separation techniques (liquid and gas chromatography), combined with mass spectrometric detection to determine concentrations of D-serine, glycine and their common precursor L-serine in biological fluids (**chapter 2**).

In the second part of the thesis (**D-serine in human physiology**), these analytical techniques were employed to determine reference ranges in human cerebrospinal fluid (CSF). We observed very high D-serine concentrations directly after birth, both absolutely and relative to glycine and L-serine, followed by a rapid decline over the first 3 years to low micromolar adult values (**chapter 3**). These specifically elevated D-serine concentrations in human CNS during the intense period of embryonic and early postnatal CNS development, which coincides with a transient expression and increased activity of NMDArs, led us to hypothesize that the NMDAr co-agonist D-serine plays an important role in

CNS development. To address this hypothesis, we investigated D-serine concentrations in CSF from patients with 3-phosphoglycerate dehydrogenase (3-PGDH) deficiency, a rare inherited disorder in L-serine and hence D-serine synthesis. These patients display severe neurological symptoms, including microcephaly, mental retardation and intractable seizures. In fact, we found that these patients failed to achieve the early postnatal peaking CSF D-serine concentrations. Conversely, the one patient identified and treated pre- and postnatally did achieve the peaking D-serine concentrations after birth and showed a complete reversal of the severe disease phenotype. As an NMDAr co-agonist, D-serine deficiency might well be responsible for the severe CNS abnormalities observed in patients and mutant mice with 3-PGDH deficiency. In **chapter 4**, we elaborate on the role of the NMDAr co-agonist D-serine in CNS development, focusing on the clinical implications. In **chapter 5**, we attempt to gain more mechanistic insight in the role of D-serine in CNS development by studying rat teratoma P19 cells. These cells can be differentiated into glia and neurons, expressing glutamatergic receptors, including the NMDAr. They represent an established model for neuronal and glial differentiation. Upon differentiation, P19 cells expressed the D-serine synthesizing enzyme serine racemase and synthesized and excreted D-serine. Inhibition of D-serine synthesis or prevention of D-serine binding to the NMDAr increased intercellular connections between differentiating cells and increased synaptophysin expression, which served as a marker of synapse formation. This unexpected finding might imply a role for NMDAr activation by D-serine, synthesized by serine racemase, in shaping synaptogenesis and neuronal circuitry during CNS development by preventing premature synaptic maturation by ensuring that only punctuated bursts of activity lead to induction of functional synapses.

In the third part of the thesis (**D-serine in human pathology**), we focus on pathological conditions associated with altered NMDAr activity, potentially induced by altered D-serine concentrations. This is of particular interest since these concentrations might by pharmacologically manipulated as the synthesizing and metabolizing enzymes of D-serine are known, thereby providing interesting therapeutic targets. As pediatricians, we chose to investigate perinatal asphyxia, currently one of the greatest causes of neonatal mortality and morbidity worldwide. Perinatal asphyxia is the consequence of disturbed blood circulation between mother and fetus, for example after placental pathology or umbilical cord accidents, leading to insufficient delivery of oxygen, glucose and other blood-borne fuels to the fetal organs, including the brain. In the long term, this can lead to mental retardation, epilepsy and spasticity. Treatment is limited to supportive intensive care, recently combined with hypothermia treatment. The pathophysiology has not been fully elucidated, but overstimulation of the NMDAr appears to play a central role. In **chapter 6**, we describe our D-serine and glycine concentration studies in 3 different models: 1. Rat glioma cells, subjected to oxygen and glucose deprivation, 2. CSF from human newborns affected by perinatal asphyxia 3. CSF from piglets exposed to

hypoxia-ischemia by occlusion of both carotid arteries, combined with hypoxia. We found that extracellular concentrations of glycine and D-serine were markedly increased in rat glioma cells exposed to oxygen and glucose deprivation, presumably through increased synthesis from L-serine. Upon reperfusion glycine concentrations normalized and D-serine concentrations were significantly lowered. The *in vivo* studies corroborated the finding of initially elevated and then normalizing concentrations of glycine and decreased D-serine concentrations upon reperfusion. The significant increases of both endogenous NMDAr co-agonists, as observed in our experiments, in combination with known elevated glutamate concentrations upon global ischemia, are bound to lead to massive NMDAr activation, excitotoxicity and neuronal damage. Influencing these NMDAr co-agonist concentrations provides an interesting treatment target for this common, devastating and currently untreatable condition.

Another pathological condition strongly associated with NMDAr dysfunction and potentially with altered D-serine concentrations is schizophrenia. This is a serious and relatively common psychiatric disorder, characterized by positive symptoms (including hallucinations), negative symptoms (blunted affect, emotional withdrawal) and cognitive defects. In **chapter 7**, we show no differences in D-serine or glycine concentrations between control CSF and CSF from patients with schizophrenia before and/or after treatment with the antipsychotic drug olanzapine. This implies that CSF D-serine and glycine analysis does not represent an easy diagnostic marker for schizophrenia. Potentially, CSF concentrations do not reflect subtle differences in specific brain regions that might exist in schizophrenia.

Finally, in **chapter 8**, the results of the studies presented in this thesis together with some preliminary data (from ALS patients) are discussed, emphasizing the role of D-serine as an essential endogenous regulator of NMDAr activity in human physiological and pathological processes, with a view towards the future.

Nederlandse samenvatting

Als bouwstenen voor peptiden en eiwitten, zijn aminozuren essentieel voor alle levende wezens. Afhankelijk van hun ruimtelijke positie, kunnen aminozuren voorkomen in een D- en een L-vorm. Deze ruimtelijke configuratie is bepalend voor de structurele interacties en is essentieel voor de functie van peptiden en eiwitten in een complexe chirale omgeving als het menselijk lichaam. Er is lang gedacht dat in de natuur alleen L-aminozuren voorkwamen. In dit opzicht was de identificatie van D-aminozuren in verschillende organismen, waaronder zoogdieren, revolutionair. In **hoofdstuk 1** wordt een overzicht gegeven van de verschillende D-aminozuren, waarvan op het moment van schrijven bekend was dat ze voorkwamen in zoogdieren, met hun bijbehorende functie. De nadruk lag in dit overzicht op D-serine, aangezien hierover het meest bekend was. D-Serine wordt gevormd (uit L-serine) en afgebroken in het menselijk centraal zenuwstelsel. Bovendien bleek D-serine in de hersenen te fungeren als een neurotransmitter door te binden aan de N-methyl-D-aspartaat receptor (NMDAr). De NMDAr is een ionkanaal dat zich bevindt op de rand van een zenuwcel, waar deze samenkomt met een andere zenuwcel, hetgeen de synaps genoemd wordt. Door stofjes uit te scheiden en receptoren zoals de NMDAr te activeren, kunnen zenuwcellen met elkaar communiceren en kunnen signalen doorgegeven worden. Het was al lang bekend dat de NMDAr een bijzondere receptor was, omdat deze pas geactiveerd wordt na binding door twee verschillende stoffen. Naast agonist glutamaat, werd lang gedacht dat glycine de noodzakelijke co-agonist was. Recente studies laten zien dat ook D-serine een co-agonist voor de NMDAr is in het menselijk centraal zenuwstelsel. De NMDAr komt in het menselijk centraal zenuwstelsel veelvuldig voor en lijkt een rol te spelen in fysiologische processen zoals centraal zenuwstelsel ontwikkeling, geheugen en leren, maar ook in pathologische processen, zoals neurodegeneratieve aandoeningen (zoals amyotrofe lateraal sclerose (ALS), de ziekte van Parkinson, Alzheimer en Huntington, polyneuropathieën), maar ook in infarcten, epilepsie, chronische pijn en psychiatrische ziekten zoals schizofrenie. Aangezien nu algemeen aangenomen wordt dat D-serine een essentiële NMDAr co-agonist is in de meeste gebieden van de menselijke hersenen, zou D-serine een rol kunnen spelen in alle processen die geassocieerd worden met NMDAr (dis-) functie.

Het eerste deel van dit proefschrift (**D-serine and L-serine analysis**) beschrijft de ontwikkeling van twee verschillende chirale chromatografische scheidingstechnieken (vloeistof en gas chromatografie), gecombineerd met massa spectrometrische detectie om concentraties van D-serine, glycine en hun gemeenschappelijke precursor L-serine in biologische vloeistoffen te bepalen (**hoofdstuk 2**).

In het tweede deel van dit proefschrift (**D-serine in human physiology**), zijn deze analytische technieken gebruikt om referentiewaarden in menselijk hersenvloeistof te bepalen. We vonden zeer hoge D-serine concentraties direct na de geboorte, zowel absoluut als ten opzichte van glycine en L-serine, gevolgd door een snelle daling in de eerste 3 jaar tot lage micromolaire volwassen waarden (**hoofdstuk 3**). Deze specifiek verhoogde D-serine concentraties in het menselijk centraal zenuwstelsel gedurende de intensieve periode van embryonale en vroeg postnatale hersenontwikkeling, die overeenstemt met een voorbijgaande verhoogde expressie en activiteit van NMDArs, deed ons vermoeden dat de NMDAr co-agonist D-serine een belangrijke rol speelt in hersenontwikkeling. Om deze hypothese te onderzoeken, hebben we D-serine concentraties bepaald in hersenvloeistof van patiënten met 3-phosphoglyceraat dehydrogenase (3-PGDH) deficiëntie, een zeldzame aangeboren stoornis in de synthese van L-serine en dus ook van D-serine. Deze patiënten hebben ernstige neurologische symptomen, waaronder microcefalie, mentale retardatie en moeilijk behandelbare epilepsie. Deze patiënten hadden geen hoge D-serine concentraties in hun hersenvloeistof na de geboorte. De enige patiënt die tijdens de zwangerschap al geïdentificeerd en behandeld was, bereikte wel de piekende D-serine concentraties kort na de geboorte en deze patiënt had een normale schedelomtrek en geen epilepsie. Zij volgt inmiddels regulier onderwijs. Als een NMDAr co-agonist, zou het gebrek aan D-serine verantwoordelijk kunnen zijn voor de ernstige centraal zenuwstelsel afwijkingen van patiënten en genetisch gemanipuleerde muizen met 3-PGDH deficiëntie. In **hoofdstuk 4** wijden we uit over de rol van D-serine als NMDAr co-agonist in hersenontwikkeling, met de nadruk op de klinische implicaties. In **hoofdstuk 5**, proberen we meer inzicht te krijgen in de mechanismen waarmee D-serine hersenontwikkeling beïnvloedt door P19 cellen te bestuderen. Dit zijn teratoomcellen, oorspronkelijk van de rat, die gedifferentieerd kunnen worden tot glia en neuronen, die receptoren als de NMDAr tot expressie brengen. Dit is een gevalideerd experimenteel model voor zenuwcel ontwikkeling voor zowel neuronen (zenuwcellen) als glia, die lang beschouwd werden als ondersteunende cellen voor zenuwcellen, maar die steeds meer als belangrijke signalerende partners van zenuwcellen gezien worden. Na differentiatie bleken P19 cellen het D-serine synthetiserende enzym serine racemase tot expressie te brengen, hetgeen leidde tot synthese en uitscheiding van D-serine. Remming van D-serine synthese en blokkade van D-serine binding aan de NMDAr intensiveerde intercellulaire connecties tussen gedifferentieerde cellen en verhoogde de expressie van synaptophysine, een eiwit van de synaptische membraan. Mogelijk zorgt NMDAr activatie door D-serine, gesyntheseerd door serine racemase, ervoor dat alleen specifieke activiteit leidt tot de inductie van functionele synapsen, waardoor premature synaptische rijping wordt voorkomen. Deze onverwachte bevinding zou impliceren dat D-serine een rol speelt in het vormen en modelleren van synaptogenese en neuronale netwerken tijdens de vroege hersenontwikkeling.

In het derde deel van dit proefschrift (**D-serine in human pathology**) wordt aandacht besteed aan ziekten, die geassocieerd zijn met veranderde NMDAr activiteit, mogelijk door veranderde D-serine concentraties. Dit is bijzonder interessant omdat deze concentraties farmacologisch gemanipuleerd zouden kunnen worden, aangezien de enzymen die D-serine vormen en afbreken bekend zijn en dus potentiële therapeutische targets vormen. Als kinderartsen hebben we gekozen voor het bestuderen van perinatale asfyxie, momenteel wereldwijd een van de belangrijkste oorzaken van neonatale sterfte en morbiditeit. Perinatale asfyxie is het gevolg van verstoorde bloed circulatie tussen moeder en foetus, bijvoorbeeld bij problemen met de placenta of navelstreng. Hierdoor wordt onder andere te weinig zuurstof, suiker en andere voedingstoffen afgegeven aan de foetale organen, waaronder de hersenen. Op de lange termijn kan dit leiden tot mentale retardatie, epilepsie en spasticiteit. Behandeling is beperkt tot intensive care ondersteuning, waarbij sinds kort hypothermie wordt toegepast. De pathofysiologie is nog niet volledig opgehelderd, maar overstimulatie van de NMDAr lijkt een centrale rol te spelen. In **hoofdstuk 6** beschrijven we onze analyses van D-serine en glycine concentraties in 3 verschillende modellen: 1. Ratten glioma cellen, blootgesteld aan zuurstof en glucose gebrek, 2. hersenvloeistof van humane pasgeborenen na het doormaken van perinatale asfyxie 3. hersenvloeistof van biggetjes, blootgesteld aan hypoxie-ischemie door occlusie van beide halsslagaders, gecombineerd met verlaagde zuurstofconcentratie in de ingeademde lucht. We zagen dat extracellulaire glycine en D-serine concentraties duidelijk stegen in de glioma cellen blootgesteld aan zuurstof en glucose gebrek, klaarblijkelijk door verhoogde biosynthese vanuit L-serine. Na reperfusie normaliseerden glycine concentraties en D-serine concentraties waren significant verlaagd. De *in vivo* studies bevestigden de bevinding van initieel verhoogde en later normaliserende glycine concentraties en verlaagde D-serine concentraties na reperfusie. Deze significante stijging van beide endogene NMDAr co-agonisten in onze experimenten, in combinatie met de bekende stijging van glutamaat concentraties na globale ischemie, moet leiden tot massieve NMDAr activatie, excitotoxiciteit en neuronale schade. Beïnvloeding van deze NMDAr co-agonist concentraties biedt een interessante therapeutische optie voor deze veelvoorkomende, verwoestende en momenteel onbehandelbare aandoening.

Schizofrenie is ook een ziekte die sterk geassocieerd wordt met NMDAr disfunctie en mogelijk met veranderde D-serine concentraties. Dit is een ernstige en relatief veel voorkomende psychiatrische ziekte, gekarakteriseerd door positieve symptomen (waaronder hallucinaties), negatieve symptomen (afgevlakt affect, emotionele terugtrekking) en cognitieve defecten. In **hoofdstuk 7** laten we zien dat wij geen verschil vonden in D-serine of glycine concentraties tussen controle hersenvloeistof en dat van patiënten met schizofrenie voor en/of na behandeling met het antipsychotische geneesmiddel olanzapine. Dit impliceert dat bepaling van D-serine of glycine concentraties in hersenvloeistof niet een eenvoudige diagnostische marker is voor patiënten met schizofrenie.

Mogelijk is in dit geval hersenvloeistof een te globale marker om potentieel subtiele verschillen in specifieke hersengebieden te detecteren.
Tot slot worden in **hoofdstuk 8** de resultaten van de studies in dit proefschrift samen met enige preliminaire data (van patiënten met ALS) bediscussieerd, met de nadruk op de rol van D-serine als essentiële endogene modulator van NMDAr activiteit in fysiologische en pathologische processen in het menselijk lichaam, met een blik naar de toekomst.

Contributors

M.M.J. de Barse
Department of Metabolic and Endocrine Diseases and Department of Biomedical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands.

R. Berger
Department of Metabolic and Endocrine Diseases and Department of Biomedical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands.

W. Cahn
Department of Psychiatry, University Medical Center Utrecht, Utrecht, the Netherlands.

L. Dorland
Department of Inherited Metabolic Diseases, Academic Hospital Maastricht, Maastricht, the Netherlands.

M. Hendriks
Department of Metabolic and Endocrine Diseases and Department of Biomedical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands.

R.S. Kahn
Department of Psychiatry, University Medical Center Utrecht, Utrecht, the Netherlands.

L.W.J. Klomp
Department of Metabolic and Endocrine Diseases and Department of Biomedical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands.

T.J. de Koning
Department of Metabolic Diseases, University Medical Center Utrecht, the Netherlands.

C.M.P.C.D. Peeters-Scholte
Department of Pediatric Neurology, Leiden University Medical Center, Leiden, the Netherlands.

M.W. Roeleveld
Department of Metabolic and Endocrine Diseases and Department of Biomedical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands.

M.G.M. de Sain-van der Velden
Department of Metabolic and Endocrine Diseases and Department of Biomedical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands.

F.E. Scheepers
Department of Psychiatry, University Medical Center Utrecht, Utrecht, the Netherlands.

Dankwoord

"LESS IS MORE"

Een van de belangrijkste dingen die ik geleerd heb tijdens het schrijven van dit proefschrift is "less is more". Ik zal proberen een kort dankwoord schrijven, waardoor hopelijk de waardering en dank voor alle hulp des te groter zal lijken.

Allereerst mijn promotoren en co-promotoren: Ruud (Berger), aanvankelijk op de achtergrond en in de laatste maanden direct betrokken bij mijn project door alle ontwikkelingen op het laboratorium: veel dank voor je betrokkenheid, je visie en het gevoel dat ik altijd bij je kon aankloppen - voor wat dan ook. Ik hoop dat je snel kan genieten van je verdiende "volgende fase". Jan (Kimpen), zowel als opleider en als promotor heb jij mij altijd de indruk gegeven veel vertrouwen in mij en het project te hebben, zelfs al kwam ik alleen om te vertellen dat ik zwanger was. Tom (de Koning), toen ik jou ontmoette dacht ik nog chirurg te worden en was ik niet - zeker niet concreet - van plan te promoveren. Ik weet niet precies wat jouw rol hierin is geweest maar voor ik het wist kreeg ik thuis een rooster met een gedetailleerd plan tot 2011 om een gepromoveerde kinderarts te worden. Door alle vrijheid die je me gaf heb ik er een "eigen" project van kunnen maken. Dankzij je gezellige optimisme heb ik altijd het gevoel gehad dat het goed zou komen met ons project. Zou het ook goed komen met het apple peel atresia stuk? Leo (Klomp), aanvankelijk direct betrokken en in de laatste maanden helaas meer op de achtergrond, dank voor je kritische en enthousiaste blik op het onderzoek. Sterkte de komende tijd!
Cacha (Peeters), dank voor het meedenken, het gebruik van je biggenliquor, het verkrijgen van die bizarre Zweedse monsters, je hulp tijdens het schrijven van de NWO aanvraag en het kritisch beoordelen van het asfyxie manuscript! Ik ben benieuwd naar de toekomst van je asfyxie behandeling, wellicht is het raadzaam je ooit eens op D-serine te richten?!
Tijdens het eerste deel van mijn onderzoek heb ik me in het diagnostisch laboratorium van de Metabole en Endocriene ziekten bezig gehouden met chromatografie / massa spectrometrie, hetgeen me vaag deed denken aan een practicum farmacie. Graag wil ik alle medewerkers van de diagnostiekafdeling bedanken voor hun geduld, de bemoedigende woorden en adviezen. In het bijzonder wil ik Bert (Dorland) en Monique (de Sain) noemen vanwege de inhoudelijke discussies en analysetijd. Daarnaast wil ik Martina (de Barse) en Martin (Roeleveld) bedanken voor al hun analyses en meer nog voor de gezelligheid! En natuurlijk ook Margriet (Hendriks) voor de statistische adviezen, Astrid (Uyttewaal) voor het halen van mijn liquormonsters toen ik in Tilburg werkte en Monique (de Gooijer) voor alle kopjes koffie en de ondersteuning vooral

tijdens de afronding van het proefschrift. Voor mij was het hoogtepunt van deze tijd de wintersportweek (met roomie Maria (van der Ham), Martina (de Barse), Ans (Geboers), Berthil (Prinsen), Siebolt (de Boer) en Gerda (Vaarkamp): wat een heerlijke week, waarin ik (in tegenstelling tot Berthil) geen seconde aan "injectienaaldjes" heb gedacht, maar meer aan goudvisjes, waterscooters en Pictionary. Het tweede deel van mijn onderzoek zat ik op de research afdeling van het laboratorium Metabole en Endocriene ziekten en ook deze afdeling zou ik graag als gehele groep bedanken voor alle hulp aan deze - op dit laboratorium - ongebruikelijke onderzoeker: een parttime werkende arts, die nog niet veel van doen had gehad met moleculaire celbiologie. Speciaal wil ik mijn kamergenoten Olivier (van Beekum), Wendy (van Veelen), Ellen (Jeninga), serine-maatje Leyla (Tabataie) en Yuan (Gao) bedanken voor alle gezellige gesprekken en inhoudelijke discussies (zelfs af en toe werkgerelateerd). Verder ben ik veel dank verschuldigd aan Bart (van de Sluis) voor de gezellige en voor mij nuttige tochtjes naar de hypoxie stoof in het UMC-Utrecht!

Voor mijn onderzoek heb ik vele liquormonsters gemeten. Allereerst natuurlijk veel dank aan alle patiënten en controle personen zelf. Via het laboratorium medische microbiologie (via dr. A. Fleer) had ik toegang tot een unieke collectie liquor van patiënten van alle leeftijden waarbij gedacht was aan meningitis, maar waarbij liquoranalyse geen bijzonderheden opleverde. Dank voor het bewaren van de monsters en de wekelijkse overdracht hiervan! Floor (Scheepers), dank voor je liquormonsters van jouw studies bij patiënten met schizofrenie, waarvoor ik tijdens mijn zwangerschapsverlof nog eindeloze mappen heb doorgespit. Michael (van Es), een huwelijksfeest blijkt een mooie gelegenheid om liquormonsters van ALS patiënten te verzamelen. Helaas nog geen spannende resultaten, maar wat mij betreft blijft de hypothese staan!

Ook de arts-assistenten en artsen van het WKZ wil ik graag als groep bedanken voor de fijne samenwerking. De combinatie onderzoek, kliniek en ook nog een druk sociaal leven (vooral inclusief jonge kinderen) blijft uitdagend en het is altijd fijn om hierover van gedachten te kunnen wisselen!

Lieve vrienden, schoonfamilie en allen die ik graag meer had gezien in de afgelopen jaren: hopelijk komt daar verandering in! Helaas zitten er maar 24 uur in een dag en zijn er zoveel leuke dingen in het leven… Maar ik ga mijn best doen!

En dan mijn paranimfen Sanne (Hoeks) en Ilse (Jansen). Sanne, jouw interesse in mijn onderzoek werkt altijd aanstekelijk! Op de een of andere manier is niet iedereen zo geïnteresseerd in D-serine. Jij bent vast binnenkort in deze positie en ik ben heel benieuwd naar jouw boekje. Dat alles maar mag lukken! Ilse, met jou heb ik voor het eerst aan onderzoek geroken; samen in Edinburgh heb ik het gevoel een half jaar vakantie te hebben gehad ondanks onze dubbele baan in het ziekenhuis en de pub. Naast farmacie daarna samen toch nog geneeskunde gestudeerd, samen gewoond op de Zandhofse straat, samen door Thailand gereisd, samen je - inmiddels - man ontmoet en samen je

huwelijk gevierd. Het mag duidelijk zijn: ik kon me niet voorstellen dat ik dit zonder jou zou doen!
Lieve familie: Make; op uw 96e bent u me voorgegaan in het schrijven van een wetenschappelijk boek. Klein verschil is dat dit boek daadwerkelijk verkocht wordt. In alles was u ruimdenkend en geïnteresseerd en zo ook in mijn onderzoek. Jammer dat u de afronding van mijn proefschrift niet meer mee heeft kunnen maken. Papa en mama, dank voor jullie interesse in een niet zo toegankelijk onderzoek. Maar meer nog, dank voor jullie open positieve blik naar de wereld, waarin elke verandering nieuwe kansen of verrassingen biedt. Ik hoop dat ik onze kinderen net zo'n gelukkige liefdevolle basis mee kan geven als jullie aan ons! Zusjes Saskia en Coralie: met de eerste heb ik – ondanks 4 luttele jaren leeftijdsverschil – samen geneeskunde gestudeerd en met de tweede in hetzelfde ziekenhuis aan een promotieonderzoek gewerkt. Meer verstrengelde levens zullen niet veel zussen hebben. Daarnaast is het altijd een feest om jullie en Pieter en Pim te zien! Coralie, ik hoop dat je binnenkort ook zo enthousiast over mij schrijft in jouw proefschrift! En Sas, je wordt een geweldige plastisch chirurg en ik hoop dat al je dromen uitkomen! Lieve Bas, dank voor het aanhoren van mijn D-serine frustraties, presentaties, publicaties, bijna succesjes en net-niet-revolutionaire ontdekkingen, terwijl je heel gelukkig had geleefd zonder ooit over D-serine gehoord te hebben. Dank ook voor je IT-ondersteuning. Volgens mij heb je in de afgelopen jaren Reference Manager wel 10x geïnstalleerd. Maar meer nog, dank voor alle kleine en grote dingen, waardoor ik geniet van elke dag! Sophie, Job en Pepijn; als enigen hebben jullie alle 3 van binnenuit ervaren hoe dit proefschrift tot stand kwam. Voor mij zijn jullie verreweg mijn meest translationele en indrukwekkende resultaat van de afgelopen jaren!

Onder het mom "less is more" blijkt er - zelfs aan het einde van dit proefschrift - nog veel te leren!

Curriculum Vitae

Sabine Annemijn Fuchs was born the 27th of February in 1974 as the eldest of 3 daughters in Heerlen, a small town in the south of Holland. From 9-14 years of age, she went to an international School (requiring bilinguality) near Paris, where the family lived for her father's work. She finished high school at the St. Maartens College in Maastricht in 1992 (gymnasium, 8 subjects, *cum laude*) and then went to Granada (Spain) to study Spanish for a year. Because of the Dutch lottery system for admission to medical school, she studied pharmacy at the University of Utrecht, where she graduated in 1998 (*cum laude*) after performing a 6 month research project on a new technique to determine vascular compliance and predict cardiovascular disease at the clinical pharmacology unit / clinical research center (prof. dr. D.J. Webb, Western General Hospital, Edinburgh (Scotland)). During her subsequent 2-year post graduate pharmacy course, the lottery system for medical school was changed, which allowed her to combine the final 6 months of this post graduate course (graduation 2000) with medical school (graduation 2001, *cum laude*) at the University of Utrecht, followed by 2 years of internships (ENT at Harvard medical school, Boston, USA) (graduation 2003 (*cum laude*)). Subsequently, she started an alternating program of specialist registrar training in paediatrics (prof. dr. J.J. Kimpen and currently dr. J. Frenkel and prof. dr. E. Nieuwenhuis) in the Wilhelmina Children's Hospital in Utrecht, combined with a clinical research fellowship in the laboratory of Metabolic and Endocrine Diseases (prof. dr. R. Berger), which resulted in this thesis. She is planning to finish her paediatric training in 2012, after which she hopes to be able to continue combining clinical work with research and a busy family life (Bas 1974, Sophie 2005, Job 2007, Pepijn 2009), while also enjoying the good things of life!

Sabine Annemijn Fuchs is op 27 februari 1974 geboren in Heerlen als oudste van 3 dochters. Toen ze 9 jaar oud was, verhuisde de familie naar Parijs, waar ze tot haar 14e naar het Lycée International ging voor tweetalig onderwijs (Frans / Nederlands). Ze behaalde haar eindexamen aan het St. Maartens College in Maastricht in 1992 (gymnasium, 8 vakken, *cum laude*) en ging een jaar naar Granada (Spanje) om Spaans te studeren. Vanwege ongunstige loting voor de studie geneeskunde, is ze farmacie aan de Universiteit van Utrecht gaan studeren. Ze studeerde in 1998 af (*cum laude*), na een 6-maanden durend onderzoeksproject naar een nieuwe techniek om vasculaire compliantie te bepalen en daarmee het risico op cardiovasculaire ziekte te voorspellen. Dit onderzoek vond plaats in de clinical pharmacology unit / clinical research center in het Western General Hospital in Edinburgh, Schotland (prof. dr. D.J. Webb). Tijdens de 2-jarige postdoctorale apothekersopleiding is het lootsysteem voor de studie geneeskunde veranderd, waardoor ze

de laatste 6 maanden van haar postdoctorale opleiding (apothekersdiploma 2000) kon combineren met de studie geneeskunde (doctoraal 2001, *cum laude*) aan de Universiteit van Utrecht. Na 2 jaar co-schappen (waaronder KNO op Harvard medical school, Boston, USA) behaalde ze in 2003 haar arts examen (*cum laude*). Aansluitend startte ze in het Wilhelmina Kinderziekenhuis (UMC-Utrecht) als assistent-geneeskunde in opleiding tot klinisch onderzoeker (AGIKO) met een combinatie van opleiding kindergeneeskunde (prof. dr. J.J. Kimpen, thans dr. J. Frenkel en prof. dr. E. Nieuwenhuis) en promotie-onderzoek in het laboratorium Metabole en Endocriene Ziekten (prof. dr. R. Berger), hetgeen geresulteerd heeft in dit proefschrift. Ze is van plan haar opleiding tot kinderarts af te ronden in 2012, waarna ze klinisch werk hoopt te kunnen blijven combineren met onderzoek, een gezellig druk familie leven (Bas 1974, Sophie 2005, Job 2007, Pepijn 2009) en alle andere leuke dingen van het leven!

Publications

Wilkinson IB, Fuchs SA, Jansen IM, Spratt JC, Murray GD, Cockcroft JR, Webb DJ. Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis. *J Hypertens* 1998;**16**:2079-2084.

Fuchs SA, Koopmans RP, Guchelaar HJ, Brodie-Meijer CC, Meyboom RH. Are angiotensin II receptor antagonists safe in patients with previous angiotensin-converting enzyme inhibitor-induced angioedema? *Hypertension* 2001;**37**:E1.

Fuchs SA, Meyboom RHB, van Puijenbroek EP, Guchelaar HJ. Caution remains warranted with angiotensin receptor antagonists in patients with a history of angioedema caused by angiotensin-converting-enzyme inhibitors. *Pharm World Sci* 2004;**26**:191-192.

Fuchs SA, Langius E, Rijnvos WPM, Nikkels PGJ, Wijburg FA, Visser G. De ziekte van Niemann-Pick B: behandeling in zicht. TvK 2004;**72**:235-238.

Fuchs SA, Berger R, Klomp LW, de Koning TJ. D-amino acids in the central nervous system in health and disease. *Mol Genet Metab*. 2005;**85**:168-180.

Fuchs SA, Dorland L, de Sain-van der Velden MG, Hendriks M, Klomp LWJ, Berger R, de Koning TJ. D-serine in the developing human central nervous system. *Ann Neurol.* 2006;**60**:476-480.

de Koning TJ, Fuchs SA, Klomp LWJ (2007) Serine, glycine and threonine.
In: Oja S, Schousboe A, Saransaari P (eds). The handbook of Neurochemistry and molecular neurobiology, 3rd edition, volume 6. Springer, New York:23-45.

Fuchs SA, Klomp LWJ, de Koning TJ (2008) D-serine in the developing human central nervous system: clinical implications. In: Heresco-Levy U and Javitt DC (eds). Glutamate in neuropsychiatric disorders. Research Signpost, Kerala; Chapter 4:75-97.

Fuchs SA, de Barse MMJ, Scheepers FE, Cahn W, Dorland L, de Sain-van der Velden MG, Klomp LWJ, Berger R, Kanh RS, de Koning TJ. Cerebrospinal fluid D-serine and glycine concentrations are unaltered and unaffected by olanzapine therapy in male schizophrenic patients. *Eur Neuropsychopharmacol*. 2008;**18**:333-8.

Fuchs SA, de Sain-van der Velden MG, de Barse MMJ, Roeleveld M, Hendriks M, Dorland L, Klomp LWJ, Berger R, De Koning TJ. Two mass-spectrometric techniques for quantifying serine enantiomers and glycine in cerebrospinal fluid: potential confounders and age-dependent ranges. *Clin Chem* 2008;**54**:1143-50.

Moat S, Carling R, Nix A, Henderson M, Briddon A, Prunty H, Talbot R, Powell A, Wright K, Fuchs S, de Koning T. Multicentre age related reference intervals for cerebrospinal fluid serine concentrations: Implications for the diagnosis and follow-up of serine biosynthesis disorders. *Mol Genet Metab, accepted for publication.*

List of abbreviations

ARA-C	cytosine arabinofuranoside
CNS	central nervous system
CSF	cerebrospinal fluid
GC-MS	gas chromatography-mass spectrometry (separation & quantification method)
DAO	D-amino acid oxidase (D-serine metabolizing enzyme), EC 1.4.3.3
DCKA	Dichlorokynurenic acid (antagonist of the NR1 subunit of NMDArs)
LC-MS	liquid chromatography-mass spectrometry (separation& quantification method)
LSOS	L-serine-O-sulphate (SR inhibitor)
LTP	Long-term potentiation (experimental model to study memory)
NMDAr	N-methyl D-aspartate receptor
NO/NG	normoxia / normoglycemia
OGD	oxygen and glucose deprivation
NR1	glycine / D-serine binding subunit of the NMDAr
NR2	glutamate binding subunit of the NMDAr
3-PGDH	3-phosphoglycerate dehydrogenase (L-serine synthesizing enzyme), EC 1.1.1.95
RA	retinoic acid (differentiation inducer for P19 cells)
SR	serine racemase (D-serine synthesizing enzyme), EC number 5.1.1.18