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PH.D. DISSERTATION

Mammalian Serine Racemase as a Pharmaceutical Target

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Contents

Abstract

Serine racemase (SR) is a pyridoxal 5' phosphate (PLP) dependent enzyme that is responsible for the biosynthesis of the neurotransmitter D-serine (DS). DS is an agonist of the *N*-methyl-D-aspartate (NMDA) glutamate receptors. NMDA receptors are important in maintaining many physiological brain functions, like synaptic plasticity, which is important for memory formation. However, the overactivation of NMDA receptors is associated with severe neurodegenerations, for example, acute brain stroke or chronic diseases including Alzheimer's disease and amyotrophic lateral sclerosis (ALS). Aberrant NMDA receptor function is placed among the causal agents of epilepsy and schizophrenia. Recent studies with serine racemase deficient mice revealed that DS and SR might be involved in ALS. SR knock-out mice were protected against β-amyloid plaque formation, supporting the link between SR and Alzheimer's disease. Serine racemase is thereby interesting as a potential pharmaceutical target.

SR is also interesting from the biochemical point of view. It is a multifunctional enzyme capable of serine racemization and deamination with comparable efficiencies. The enzyme is activated by nucleotides like ATP, by divalent cations, and by reducing agents.

The close sequence and functional similarity of mouse and human SR allows the use of mouse models and recombinant mouse SR for the study of human SR. This work presents the results of a mouse SR inhibition study.

Abbreviations

ALS	amyotrophic lateral sclerosis (Lou Gehrig's disease)
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMP-PCP	adenosine-5'-(β, γ-methylene) triphosphate
ASCT	alanine-serine-cysteine transporter
ATP	adenosine-5'- triphosphate
DAAO	D-amino acid oxidase
DPFC	dorsolateral prefrontal cortex
DS	D-serine
DTT	1, 4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
GRIP	glutamate receptor interacting protein
L-EHA	L-*erythro*-3-hydroxyaspartate
LS	L-serine
LSOS	L-serine-*O*-sulfate
L-THA	L-*threo*-3-hydroxyaspartate
LTP	long term potentiation
NMDA	*N*-methyl-D-aspartate
PICK1	protein interacting with kinase C 1
PLP	pyridoxal-5'-phosphate
SR	serine racemase (h, human; m, mouse; r, rat)
TCEP	tris-carboxyethlylphosphine

Chapter 1 Introduction

1.1 The World of D-Serine

1.1.1 L or D?

In 1848 Louis Pasteur (*1822 - †1895) first described the phenomenon of molecular chirality after his observations of tartrate crystals and solutions in polarized light [1]. Chiral, or optically active, molecules rotate the plane of polarized light, each enantiomer in a different sense: clockwise or counterclockwise. On the molecular level such compounds lack an internal plane of symmetry and have non-superimposable mirror images. The word “chirality” was derived from the Greek *cheir* (hand) as human hands are the first obvious examples of objects with non-superimposable mirror images.

The knowledge of chirality preceded the discovery of the amino acid sequence of proteins, which was revealed around 1950 [2-4]. The α-amino acids (except glycine) also exist as two optically active isomers. The proteosynthetic machinery uses exclusively L-amino acids. From the evolutionary point of view this fact remains very exciting. Many chemists, theoreticians, and geologists have come up with interesting explanations. For example salt-induced peptide formation favoring L-α-amino acids [5], or single chirality formed from a nearly racemic mixture of crystals that are grinded in saturated solution [6-7].

It is, however, not true that D-amino acids are not naturally occurring, as is often taught in high schools and believed in public. However, the detection of naturally present D-amino acids

first required advances in separation and detection techniques that have now been available for several decades.

In proteins D-amino acids occur as a result of posttranslational modification [8]. They are common composites of bacterial cell wall peptidoglycans, peptide antibiotics, or peptide products of mollusks, frogs, and snails [9]. Moreover all protein amino acids undergo spontaneous racemization during aging and upon the death of the organism, in other words, once the tissue is metabolically inert [10]. This phenomenon is used in dating of fossils or determination of the death time [11].

The detection of the free D-amino acids was again preceded by the discovery of kidney D-amino acid specific enzyme D-amino acid oxidase (DAAO) back in 1935 [12]. The reason for the presence of such an enzyme was not clear for many years. Only in 1986 Dunlop *et al.* detected high amounts of free D-aspartic acid in various mammalian tissues [13]. A couple of years later Hashimoto *et al.* reported the presence of free D-serine and D-aspartic acid in human and rat brain [14-15]. Subsequently other proteinogenic amino acids in their D-form were also detected in the mammalian tissues, but their function is largely unknown [16]. In some cases the level is so low that the potential role of these compounds can be debated. In contrast, the amount of D-serine and D-aspartic acid represent 5-30% of the L-form, depending on the tissue and developmental stage. The role for both D-amino acids has already been assigned. D-Asp regulates endocrine functions and tissue development and D-ser serves as a neurotransmitter [17].

1.1.2 D-serine in *N*-methyl-D-aspartate Receptor Neurotransmission

N-Methyl-D-aspartate (NMDA) receptors are a subclass of excitatory (or activating) ionotropic glutamate receptors in the mammalian nervous system. They are located in the membranes of neurons, mostly in the synapses and serve as sodium/potassium/calcium ion-channels that upon opening cause excitation of the target cell. Among the ion-channels are NMDA receptors outstanding in their mechanism of function, because they require two different agonists for the opening of the channel. One agonist is glutamate and glycine or D-serine act as the co-agonists [18]. NMDA receptors are predominantly heterotetramers consisting of two distinct subunit types each bearing one agonist site. The NR1 subunits display the glycine/D-serine while the NR2 subunits the glutamate binding site. NMDA receptors undergo complex

regulation by various other activators, like polyamines, and blockers, for example Mg^{2+} ions [19] (see Fig. 1).

It is not known how the NMDA receptor activation is split between glycine and D-serine. Several studies occupied with this subject showed that D-serine has the same or higher affinity to the NMDA receptor and is more potent in the signal triggering [20-21]. Enzymatic depletion of DS leads to significant decrease of NMDA receptor signaling [22]. Furthermore the spatiotemporal distribution of D-serine with its synthesizing enzyme, serine racemase (SR), resemble that of NMDA receptors, while glycine with serine hydroxymethyltransferase do not [23]. It is not surprising that glycine distribution is distinct, because it is a recognized inhibitory neurotransmitter. It acts through the strychnine-sensitive glycine receptors [24]. Recently the serine racemase deficient mice with 90% reduction in DS level show reduced NMDA receptor function [25]. These observations suggest what is now generally accepted: D-serine is the major agonist of the NMDA receptor co-agonist binding site. However, Oleit *et al*. showed that D-serine might act preferentially through the synaptic NMDA-receptors while glycine acts through the extrasynaptic receptors [26].

Initially DS was considered an atypical messenger, sometimes referred to as a gliotransmitter, because it was only observed in the glial cells and thought to participate in the synaptic transmission being released from glia only [27-28]. Later it was shown that DS and serine racemase are present in neurons as well [29]. It remains to be clarified how D-serine circulates in the synapse and the surrounding cells (for further details see Chapter 1.1.4).

NMDA receptors are major glutamatergic receptors allowing synaptic transmission in many parts of the brain. They participate in processes like neuronal development, plasticity, learning, and memory. On the account of being crucial they serve as a crossroad for many pathological processes. Their improper activation switches them to excitotoxic and neurodegenerative agents. The pathophysiological contribution of NMDA receptors has been demonstrated for many acute and chronic diseases, for example Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Huntington's and Parkinson's diseases, brain ischemia, schizophrenia, and epilepsy. NMDA receptors further participate in nociception, depression, anxiety, and drug tolerance [30]. Therefore, they serve as a molecular site for pharmacological intervention. Unfortunately the NMDA receptor antagonists often bring notwithstanding side

effects that make them useless for the treatment [30-31]. The potential drug targets are now searched for among the NMDA receptor signaling molecules upstream or downstream of the receptor. Serine racemase, as the co-agonist D-serine producing enzyme, is thus potentially very interesting.

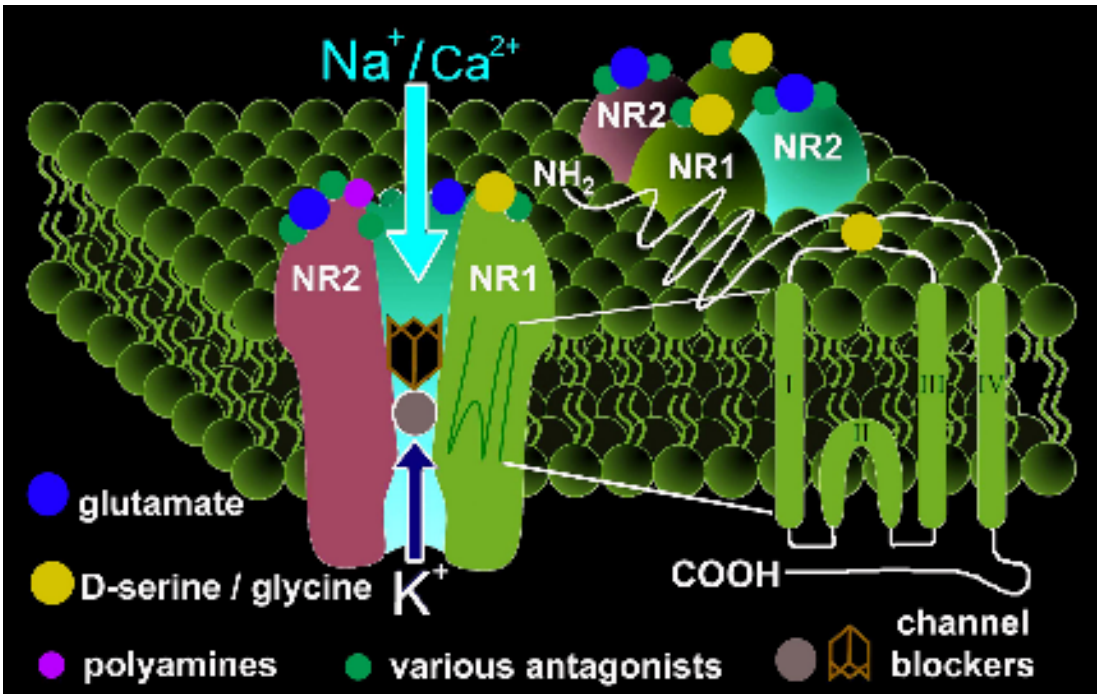


Fig. 1. NMDA Receptor Scheme

NMDA receptor channel scheme showing its subunit structure and composition and positions of various modulators. For example Zn^{2+} ions serve as naturally occurring antagonists, while Mg^{2+} ions act as channel blockers. NMDA receptor subunits NR2 are shown in different colors, because they exist in several distinct subtypes that usually combine in one receptor. The picture was adapted from [32].

NMDA receptors are found in the central (CNS) and peripheral nervous system (PNS) as well as in the peripheral non-neuronal tissues. D-Serine was also detected outside the nervous system and is thought to occupy the peripheral NMDA receptors too. The role of NMDA receptors in the pathology of peripheral organs was already shown for the heart and kidneys [33-34].

Recently, phosphatidyl-D-serine was isolated from rat brain. Phosphatidyl-L-serine is a frequent component of brain cell membranes. Here, the authors showed that the D-form represents about 1% of the amount of the L-form [35]. This is the first demonstration of another role for D-serine aside from NMDA receptor signaling.

1.1.3 Source of DS – Is Serine Racemase the Only One?

Serine racemase (SR), a cytosolic pyridoxal-5'-phosphate dependent enzyme (see below), was identified as the key enzyme in DS production. Recently, an important contribution in the field of SR was achieved by several groups: the breeding of SR knock-out mice [36-39]. It was surprising to realize that the animals still possess a significant amount of DS. The total DS amount represented 10% of the amount in the wild type animals. It is not clear which pathway, except for the SR-catalyzed, might lead to the production of DS [36-37].

Before SR was first isolated, various metabolic routes to DS in the mammalian tissue were discussed. One obvious source is intestine. DS can come from digested food or it can be produced by endogenous bacteria. Experiments with radiolabeled DS revealed that it can be transported to the brain through the blood brain barrier [40].

Phosphoserine phosphatase is able to produce D-serine from D-phosphoserine, which has, however, not been detected in the mammalian brain tissue [41]. In 1997 two groups independently demonstrated a direct metabolic link between L- and D-serine and suggested the existence of mammalian brain serine racemase. They measured if DS is elevated upon administration of glucose, glycine, L-threonine, or L-serine. Takahashi *et al.* used non-labeled compounds and observed elevation of DS level after LS administration and elevation of LS together with DS after glycine administration [42]. Dunlop and Neidle used radiolabeled compounds and observed formation of D-serine only after L-serine administration [43]; they thus ruled out the direct metabolic link between glycine and D-serine suggested previously [44].

After mammalian serine racemase was finally identified in 1999 [45], the search for other possible sources of DS ceased.

1.1.4 DS Release and Transport in Tripartite Synapse

Lot of research was done in the past decade on the contribution of astrocytes and other types of glial cells in the central nervous system (CNS) to the neuronal signaling (for reviews see [46] or [47]). Structurally, the synapse is formed by presynaptic and postsynaptic neurons and surrounded by a glial cell, for example an astrocyte (see Figure 2). Astrocytes are known to release numerous transmitters, such as nucleotides, NO, glutamate, and D-serine [27, 48-49]. They play important roles in neuronal tissue development and regulation [50-51].

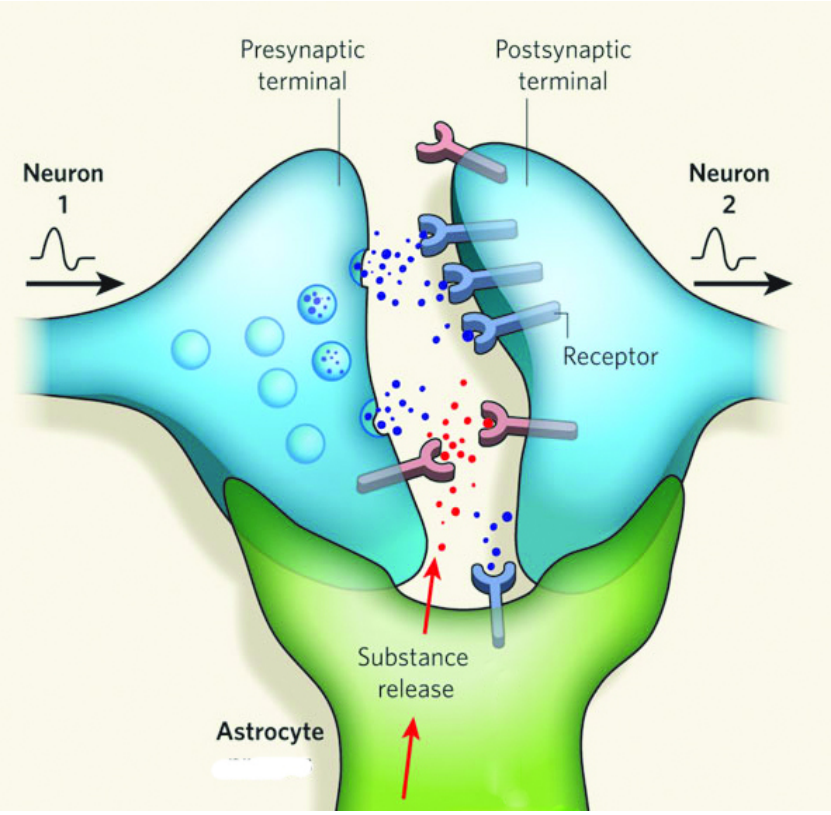


Fig. 2. Scheme of Tripartite Synapse

Synapse formed by a presynaptic and a postsynaptic neuron and an astrocyte. The release of gliotransmitters and neurotransmitters is schematically outlined by the red and blue dots, respectively. The picture was adapted from [47].

As a neurotransmitter, DS must be released to the synapse and removed from the synapse in an efficient way. It was shown that in glia DS accumulates in secretory vesicles and can be released through vesicular fusion with the plasmatic membrane upon Ca^{2+} stimulation, similar to

other neurotransmitters [52-53]. It is possible that D-serine and glutamate use the same vesicles and are released to the synapse together [52]. At the same time, DS was shown to be released upon membrane depolarization from the cytosol of neurons in a non-vesicular manner [29].

The clearance of DS from the synapse may be provided by the neutral amino acid alanine-serine-cysteine transporter (ASCT) [54-55]. D-Serine has high affinity to the ASCT2 transporter type [56], which can efficiently control the DS homeostasis in the C6 glioma cell line [57]. The ASCT2 transporter is present on the glial as well as the neuronal cell membrane [56].

1.1.5 DS Degradation – Is D-Amino Acid Oxidase the Main Actor?

Nothing was known about DS and its role in the mammalian brain when its putative degrading enzyme, D-amino acid oxidase (DAAO, also abbreviated DAO), had already been well characterized. DAAO was first isolated and described as early as 1935 [12].

DAAO is a flavine-adenine dinucleotide (FAD) enzyme that catalyzes oxidative deamination of D-amino acids to iminoacids with the concurrent release of H_2O_2 [58]. The produced iminoacid is subsequently hydrolyzed to an α-ketoacid and ammonia. D-serine is thus converted to hydroxypyruvate. The effectiveness of DS clearance by DAAO is well demonstrated by the inverse spatiotemporal distribution of DAAO and DS [59]. In the same time this means that the site of DS production (SR) and degradation (DAAO) do not appear alongside each other. It remains unanswered how DS accesses the DAAO regions for its degradation.

Being the putative DS degrading enzyme, DAAO has also been studied in the NMDA receptor related pathological conditions. Broad study with Canadian schizophrenic patients revealed an association of G72 gene polymorphism with the illness [60]. The product of G72 gene is a potential interaction partner of DAAO [60]. This discovery led to a boom of DAAO investigations, especially its correlations to schizophrenia [61-65]. The results from experiments with DAAO deficient mice further support the possibility of such a link [66-67].

Another possibility for DS degradation is catalysis by serine racemase alone. SR deaminates serine to pyruvate through β-elimination, its second activity [68-69] (for further details see Chapter 1.2). Researchers speculate about some intracellular means of regulation *via*

posttranslational modification, an interaction partner, or a spatio-temporal distribution that could determine the SR reaction pathway [70]; however, none of these speculations have yet been confirmed.

1.1.6 DS Detection

A major milestone in the field of serine racemase and D-serine was the establishment of D-amino acid detection methods.

Detection of D-serine has been performed using separations techniques, coupled enzyme assays, and immunochemical methods. Capillary electrophoresis (CE) [71-72], HPLC [14, 73], and gas chromatography GC [74-75] are suitable techniques for DS resolution. To distinguish between LS and DS, an optically active derivatization agent or chiral resin must be used. Depending on the detection method, the derivatization compounds typically used are 2-fluorodinitrophenyl-L-alanine amide (FDAA, Marfey's reagent) for UV/vis or MS detection [76-77], or *ortho*-phthaldialdehyde (OPA) with chiral SH-donors, such as isobutyryl-L-cysteine or *N*-*tert*-butyloxycarbonyl-L-cysteine (*t*-Boc-L-cysteine), for fluorescent detection [78] . The most widely used technique is a conventional reversed-phase HPLC fluorescence method with precolumn derivatization with *o*-phthaldialdehyde (OPA) and a chiral thiol.

1.2 Biochemistry of Serine Racemase

The enzyme responsible for the neurotransmitter DS production is serine racemase (SR). This thesis describes mammalian serine racemase, especially its mouse and human orthologs. However, first it should be briefly clarified which other serine racemases are known. Literature and on-line biochemical databases, such as BRENDA [79] or NCBI [80], mention several distinct serine racemases: general amino-acid epimerase (EC number 5.1.1.10) typical for bacteria, protein-serine racemase (5.1.1.17) found in funnel web spider venom [81], and the free serine racemase (EC number 5.1.1.18) that is the subject of the thesis. The NCBI protein database reveals numerous free serine racemases of bacterial origin that usually have about 30-40% sequence identity to mammalian SR, similar to serine/threonine dehydratases that belong to the same family of PLP-dependent enzymes (discussed later in text). There are several types of serine racemases in bacteria, some of which are membrane bound and contribute to antibiotic resistance [82]. The bacterial serine racemases are distinct from the mammalian serine racemases in most cases. However, SR from the hyperthermophilic archeon *Pyrobaculum islandicum* is highly similar to the mammalian orthologs in its enzymatic behavior [83]. The relation between prokaryotic and eukaryotic SRs remains to be clarified.

Serine racemase homologs and orthologs are represented throughout all biological kingdoms. SR was already described in yeast *Schizosaccharomyces pombe* [84] and *Saccharomyces cerevisiae* [85]; in plants, such as rice (*Oryza sativa*) [86], barley (*Hordeum vulgare*) [87], or *Arabidopsis thaliana* [88]; and in high vertebrates like salamander (*Ambystoma trigrinum*) [89], mouse (*Mus musculus*) [68], rat (*Rattus norvegicus*) [45], and human (*Homo sapiens*) [90]. The sequence alignment revealed 90% identity among the mammalian SRs and 35-48% identity of yeast and plant SRs with the mammalian SRs.

1.2.1 Serine Racemase Is a Pyridoxal-5'-Phosphate Dependent Enzyme

Serine racemase employs pyridoxal-5´-phosphate (PLP) as a cofactor. PLP-dependent enzymes are widespread throughout all the kingdoms of organisms, from archaebacteria, plants, and fungi to animals [91]. PLP is one of the active forms of pyridoxine, vitamin B6. It has a reactive aldehyde moiety that interacts with primary amines. PLP-dependent enzymes therefore

act predominantly on amino acids, and they participate in an enormous variety of reactions, including deaminations, decarboxylations, β-replacements, transaminations, and racemizations. The reaction versatility typical for PLP-dependent enzymes is mirrored in the fact that they can be found in 5 out of 6 enzyme classes [92].

The first steps of the reaction mechanism are common to all PLP-dependent enzymes. In the resting state PLP is covalently bound to the ε-amino-group of an active site lysine, forming a Schiff base commonly called the internal aldimine. When substrate enters the active site, PLP swaps the lysine ε-amino-group for the α-amino group of the amino acid substrate, thus forming the external aldimine. The fate of the external aldimine now relies on the enzyme active site architecture, which controls the spatial arrangement of the intermediate. It has been shown that initial cleavage takes place on the α-carbon bond that projects perpendicular to the pyridoxal ring plane; this observation is referred to as the Dunathan stereoelectronic hypothesis [93]. Most frequently, the α-hydrogen lies in the perpendicular position, leading to cleavage of the hydrogen-carbon bond. The thus formed quinoid intermediate is common to racemization, transamination, β-elimination (deamination), and β-replacement reactions [94]. This pathway has been proposed also for serine racemase (Fig. 3).

1.2.2 Reactions Catalyzed by Serine Racemase

Because we mapped SR activity in great detail, some of the results from Publication I are already mentioned here in the introduction.

PLP-dependent enzymes are known to catalyze various side reactions; some of them can be physiologically relevant [95-96]. Most probably, the reason for the low reaction specificity is PLP reactivity and catalytic versatility. However, it should be noted that not only PLP-dependent enzymes were shown to catalyze side reactions; Copley *et al.* discuss that enzymes in general are less specific than we thought [97].

Serine racemase is able to catalyze racemization of serine and β-elimination of both L- and D-serine. The reactions share a common enzymatic active site [98], as expected from the proposed mechanism of action and further supported by the recently available human and rat SR X-ray structures [99]. In the case of human and mouse SR, the β-elimination of L-serine has comparable efficiency with racemization. The β-elimination (also called α,β-elimination,

dehydration, or deamination) of D-serine to pyruvate is significantly slower (See Table 1). This is, however, not true for SR from other species, such as yeast or archebacteria (Table 1), where, based on the reaction velocities, SR serves as a β-eliminase rather than racemase. It is attractive to speculate that in mammalian SR the β-elimination activity is a residual of evolution from a common ancestral enzyme with serine/threonine dehydratases [100]. Supportive to this speculation might be the fact that in plants SR is, so far, the only known D-serine degrading enzyme; plants lack D-amino acid oxidase [58, 87]. Fujitani *et al.* thus suggest that SR catalyzed β-elimination of DS is more important than DS production in plants [87-88].

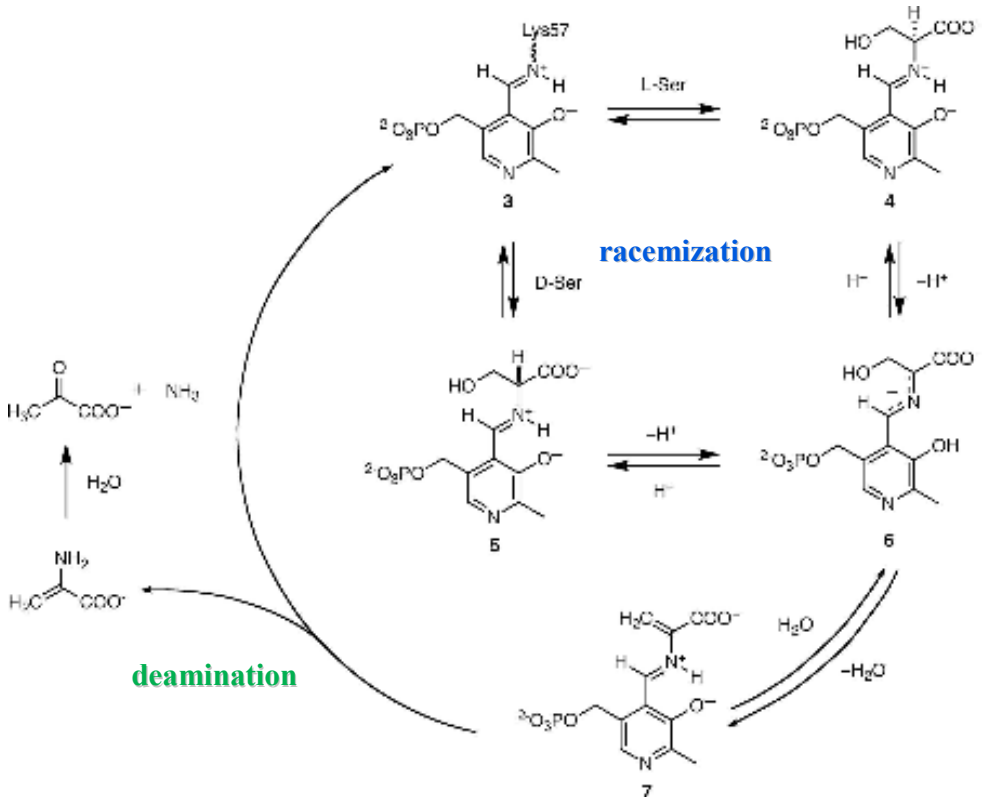


Fig. 3 SR Reaction Mechanism

Proposed SR reaction scheme, taken from [101]. Lys57 (*S. pombe* SR numbering) corresponds to Lys56 of hSR. Internal aldimine (3) is replaced by external aldimine formed with LS (4) or DS (5) as a substrate. When the α-hydrogen is removed to form the quioid intermediate (6) the reaction pathway can follow two directions, one is that the hydrogen is attached back from the same or opposite direction to form the same or opposite enantiomer, respectively (racemization); or it undergoes dehydration to form aminoacrylate aldimine intermediate (7) that is released from PLP and undergoes hydration to form the corresponding keto-acid (pyruvic acid in the case of serine) and ammonia (deamination).

human SR	K_M [mM]	k_{cat} [min^{-1}]	k_{cat} / K_M [min^{-1} mM^{-1}]
LS rac.	4.1	41.5	**10.0**
DS rac.	10.8	84.9	**7.9**
LS ß-el.	4.7	100	**21.2**
DS ß-el.	9.5	7.7	**0.81**

mouse SR	K_M [mM]	k_{cat} [min^{-1}]	k_{cat} / K_M [min^{-1} mM^{-1}]
LS rac.	3.8	45.5	**12.0**
DS rac.	14.5	113	**7.8**
LS ß-el.	4.0	81.3	**20.3**
DS ß-el.	3.2	8.8	**2.7**

barley SR	K_M [mM]	Vmax [nmol $mg^{-1}min^{-1}$]	Vmax / K_M
LS rac.	2.6	21	**8.1**
DS rac.	9	455	**51**
LS ß-el.	2.7	2.8	**1.0**
DS ß-el.	8.3	161	**19.4**

fission yeast SR	K_M [mM]	Vmax [nmol $mg^{-1}min^{-1}$]	Vmax / K_M
LS rac.	32	30	**0.9**
DS rac.	9.6	7.1	**0.7**
LS ß-el.	36	870	**24**
DS ß-el.	10	52	**5.2**

***P.island.* SR**	K_M [mM]	k_{cat} [min^{-1}]	k_{cat} / K_M [min^{-1} mM^{-1}]
LS rac.	185	888	**4.8**
DS rac.	95.4	313	**3.3**
LS ß-el.	2.16	3660	**1694**
DS ß-el.	-	-	**-**

Table 1 Serine Racemase Catalytic Parameters

Summary of K_M and k_{cat} or V_{max} (in red lettering) values and the corresponding catalytic efficiencies of L- and D-serine racemization (rac.) and β-elimination (β-el.) reactions catalyzed by various recombinant SR orthologs. Data for human SR are from [102], for mouse SR from [98] (Publication I of the thesis), for barley (*Hordeum vulgare*) from [87], for fission yeast (*Schizosaccharomyces pombe*) from [101] suppl., and for the archeon *Pyrobaculum islandicum* from [83].

It is not clear, whether, or not mammalian SR catalyzed β-elimination is physiologically important. It was proposed that SR can serve as the site of DS synthesis as well as degradation [69]. Human and mouse SR, which share 89% sequence identity and have comparable kinetic parameters [102], bear one interesting enzymatic difference in their K_M and k_{cat} values for D-serine β-elimination (see Table 1). Human SR can catalyze DS deamination with the K_M value, as expected, corresponding to DS racemization, but with very low k_{cat}. On the other hand, mSR can catalyze DS deamination seemingly faster than hSR, but the K_M value does not correspond to DS racemization but to the LS reactions. These observations, together with the fact that all reactions occur in the same active site [98], lead us to the conclusion that mSR, in contrast to hSR, is not able to catalyze direct DS deamination. The pyruvate formed when DS is used as substrate of mSR is probably the product of deamination of LS previously formed from DS by racemization. (note: these observations belong to the Publication I in the thesis).

The energetic consequence of SR catalyzed serine deamination has been discussed several times. The authors mostly agree that the observed reaction velocity is so low that it cannot cause significant changes in the energetic milieu of the cell. For comparison, rat liver serine dehydratase, also catalyzing L-serine to pyruvate deamination, has velocity several orders of magnitude higher [98]; all together the SR pyruvate production represents less than 0.1% of pyruvate production from glucose [103]. However, SR could contribute to the intermediary metabolism if its activity is up-regulated *in vivo*, or in nonstandard cases, such as in the energetically deficient long sciatic nerve axons that are dependent on the energy supply from surrounding Schwann cells, type of glial cells that, among others, express also SR [104].

Interestingly, it was recently shown that D-serine is capable of rat cortex citrate synthase inhibition [105]. At the same time we have revealed that citrate together with other tricarboxylic acid cycle intermediates can inhibit SR [98, 106]. The observations suggest that D-serine and serine racemase might be somehow interconnected with the intermediary metabolism of the neuronal tissue cells.

1.2.3 Other Serine Racemase Substrates

Panizzutti *et al.* first observed that serine racemase can transform other substrate than L- or D-serine [107]. They identified L-serine-*O*-sulfate as new serine racemase substrate. Subsequently we identified several other SR substrates, most of which are mentioned in

Publication I of the thesis. For the purposes of completeness are the results of Publication I mentioned here.

Mammalian serine racemase is capable of transformation of amino acids other than serine. SR in its "racemization mode" is serine specific, with the only known exception being threonine that is converted as follows: L-*allo*-threonine to D-threonine and D-*allo*-threonine to L-threonine and *vice versa* [98]. It is not stunning that SR can adopt threonine as a substrate since it is sequentially and evolutionary related to serine/threonine deaminases [92, 108]. This activity is, however, likely not physiologically relevant because the threonine isomers have very low affinity to SR; moreover, in the case of L-Thr, the initial velocity of its racemization is less than 10% of its deamination [98] (Table 2).

mouse SR	**product**	K_M [mM]	k_{cat} [min^{-1}]	k_{cat} / K_M [$min^{-1} mM^{-1}$]
L-Thr	2-ketobutyrate	48	627	**13.1**
L-Ser-O-sulfate	pyruvate	0.43	807	**1879**
L-*threo*-3-OH-Asp	oxalacetate	1.0	1860	**1788**
L-Cl-ß-Ala	pyruvate	1.6	155	**97**

Table 2 SR Substrates

Kinetic parameters of known mSR β-elimination substrates [98]. Compare the data with mSR parameters for serine in Table 1. Except for L-threonine the substrates are unnatural to mammalian tissue [109]. L-*threo*-3-hydroxyaspartate has been detected in proteins as a result of posttranslational modification but it was never confirmed to occur in the free form. The other presented amino acids are non-natural to mammalian tissue.

The experiments have revealed that SR is not very "picky" in terms of the substrates for β-elimination. It accepts various amino acids and converts them to the corresponding keto-acid products through the mechanism shown in Fig.3. The deamination substrates, however, share

some common features; they are all small L-α-amino acids with an electronegative leaving group attached to the β-carbon [98]. Recently, we identified the 2- and 3-hydroxyglutamic acids as another class of potent mSR β-elimination substrates (unpublished observations).

Perhaps even more striking than the actual low substrate specificity is the fact that SR catalyzes the deamination of these compounds significantly faster than its natural substrate serine. Serine racemase thus acts as a broad-specificity β-eliminase. However, the deamination activity with these other substrates may again be a residual of the evolution (as discussed in Chapter 1.2.2) and bear no physiological significance. Supportive to this are the observations that SR homologs from soil bacteria *Pseudomonas* sp. T62 [100] and from yeast *Saccharomyces cerevisiae* [85] have only L-*threo*-3-hydroxyaspartate (L-THA) deamination activity and lack serine racemization activity. Furthermore, the substrates, with the exception of the low affinity L-threonine, are all unnatural compounds, which thus cannot compete with serine *in vivo*.

1.2.4 Serine Racemase Activators

In addition to PLP, mammalian serine racemase requires divalent cations, such as Mg^{2+}, Mn^{2+}, or Ca^{2+}, for its activity [68, 110]. One atom of the cation is embedded inside the enzyme (one per monomer), in the vicinity of the active site as shown by the X-ray structures of yeast [84], rat, and human SRs [99] (see Fig. 6). It probably preserves proper architecture of the enzyme active site. Addition of chelators, or mutations close to the cation site lead to loss of SR activity [68, 111]. The deactivation by chelators is reversible; SR can be reactivated with a sufficient supply of cations. Fe^{2+} and Ni^{2+} ions can also partially reactivate SR. In contrast Cu^{2+}, Co^{2+}, and Ni^{2+} ions inhibit SR. It was speculated that calcium, as an abundant second messenger in the neuronal tissue, could regulate SR activity *in vivo*. More probably, however, the cation site is constantly occupied by Mg^{2+} ions that are present in cytosol at a sufficient concentration of 0.6 mM [112-113].

Another surprising feature of mammalian and yeast, but not plant, SRs is that they are activated by various nucleotides, with the largest effect performed by adenosine-5'- triphosphate (ATP), followed by less effective guanosine, cytidine, and uridine analogs [113-114]. The activation does not require ATP hydrolysis; adenosine-5'-diphosphate or a non-hydrolyzable ATP analog adenosine 5'-*O*-(3-thiotriphosphate) can equally activate SR [114]. The molecular

mechanism of the activation is not very clear. Some authors demonstrate the increase of k_{cat} without an effect on K_M [69]; others show a 10 fold decrease in K_M with k_{cat} remaining the same suggesting pure allosteric activation [98, 114]. It is possible that the different results are due to the presence or absence of reducing agents, such as 1,4-dithiothreitol (DTT), tris-carboxyethylphosphine (TCEP), reduced glutathione (GSH), or mercaptoethanol. Reducing agents significantly alter SR activity and temperature stability [111]. It has been demonstrated that the effects of ATP and DTT are interconnected [111, 115]. Reducing conditions are important for ATP binding, perhaps to protect Cys 113 (hSR numbering) in the vicinity of the ATP binding site from oxidative modification [115]. The cell cytosole is rich in reduced glutathione, which is able to reduce majority of cysteines [116], as well as in ATP (2-3 mM) [19], therefore mammalian SR, as a cytoplasmic protein, is stabilized and fully activated *in vivo*.

ATP molecules often chelate Mg^{2+} ions in a 1:1 ratio; thus, the formed complex then functions as the actual allosteric activator. The 3D structure of yeast SR (PDB code 1WTC) first revealed two independent sites for the activators; one for the ATP/Mg^{2+} complex, and the other one for Mg^{2+}. The structural data correspond with the biochemical observations that Mg^{2+} and ATP/ Mg^{2+} have additive activation effects [113].

1.2.5 Posttranslational Modification and Interacion Partners

Recombinant serine racemase catalytic efficiency for serine as a substrate is surprisingly low. The reaction velocity corresponds to one molecule of product per 7 seconds [68]. Possibly SR is somehow upregulated *in vivo*. Several interaction partners and possible posttranslational modifications have been described. The available results, however, have not demonstrated a significant increase in catalysis.

The fact that active mammalian SR has been produced in *E. coli* cells (which do not possess eukaryotic posttranslational apparatus) demonstrates that posttranslational modifications are not necessary for SR activity [117].

So far, it has been shown that SR can undergo polyubiquitination prior to degradation [118]. Human and mouse SRs are predicted to have several serine, threonine, and tyrosine phosphorylation sites (analyzed in NetPhos 2.0 server [119]). Thr 227 present in mSR was shown

to be phosphorylated [120] (in hSR this residue is Met, see Fig. 4). The authors further suggested that SR could be regulated by acylation [120].

Human SR could be *S*-nitrosylated on its surface exposed cysteine residues (Cys 2, 6, 113, 127, and 269). Mustafa *et al.* demonstrates specific inhibition of SR through Cys 113 nitrosylation in the presence of *S*-nitrosoglutathione [115]. The compound could, however, as well serve as a glutathione donor [70]. Most of the cysteine residues are not highly conserved among SRs from other species, with exception of Cys 113 and Cys 46, which are conserved in mammalian and plant SRs. It remains to be clarified if modification of cysteine residues can regulate SR activity.

Several SR interaction partners have been identified (for reviews see [70, 117]); two of them, glutamate receptor interacting protein (GRIP) [121-122] and protein interacting with kinase C (PICK1) [123], are thought to interact with the 4 C-terminal amino acid residues SVSV (hSR, or TVSV in mSR). The interaction with these proteins is important for SR targeting and activation. Interestingly, other mammalian SRs are truncated at the C-terminus and do not contain this motif. Another described interaction partner, golgin subfamily A member 3 (Golga 3), is supposed to interact with the 66 N-terminal amino acid residues. Golga3 probably prevents SR polyubiquitination and degradation [118].

1.2.6 Serine Racemase Structure

1.2.6.1 Primary Structure

Human SR sequence is formed by 340 residues, giving it a size of 36.5 kDa. The primary structure of serine racemase is depicted in Fig 4. As already mentioned, SR is sequentially related to serine/threonine deaminases and was thus predicted to belong to the fold type II family of PLP-dependent enzymes [92, 108]. The correctness of the fold type prediction was later confirmed by the 3D structures [84, 99].

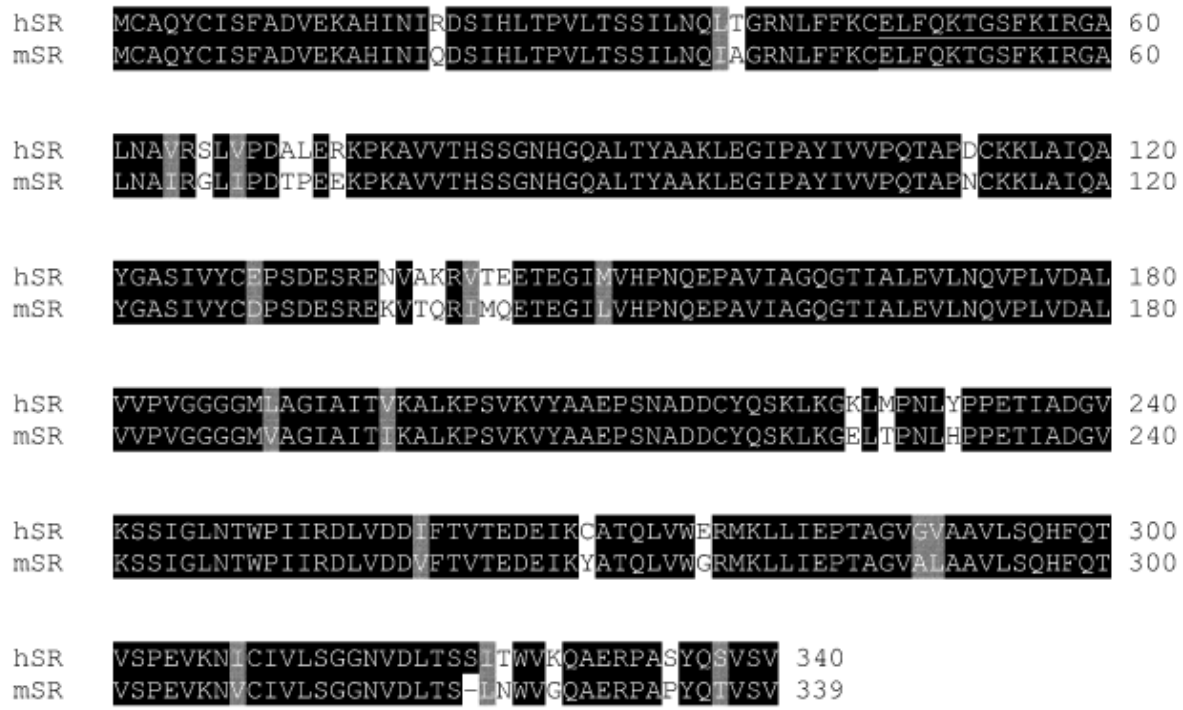


Figure 4 Sequence Alignment of Human and Mouse SR

Human and mouse SRs share 89% sequence identity. The identical residues are shown against black background, homologous residues against grey background, and the different residues are highlighted white. The PLP-binding consensus sequence as defined in Prosite under the Accession Number PS00165 is underlined. The picture is taken from [102].

1.2.6.2 Secondary Structure

SR is a globular protein. Human SR has 40.3% of residues in alfa-helices, 13.9% in beta-sheets, 15.8% in beta-turns and 27.1% in random coil. The mouse ortholog has a comparable secondary structure representation [102]. These results are derived from circular dichroism measurements with the recombinant SRs [102]. The experimental data correspond to the observed secondary structural motifs in the X-ray structure of human SR depicted in Fig. 5.

1.2.6.3 Tertiary Structure

Serine racemase is built with two domains that both contribute to the formation of the active site. The large domain is made up of residues 1-68 and 157-340 (hSR numbering), and the small domain of residues 78-155. The linkers are formed by flexible loops. As has been described for other PLP-dependent enzymes, SR adopts an open (free) and a closed (occupied) conformation. Ligand binding induces a substantial movement of the small domain [99].

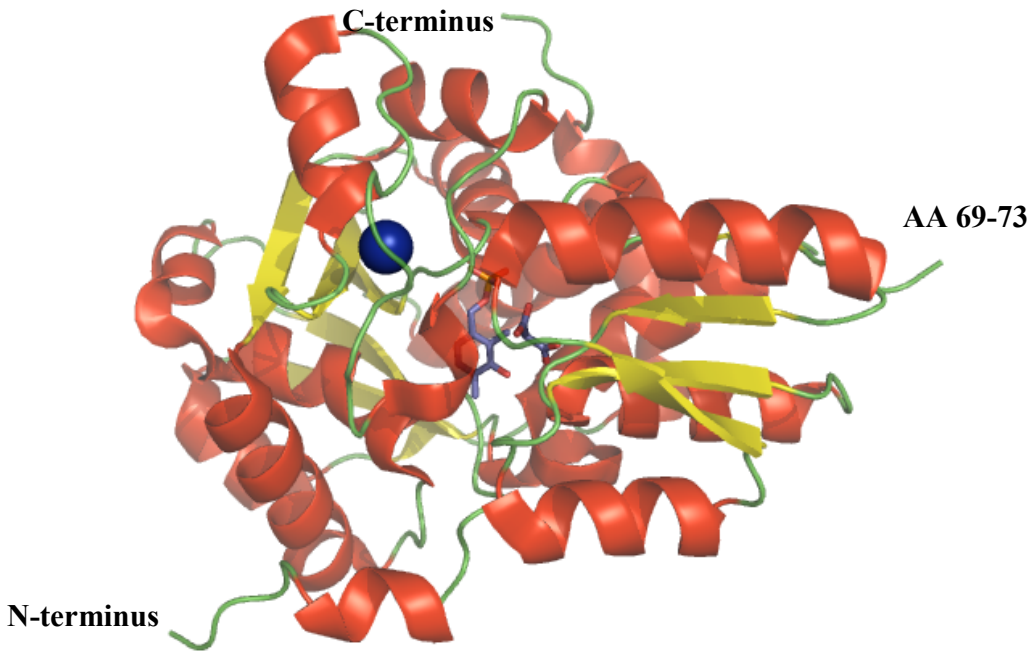


Figure 5 Overall Structure of hSR

Human SR monomer structure in cartoon representation with the basic secondary structure motifs further distinguished by color: α-helices (red), β-sheets (yellow), and flexible loops (green). The picture was prepared in the PyMol program [124] using the structure under the 3L6B PDB code. Active site is occupied by PLP and malonate shown in blue sticks representation. The cation site is occupied by Mn^{2+} shown as a blue sphere. 11 C-terminal flexible residues and residues 69-73 are not visible in this structure.

1.2.6.4 Quaternary Structure

In solution SR forms dimers with a small fraction of tetramers [102]. The multimerization state is not influenced by reducing agents; therefore, it is probably not stabilized by disulfide bridges. The 3D-structure of hSR further supports this observation, because there are no cysteine residues pointing at the dimerization interface of SR. Surprisingly, despite the observation that ATP/Mg^{2+} molecules bind between the two monomeric units [115], the experiments show that the multimerization state of SR does not depend on ATP *in vitro* [114]. Dimerization is also independent of the divalent cations [68, 110]. Human SR dimer is shown in Fig. 7.

1.2.6.5 Available X-ray Structures

The three-dimensional X-ray structures of yeast SR, and recently also rat and human SRs, are now available. Two yeast SR structures (PDB codes 2ZR8 and 2PZU) contain modified PLP that is created upon prolonged incubation with the substrate L-serine and adopt the closed conformation. Additionally, 2ZR8 contains L-serine bound in the active site. Two other yeast SR structures (PDB codes 1WTC and 1V71) are in open conformations, have unmodified PLP, and contain neither substrate nor inhibitor in the active site. 1WTC structure includes a nonhydrolyzable analogue of ATP, adenosine-5'-(β, γ-methylene) triphosphate (AMP-PCP), bound in the allosteric site.

The rat structures (PDB codes 2L6C and 3HMK) appear, unlike the others, as dimers in asymmetric unit, mapping the SR dimerization interface clearly. The models contain Mn^{2+} in the metal binding site. Additionally the structure 2L6C contains the inhibitor malonate in the active site, and thus adopts the closed conformation.

Finally, the human SR structures (PDB codes 2L6R and 2L6B) contain Mn^{2+} and malonate and are, therefore, both in the closed conformation. SR under the 2L6R code is a selenomethionine protein that was solved to 1.7 Å resolution. The 2L6B structure was solved to 1.5 Å resolution and is used in the presented Figures.

All the structures include Mg^{2+} or Mn^{2+} cation in the metal binding site. Thereby the cation site has been mapped in detail. The cation is octahedrally coordinated by three conserved

residues and three structural water molecules. The residues that coordinate the cation are Glu 210, Ala 214, and Asp 216 (Fig. 6)

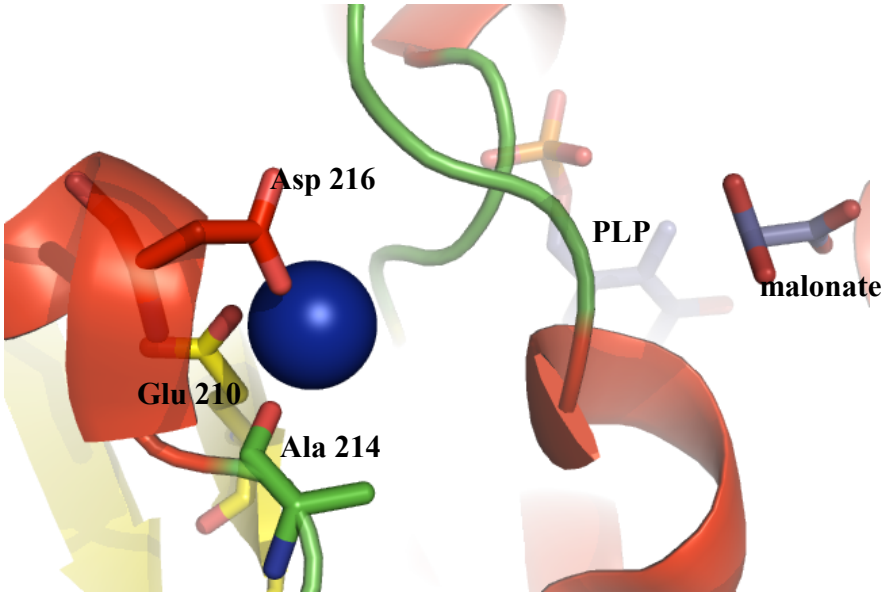


Figure 6 Divalent cation binding site

Close-up of the hSR divalent cation site occupied by Mn^{2+} (blue sphere). Mn^{2+} is octahedrally coordinated with amino acid residues Glu 210, Ala 214, and Asp 216, highlighted in sticks, and three structural water molecules (not shown). The PLP and malonate molecules mark the active site in the vicinity. The picture was prepared with PyMol [124].

1.2.6.6 ATP/Mg^{2+} Binding Site

The mammalian SR structures do not contain ATP/Mg^{2+} complex. We therefore docked the complex into the 3L6B hSR structure (Fig. 7). The following data are a part of results in Publication IV of the thesis.

The docking analysis reveals that the aromatic stacking of the adenosine moiety with Tyr121 and the anchoring of the phosphate to Gln50 *via* the magnesium ion play key roles in ATP binding. The sugar moiety is stabilized by interactions with Gln 89, Thr 52, and Gly 53

[106]. Except for Thr 52 and Gln 89 all the residues conserved in all eukaryotic SR orthologs [111]. Thr 52 is conserved only in mammalian SRs; interestingly Gln 89 is conserved in mammalian and yeast SRs but not in plant SRs, which are not activated by ATP.

Since the ATP/Mg^{2+} complex binds within the dimerization interface it also interacts with the other monomeric unit. These interactions are mediated by residues Lys 279, Arg 277, also conserved in yeast and mammalian SRs only, and Ser 32, conserved in mammalian and *S.pombe* SRs [106, 111].

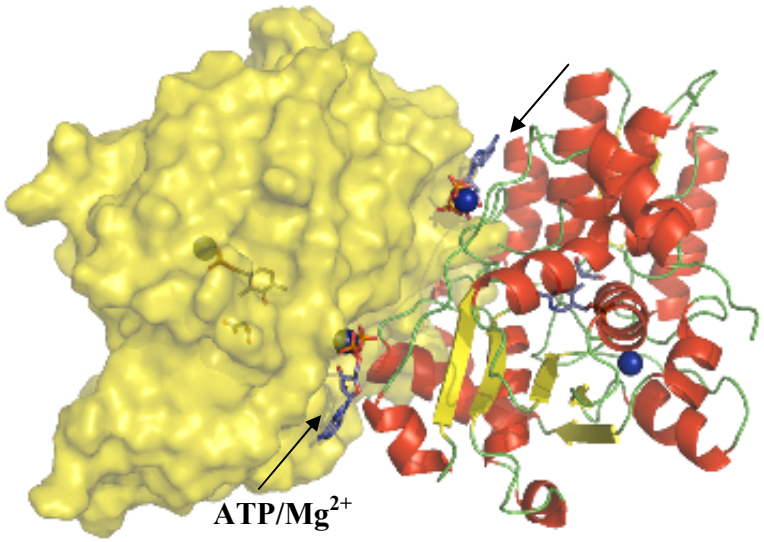


Figure 7 hSR Dimer with ATP/Mg^{2+} Complex

Two ATP/Mg^{2+} complexes bound in the interface of human serine racemase (PDB code 3L6B) dimer. The overall structure shows secondary structure motifs and PLP, malonate and Mg^{2+} in the cation binding site as in Fig 5. The position of ATP/Mg^{2+} was set by docking as described in Publication IV. The picture was prepared with PyMol [124].

1.2.6.7 The Active Site

The active site of hSR resembles that of other SRs and other PLP-dependent enzymes: PLP phosphate is anchored by the backbone of the so-called “tetraglycine loop” (Gly 185-188) and by the backbone of adjacent Met 189, pyrimidine ring nitrogen is bound to Ser 313, and the 5-hydroxy group interacts with Asn 86.

The human and rat SR structures contain the inhibitor malonate (see below) bound in the active site [99]. Both carboxylates participate in several H-bonds that stabilize the molecule within the active site. The active site residues forming the H-bonds are Ser 84, His 87, Arg 135, and Ser 242. Ser 242 is only conserved in mammalian SRs, while the other residues are conserved in all other SR orthologs too [111].

1.3 Physiological and Pathophysiological Roles of Serine Racemase

The physiological and pathophysiological functions of serine racemase have been mentioned in connection with NMDA receptors. As already mentioned, serine racemase is the major source of the NMDA receptor glycine-site agonist D-serine in mammalian tissue. It is also possible that it contributes to DS degradation. As such, it is a key enzyme in DS metabolism and potentially interesting for pharmaceutical intervention in NMDA-receptor related diseases. The neuropathology of NMDA receptors has been covered extensively in the last two decades (for reviews see [125-128].

A few recent studies already show a direct link between DS and SR in some of these diseases - namely, schizophrenia, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease. The connection of SR with the diseases will be discussed here in more detail.

The role of DS in neurodegeneration was recently demonstrated in a broad study of rat gene expression changes upon DS administration. Among the upregulated genes were genes with important neurological functions and genes associated with the neuronal cell death, such as genes of proimflamatory cytokines or ubiquitin pathway [129]. The results suggest that aberrant regulation of DS might lead to neurodegeneration.

1.3.1 Spatiotemporal Distribution of Human and Mouse Serine Racemase

The current expressed sequence tag (EST) profile of human SR mRNA at the NCBI UniGene database [80] suggests that SR may be present in numerous other human tissues outside brain, such as kidney, liver, skeletal muscle, heart, adrenal gland, intestine, lung, stomach, mammary gland, pharynx, prostate, testis, thymus, and uterus. The function of serine racemase in most of these tissues is unknown. However, since NMDA receptors have been found in the periphery as well [33-34, 130-131], we can speculate that SR located in these tissues produces DS for the nearby peripheral NMDA receptors too.

Several observations show that SR contributes to early stage neuronal tissue development. SR is present in fetal brain as well as human placenta [132-133]. Furthermore, DS was shown to be transported through placenta to fetus [132]. Schell *et al.* demonstrate that DS is important for the NMDA receptor dependent synaptogenesis [134].

It has been demonstrated in rats that SR and DS levels change with age [59]. DS levels are high in early life stages and decrease with increasing age. Low SR and DS levels have been associated with cognitive dysfunction in the elderly [135-136].

1.3.1.1 Central Nervous System

SR is most abundant in the forebrain regions of mammalian brain. Along with DS it is found in hippocampus, amygdala, and various cortical regions [63, 90]. The cellular distribution of SR is controversial. It was first detected in glial cells only, but later it was found in neurons too [39]. SR has been detected in protoplasmic astrocytes and pyramidal neurons of the hippocampus and cortex and in Purkinje cells and Bergmann glia of the cerebellum [39, 63].

1.3.1.2 Peripheral Nervous System

SR is present in Müller cells and astrocytes of rat retina [89]. Its presence in both neurons and astrocytes of retina was further confirmed on the mRNA level [137]. SR was studied in the rat vestibular sensory system [138], and it is present in Schwann cells and epineural fibroblasts of rat sciatic spinal nerve [104]. NMDA receptors are located on peripheral nerve termini, where they are responsible for the sensory transduction of pain; the possible role of SR in spinal nerves is to contribute to nociception [131].

1.3.1.3 Other Tissues

SR has also been detected in other tissue types outside the nervous system, like liver, kidney, and heart muscle [90]. According to Xia *et al.*, SR mRNA transcripts are present in the human heart, skeletal muscle, kidney, and liver. The authors did not detect SR mRNA in human colon, small intestine, spleen, thymus, leukocytes, or lungs. Wang *et al.* observed the same distribution of SR protein in rats [59].

In human kidney SR is present in the epithelial cells of convoluted tubules [90]. DS activates the renal NMDA receptors responsible for renal reflex control [34], but in higher amounts it is nephrotoxic [130, 139].

SR is also expressed in heart muscle tissue, namely in ventricular myocytes [90]. Gao *et al.* performed a pilot study of NMDA receptor function in rat neonatal cardiomyocytes. Their

study revealed that NMDA receptors might contribute to myocardial pathogenesis [33], suggesting SR as a potential target for the treatment of some cardiac diseases.

1.3.2 Serine Racemase and D-Serine in Schizophrenia

Schizophrenia is a severe psychiatric condition of complex and not entirely known etiology. Schizophrenic patients suffer from a typical group of symptoms divided into positive (such as hallucinations), negative (such as apathy), and cognitive symptoms. Several possible molecular casual agents have been identified, and new molecular mechanisms of schizophrenia are under investigation. One of the more recently identified molecular mechanisms is NMDA receptor impairment [140].

It has been confirmed by several groups independently that schizophrenic patients have, in comparison to healthy individuals, decreased D-serine levels in cerebrospinal fluid and serum [64, 141-142]. Moreover, it was shown that additional D-serine can improve the negative and cognitive symptoms of some, but not all, schizophrenic patients [143-146]. A broad study with Canadian schizophrenic patients revealed polymorphism in one gene (G72) being associated with the illness [60]. The product of G72 is a potential interaction partner of DAAO. This discovery led to a boom of DAAO investigations, especially its correlations to schizophrenia [61-64]. The results from experiments with DAAO deficient mice further prove this link [66-67]. However, the SR deficient mice also suffer from schizophrenic-like behaviors [147].

Schizophrenia is largely a hereditary disease. Several groups therefore studied potential schizophrenia-associated polymorphisms in the genes of SR or DAAO, with contradictory results. Some observed a clear association between SR genetic variants and polymorphisms with schizophrenia [147-148], while others exclude this possibility [64, 149]. Another interesting explanation of how altered DS metabolism could be implicated in schizophrenia is *via* the SR interaction partner PICK1. PICK1 polymorphism is associated with a more severe type of schizophrenia, disorganized schizophrenia [123]. Based on the observation that PICK1 knock-out mice have decreased brain DS, PICK1 is believed to activate SR-catalyzed DS production [150]. Disturbed PICK1 activation of SR could explain the decrease of DS in at least some schizophrenic patients.

The subject of DS metabolism and its role in pathology of schizophrenia is matter of intense research. Most probably the outcome will depend on the different types of the illness. Perhaps, in the future, we will distinguish between DS-dependent and DS-independent schizophrenia, which will allow more suitable treatment.

1.3.3 SR and DS in Alzheimer's Disease and Amyotrophic Lateral Sclerosis

Alzheimer's disease and ALS are both severe chronic neurodegenerative diseases that are largely untreatable. The diseases have, among others, altered glutamate signaling; NMDA receptors might be the major glutamate affected pathway [151-153]. Hashimoto *et al.* first suggested altered DS metabolism of patients suffering from Alzheimer's disease [154]. The authors observed that the patients have increased LS and decreased DS levels in the serum.

Sasabe *et al*. showed that DS and SR, but not DAAO levels correlate with the progression of ALS in mouse models and that DS is elevated in ALS patients [155]. Furthermore, the addition of SR inhibitor phenazine methosulphate (see below) to ALS mouse spinal cord primary cultures relieved NMDA toxicity. This observations demonstrated direct link between NMDA receptor mediated toxicity and DS.

Wu *et al*. claim that amyloid β-peptide induces serine racemase and D-serine production in primary microglia cell cultures and that serine racemase mRNA is elevated in the hippocampus of Alzheimer's disease patients. The authors probed several human brain samples and found increased levels of SR mRNA in Alzheimer's disease patients relative to age-matched controls [156]. Recently, Inoue *et al.* showed that amyloid β-peptide induced neurotoxicity is significantly reduced in SR knock-out mice, providing a compelling evidence for SR being a prospective pharmaceutical target [157]. The results seem to contradict with the observations made by Hashimoto *et al.* who, upon the detected decrease of DS levels, suggested decreased SR activity [154]. The discrepancy could be explained for example by compensatory activity of DAAO or DS transporters. The role of DS and SR in the Alzheimer's disease pathophysiology remains to be clarified.

1.3.4 DS in Epilepsy

Epilepsy is a serious chronic neurological disorder manifesting as spontaneous recurrent seizures. The neurochemical background of the disease seems rather complex and has yet not been clarified. Glutamate, together with other excitatory amino acid neurotransmitters, is highly probably involved in the initiation and propagation of epileptic seizures. Therefore, also NMDA receptors in epilepsy are a subject of research [158]. It has been shown that NMDA receptor antagonists, including the glycine/DS antagonists, behave protective in some animal models of epilepsy [159-161].

Liu *et al.* observed substantial upregulation of DS in hippocampal and cortex neurons of epilepsy mice model (pilocarpine treated mice) [162]. Most of the cells with increased DS had also more prominent NMDA receptor phosphorylation leading to its activation, and underwent degenerated death. This is the first evidence that DS might be somehow involved in epileptogenesis as well as recurrent seizures.

The study with ASCT1 transporter knock-out mice that suffered from severe seizures and died within several postnatal days stresses the importance of DS (and perhaps also glycine) clearance from the synaptic cleft [163]. The results suggest that upregulated DS signaling can be involved in the seizure onset.

The role of DS and its metabolism in epilepsy is as an attractive and highly contemporary topic currently under investigation by several groups. The professional audience is expecting release of the first results soon.

1.4 Serine Racemase Inhibition

NMDA receptors have become very interesting for clinical research as important drug targets as reviewed by several groups; see refs [30, 128, 164-165]. Various antagonists of the glutamate, glycine, or the modulatory binding sites, or channel blockers with a therapeutic effect have been identified, but their use is often accompanied by adverse side effects that do not permit their clinical use [166-171]. Therefore, researchers gradually extended their interests to molecules upstream or downstream of the NMDA receptor to find new targets for treatment of the numerous neurological diseases. Putative drug targets of the NMDA system may include glutamate, glycine, and D-serine re-uptake transporters [172]; molecules of NMDA signaling like NO-synthase (NOS) [173]; and enzymes producing or degrading the neurotransmitters. At this point, serine racemase comes to the stage as a potential pharmaceutical target. Several groups have therefore searched for potent mammalian SR inhibitors.

The SR inhibition results depend on the assay setup, especially on the addition of SR activators [106]. The most potent inhibitors are small amino and carboxylic acids [98], but several moderate tripeptide inhibitors have been also described [174].

For the purpose of completeness, I cannot avoid mentioning the results of our group that are part of the Publications included in the thesis. The results are referenced as follows: Publication I [98], Publication II [72], Publication III [175], and Publication IV [106].

1.4.1 Small Amino Acid Inhibitors - Substrate Analogues

Among the proteinogenic amino acids are several SR inhibitors. Glycine is a competitive inhibitor with K_i of 150 – 1640μM. The K_i value depends on used serine racemase; with recombinant mouse SR the K_i is 1640 [98], with brain isolated mouse SR it is 150 [103], and with recombinant human SR it is 366μM [102]. The observed differences between human and mouse SR may help reveal differences within the active site of the enzymes. The higher affinity for glycine when mouse isolated SR is used may suggest another, yet unknown, natural SR effector influencing the result. The inhibition by glycine is probably physiologically relevant because the reported concentration of glycine in the cells is 500-700μM [103]. Also L-asparagine behaves as

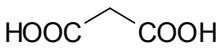
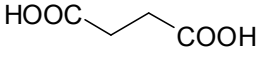
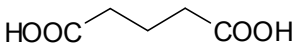
a competitive SR inhibitor with K_i of 1130μM (for recombinant mSR) [98]. Various other derivates of aspartic acid are potent SR inhibitors. The highest SR affinity has L-*erythro*-3-hydroxyaspartic acid (L-EHA) with K_i of 43 and 11 μM for mouse and human SR, respectively. The other three hydroxyaspartate stereoisomers have lower (L-THA, Table 2) or no affinity for SR.

1.4.2 Small Carboxylic Acid Inhibitors

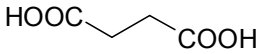
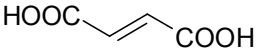
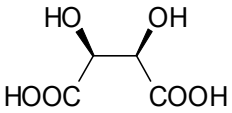
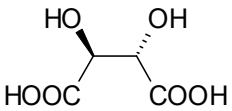
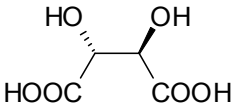
We have shown that the amino-group is not necessary for binding; several small dicarboxylic acids behave as potent SR inhibitors

The most suitable distance between the carboxylates bears malonate with the 3 carbon chain length. Succinate with the 4-carbon chain has lower affinity than malonate, but still preserves higher affinity than the SR substrate L-serine. Oxalate and glutarate with 2-carbon and 5-carbon chains, respectively, have very low affinity to SR. (Table 3a). The main chain conformation and the side chain configuration influence the affinity to SR (Table 3b). Among the tested succinic acid analogues, maleic acid has the most suitable main chain conformation, while *meso*-tartaric acid has the best side chain configuration, the (*R,S*) configuration, for SR binding. The rule of (*R,S*) or (*S,R*) configuration being the most suitable repeats with other succinic acid analogues, for example *meso*-diaminosuccinic acid or L-EHA.

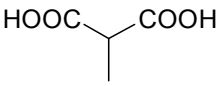
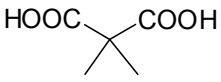
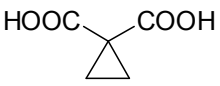
a)

acid		V_{0I} / V_0
oxalic	HOOC—COOH	0.80
malonic		0.12
succinic		0.36
glutaric		0.62

b)

acid		V_{0I} / V_0
succinic		0.36
maleic		0.20
fumaric		0.91
***meso*-tartaric (*R,S*)**		0.19
D-tartaric (*S,S*)		0.47
L-tartaric (*R,R*)		1.00

c)

malonic acid		V_{0I} / V_0
2-methyl-		0.52
2,2-dimethyl-		0.8
2,2-ethyl-1,2-diyl-		0.9

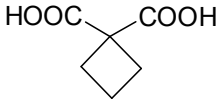
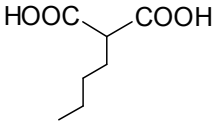
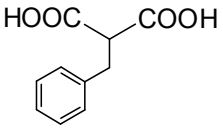
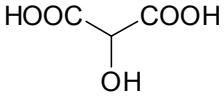
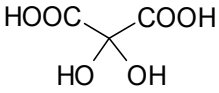
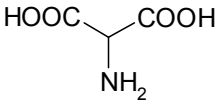
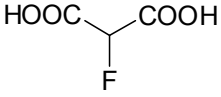
2,2-propyl-1,2-diyl-		1.01
2-butyl-		0.88
2-benzyl-		0.48
2-hydroxy-		0.11
2,2-dihydroxy-		0.23
2-amino-		0.02
2-fluoro-		0.25

Table 3 Dicarboxylic Acid Inhibitors

The tables show SR inhibitors and their corresponding potencies as ratio of initial velocity in the presence and absence of the inhibitor (V_{0I}/V_0). The experimental setup involves equimolar amounts of LS and the inhibitor (5mM). Relative velocity of 0.5 means that the inhibitor has comparable affinity with LS, below 0.5 means it is higher affinity ligand, and value above 0.5 means lower affinity than LS. Table a) shows how chain lengts influences the affinity towards DS, table b) shows the influence of the main chain configuration, and table c) presents various malonic acid analogues and their SR inhibition. The data are from [98, 106].

We tested various malonic acid analogs. Most of them lose their SR affinity comparing to the mother compound (Table 3c). Only the 2-hydroxy and 2-amino analogs retained comparable inhibition potency to malonic acid.

Recently we found that also tricarboxylic acids bear some affinity towards serine racemase. The relative velocity of racemization reaction in the presence of citric acid is 0.4 suggesting it has better affinity than LS. The observation led us to test all the tricarboxylic acid cycle analogues. We found that oxaloacetate, citrate, succinate and L-malate are moderate SR inhibitors with affinity lower than LS. The mechanism of inhibition of the compounds is not known. However, based on the observation that similar compounds *meso*-tartrate, dihydroxyfumarate and maleate are all competitive SR inhibitors, we can presume that the mentioned tricarboxylic acid cycle intermediates might also behave as competitive SR inhibitors. The physiology relevance of the inhibition is not clear. It is possible that serine racemase is somehow connected with the cell energy metabolism. Supportive to that is a recent observation that DS can regulate the citric acid cycle in rat cerebral cortex *via* citrate synthase inhibition [105]. However, the exact mechanism of SR role in the intermediary metabolism remains to be clarified, especially with respect to different compartmentalization of SR and the citric acid cycle machinery.

1.4.3 Other SR Inhibitors

In 2005 Dixon *et al.* identified several moderate surface-binding hSR peptidic inhibitors based on a tripeptide-like library of compounds [174]. The most potent compounds had K_i values of 320 and 610 μM respectively. All the active compounds were bulky hydrophobic tripeptides that bear no structural similarity to the substrate. Although the authors claim, based on the observed competitive mechanism of inhibition, that these are active site inhibitors, it is also possible that the compounds compete for some surface exposed site, such as the ATP binding sites.

In 2005 Kim *et al.* used another bulky molecules, phenazine methosulfate and ethosulfate, as inhibitors of SR in cell migration experiments [121]. The authors mention IC_{50} values of 3 and 5μM for the methosulfate and ethosulfate analogs, respectively, without specifying the inhibition

assay. In our hands, phenazine methosulfate and ethosulfate inhibit mSR with IC_{50} values of 220 and 2170 μM, respectively [106]. The compounds clearly act as SR inhibitors *in vivo* because, in the cell migration experiments, the effect of the compounds could be partially compensated by addition of D-serine. The mechanism of action of these compounds is not known.

Mustafa *et al.* describe that a certain percentage of glial SR is localized to membranes through interaction with phospholipids [176]. The authors show that phosphorylated phosphatidylinositols inhibit both SR catalyzed racemization and β-elimination. The greatest effect was observed with phosphatidylinositol (4,5)-bisphosphate (PIP2). Addition of phospholipase C, which degrades PIP2, results in loss of SR inhibition. PIP2 inhibits SR by interfering with ATP binding and behaves as a noncompetitive inhibitor with an IC_{50} of 13μM. The inhibition is physiologically relevant for serine racemase activity regulation [176].

Chapter 2 Results

2.1 Aims of the Project

The first aim of the project was to prepare and characterize recombinant mouse serine racemase and to develop methods for its activity determination (Publication I).

Secondly, we wanted to develop a less laborious method for DS detection during inhibition experiments (Publication II).

Finally, we wanted to characterize the enzyme structurally and to design potent and selective inhibitors (Publication III and IV).

2.2 List of Publications Included in the Thesis

I. Stříšovský, K.; Jirásková, J.; Mikulová, A.; Rulíšek, 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. 44(39), 13091-100. [98]

II. Koval, D.; Jirásková J.; Stříšovský, K.; Konvalinka, J.; Kašička, V., **Capillary electrophoresis method for determination of D-serine and its application for monitoring of serine racemase activity.** *Electrophoresis*. 2006. 27(13), 2558-66. [72]

III. Hoffman, H.E.; Jirásková, J.; Cígler, P.; Šanda, M.; Schraml, J.; Konvalinka, J., **Hydroxamic acids as a novel family of serine racemase inhibitors: mechanistic analysis reveals different modes of interaction with the pyridoxal-5'-phosphate cofactor.** *J Med Chem.* 2009. 52(19), 6032-6041. [175]

IV. Jirásková, J.; Ettrich, R.; Vorlová, B.; Hoffman, H.E.; Lepšík, M.; Jansa, P.; Konvalinka, J., **Inhibition of Human Serine Racemase, an Emerging Target for Medicinal Chemistry.** *Curr. Drug Targets* (submitted, May 2010) [106]

2.3 Publication I

The publication summarizes our work on the *E. coli*-based expression and a three step purification system for full-length mouse serine racemase. The publication also includes a detailed enzymatic characterization, including identification of novel, potent competitive inhibitors. Based on the observed substrate specificity of the yeast *Saccharomyces cerevisiae* SR ortholog [85], we decided to test L-*threo*-3-hydroxyaspartate (L-THA) and its other stereoisomers as mouse SR substrates. To our surprise, L-THA turned out to be a potent mSR substrate with two-fold higher catalytic efficiency than LS, while L-erythro-3-hydroxyaspartate (L-EHA) behaved as a potent competitive inhibitor. With a K_i of 43 μM, L-EHA remains the most potent SR inhibitor identified to date. The observation that aspartic acid analogues can become high affinity binders lead us to the idea of also testing various dicarboxylic acids. It turned out to be a good choice; several small dicarboxylic acids behave as competitive mSR inhibitors, with the most effective inhibitor being malonic acid.

Since no 3D structural knowledge of serine racemase was available at the time of publication, we calculated the equilibrium geometries of the active compounds in a dielectric continuum representing an aqueous environment. The calculations and screening of various other compounds for their mSR inhibition allowed us to define some attributes of successful SR inhibitors.

2.3.1 Expression and Purification of mSR

We developed an *E.coli* expression system for mSR employing the arabinose-inducible pMPM-A4 vector. Full length mSR (GenBank nucleotide accession number AF148321) was expressed in MC1061 cells growing in nutrient-rich media with a yield of 2 mg of purified mSR / L of bacterial culture. The successful three-step purification protocol encompasses hydrophobic interaction, ion-exchange, and affinity column chromatography. We chose ATP-agarose resin for the last step affinity-chromatography because of the previously published observation that ATP and other nucleotides can activate SR. The final purity of mSR was about 95%. The purified recombinant mSR was active, with kinetic parameters comparable to the results of other groups. Based on N-terminal sequencing we observed truncation of the initial two amino acid residues,

methionine and cysteine. Despite the high purity and sufficient solubility allowing concentration up to 10mg/ml, we were unable to crystallize the enzyme into well-diffracting crystals. For several years crystallization has remained the ultimate, but unreachable, goal in the project. Even the identification of potent competitive inhibitors did not help. The 3D structure of mammalian SR was recently released by a group from the company Evotec, Oxon, UK [99]. The authors also used an *E. coli* expression system, with the important difference being mutation of two N-terminal cysteine residues to more water-soluble aspartic acid residues (C2D, C6D).

2.3.2 Enzymatic Characterization

Various SR activators were revealed during our work on this part of the project. In our activity assays we could use all the required additives to fully activate the enzyme and to better mimic the *in vivo* conditions. This turned out to be important, because the additives influence the kinetic parameters of the enzyme, and they can also influence the inhibition profile of some inhibitors. In addition to buffer and PLP our activity mixture contains 5 mM DTT, 1 mM ATP and 1 mM $MgCl_2$.

The Michaelis-Menten kinetic parameters of mSR (Table 1) revealed comparable efficiencies for LS racemization and elimination and lower efficiencies when DS is used as substrate. We repeatedly observed an approximately 4-fold higher K_M for DS compared to LS. Somewhat puzzling was the observation that DS elimination has a different K_M than DS racemization; the K_M for DS elimination resembles that of LS conversions. Our explanation was that either there are two independent active sites for DS racemization and elimination, or mSR is unable to eliminate DS and what we observe is elimination of LS formed from DS by racemization. To test our assumption, we used the competitive inhibitor L-*erythro*-3-hydroxyaspartate to measure its mechanism of inhibition for all four SR-catalyzed serine conversions. L-EHA turned out to be a competitive inhibitor for all the reactions, suggesting that serine racemase uses the same catalytic site for all of its reactions. This means that mSR is probably not capable of direct DS elimination. The predicted presence of a common active site has been demonstrated by the 3D structures of other SRs [84, 99].

Later we observed that, unlike mSR, hSR is capable of DS elimination (the K_M values for both DS reactions are comparable), though the reaction has very low efficiency (Table 1). In the

same way it can be deduced that other SR orthologs, such as barley and fission yeast SRs, can also catalyze DS elimination (Table 1).

2.3.3 Novel mSR Substrates and Inhibitors

The current knowledge of serine racemase substrate specificity led us to test a panel of related compounds for their mSR affinity. We thus revealed several new elimination substrates, such as β-Cl-L-alanine, L-threonine, and L-*threo*-3-hydroxyaspartate (2*S*,3*S*) (Table 2). L-THA is one of the four stereoisomers of 3-OH-aspartic acid. The L-EHA (2*S*,3*R*) stereoisomer is a potent SR inhibitor (see below). In contrast, the D-*threo* (2*R*,3*R*) and D-*erythro* (2*R*,3*S*) stereoisomers turned out inactive, just like the D-forms of other known substrates. So, the only known D-amino acid with affinity to SR is D-serine. It is interesting to speculate whether or not the conversion of the other substrates could be physiologically relevant.

L-EHA and L-THA differ substantially in their equilibrium configurations; L-EHA is more stable due to better orientation of intramolecular H-bonds between carboxylates with the hydroxyl and amino group, respectively. The configuration, together with the architecture of the enzyme's active site, is probably the reason why L-THA serves as a SR substrate while the L-EHA is a potent inhibitor.

We further revealed that glycine, L-asparagine, and several small dicarboxylic acids are competitive SR inhibitors, with malonic acid (K_i of 71 μM) being the second most potent inhibitor after L-EHA.

The most potent SR competitive inhibitors malonic acid and L-EHA have already been successfully used in SR research; malonate as an inhibitor assisting human and rat SR crystallization [99] and L-EHA as a SR inhibitor in a recent neurophysiology study revealing the role of glial DS in the long term potentiation (LTP), a model of memory formation [177].

My contribution to the publication

I participated in the inhibitor screening, established the oxo-acid detection method,. and analyzed the oxo-acid reactions. I performed some mechanism of inhibition experiments with L-EHA.

2.4 Publication II

Serine racemase inhibition is a highly interesting topic from the pharmaceutical point of view. We are aware that many groups are searching for potent and specific hSR inhibitors. Serine racemase, however, seems to be a difficult target. Being a PLP-dependent enzyme, known for their low substrate and inhibitor specificity, it is not so surprising that the road to identifying potent, specific SR inhibitor is a challenging one. Despite the efforts of several scientific teams, the best know SR inhibitors are either non-specific compounds interacting with PLP [175] or specific competitive inhibitors with K_i values in the μM range [98, 106, 174].

Since there is no continuous assay available to monitor SR catalyzed racemization, we are limited to time consuming and laborious end-point measurements of the product. For the purpose of continuous activity monitoring, serine racemase has to be coupled for example to DAAO. DAAO in combination with horseradish peroxidase and the chromogenic substrate *O*-phenylenediamine can be used for D-serine detection [110]. Such combination of enzymes is, however, not suitable for measuring SR inhibition, because the other enzymes may interfere with the inhibitor.

In order to speed up testing of approximately 70 potential SR inhibitors selected from a large library of compounds offered by the company Guilford Pharmaceuticals, we developed a medium throughput assay for DS detection. The assay is based on OPA derivatization, chiral resin accompanied capillary electrophoresis (CE) separation, and subsequent spectrophotometric detection of the OPA-serine derivates.

2.4.1 In-Capillary Derivatization

Ortho-phtaldialdehyde (OPA) is a common amino acid derivatization agent used for UV/vis or fluorescent detection. The reaction requires a source of SH- groups; mercaptoethanol is sufficient for that purpose. For chiral separations are easily employed optically pure compounds, such as isobutyryl-L-cysteine or *N*-acetyl-L-cysteine, as the SH-group donors.

For the in-capillary derivatization the sample and derivatization reagent are injected separately, than the electric field is applied to mix the two countermoving zones, and lastly the

electrophoretic separation in higher voltage is performed. Scheme of the on-capillary derivatization and subsequent separation are shown in Fig. 2 of the publication.

The major advantage of in-capillary derivatization is that it circumvents the systematic error originating in the low stability of OPA derivates.

2.4.2 Method Application and Validation

The relative standard deviation (RSD) of the method was around 5% for both, interday and intraday analyses. When we applied the CE-based method and the previously described and more laborious HPLC-based method on the same samples, we observed comparable results. The method was thus successfully applied on inhibition screening of the large group of compounds.

The disadvantage of the method lies in the derivatization agent. We observed that not only the derivates but also the OPA/mercaptoethanol solution was unstable and had to be often replaced.

The sensitivity of the method was in low μM range, which was sufficient for the inhibition analysis; it could be significantly enlarged when coupled to laser induced fluorescence (LIF) detector.

My contribution to the publication

I prepared the activity reactions, performed the HPLC measurements and the application part, and contributed to the text.

2.5 Publication III

In continued effort to identify or develop potent SR inhibitors we identified hydroxamic acids as novel family of potent inhibitors by random screening of various analogues of known mSR substrates and inhibitors we identified hydroxamic acids as novel family of promising potent inhibitors. However, our results from the mechanistic analyses were unexpected and rather puzzling; for example, the dihydroxamic acid analogue of malonate, a competitive SR inhibitor, behaved as noncompetitive one. We analyzed the mechanism in detail. We observed interaction of several dihydroxamic acids with PLP in solution, identified the products of the reaction using mass spectrometry, ^{1}H-NMR, and UV/vis spectroscopy, and showed that some dihydroxamic acids nonspecifically inhibit several PLP-dependent enzymes by PLP-sequestration. Since hydroxamic acid moieties often appear as a part of inhibitors of other molecular targets, some of them even in clinical use, we found our findings very important for medicinal chemists.

Moreover, among the tested hydroxamic acids we did identify one SR-selective, potent, and competitive inhibitor: L-aspartic acid β-hydroxamate.

2.5.1 SR Inhibition by Hydroxamic Acids

Because we observed that hydroxamic acid moiety could improve affinity of the compound towards SR, we screened several other hydroxamic acid analogs. Surprisingly the glycine and serine analogs did not inhibit SR at all (Fig. 1 in the paper). On the other hand the malonic, succinic and glutaric analogs behaved as very potent SR inhibitors. Due to the reputation of hydroxamic acids as potent metal chelators, we first checked that the mechanism of action was not chelation of the functionally important Mg^{2+} ion. However, the inhibition of SR by these compounds was not diminished even with a large molar excess of $MgCl_2$ over the test compound, excluding metal chelation as the mechanism of action of these compounds.

We revealed that the mechanism of inhibition of these compounds is rather complex. The inhibition by malonodihydroxamic acid fitted best to a non-competitive model, while succinodihydroxamic acid and L-aspartic acid β-hydroxamate behaved as competitive inhibitors with K_i values of 3.6 and 97μM, respectively.

2.5.2 Interaction of Hydroxamic Acids and PLP

Our inhibitor specificity test with a panel of several PLP-enzymes revealed that the succino and malonodihydroxamic acids are highly nonspecific inhibiting all the enzymes, while L-aspartic acid β-hydroxamate inhibited mouse and human SRs and the homologous (20% sequence identity) serine dehydratase (SDH). These observations motivated us to investigate the reactivity of these compounds with PLP. Hydroxamic acids are derived of hydroxylamine, known to interact with the reactive PLP aldehyde moiety. Hydroxylamine is a nonspecific inhibitor of PLP-dependent enzymes through PLP-sequestration. Because the tested hydroxamic acids are re-crystalized from water we know that they do not release hydroxylamine in water. The obvious experiments to follow were the analysis of potential interaction of PLP with the hydryxamic acids. We employed spectroscopy and observed the same spectral shift (390 to 335nm) when PLP was mixed with hydroxylamine, succino-, or malonodihydroxamic acid. We analyzed the product of interaction of these compounds with PLP using ^{1}H-NMR and mass spectroscopy. Both methods revealed PLP-aldoxime product formed also upon PLP and hydroxylamine reaction. Detailed 1H-NMR analysis showed that two isomers of the PLP-aldoxime are formed. The structures of the aldoximes are shown in the Scheme 1 of the publication.

2.5.3 L-Aspartic Acid β-hydroxamate

L-Asp β-hydroxamate is a competitive, SDH and SR specific inhibitor. According to MS and ^{1}H-NMR analyses it does not form the PLP-aldoxime. However, in the visible spectra of PLP and the inhibitor appeared the 335nm signal similarly like with the dihydroxamic acids. The ^{1}H-NMR analysis ultimately revealed that it interacts with PLP in a different way than the non-specific dihydroxamic acids; it forms in solution the external aldimine corresponding to product of interaction of PLP with amino acids. L-Asp β-hydroxamate is SR inhibitior with increased potency over L-aspartic acid, which is only a moderate inhibitor with affinity comparable to LS. The specificity of L-asp β-hydroxamate is however lower than the specificity of malonate (Fig. 3 in the publication).

My contribution to the publication

I participated in the initial inhibitor screening and mass spectrometry study, performed the analysis of mechanism of inhibition, and contributed to the text.

2.6 Publication IV

The motivation for the review on mammalian SR inhibition was to summarize the current knowledge on SR inhibition and to include some of our new unpublished observations in order to provide a comprehensive guide to the expert readership as well as a starting guide to the newcomers in the field of serine racemase inhibition. Our long lasting experience with recombinant human and mouse, SR orthologs, precise activity assays, and systematic search for SR inhibitors allowed us to prepare such an overview.

The publication provides summary of the methodology; it describes the inhibition of PLP-dependent enzymes in general; it summarized known SR inhibitors; and it introduces new inhibitory compounds.

During our work on the text a 3D structure of human and rat SR was released [99]. We decided to have a close look at the ATP binding site (Fig.8) and to provide insight into the interaction of the most potent SR inhibitor L-EHA within the hSR active site (Fig. 9).

2.6.1 ATP Binding Site

SR uses ATP as an allosteric activator. One of the yeast SR structures (PDB code 1WTC) contains SR with bound non-hydrolyzable ATP analogue AMP-PCP [84]. The authors suggested that ATP binding site lies at the putative dimarization interface. Baumgart *et al.*, who later modeled SR dimer with ATP, support the hypothesis [122].

Only recently, rat SR crystallized with a dimer in the asymmetric unit [99]. The dimer resembles that shown in the model by Baumgart *et al.* However, neither the human nor the rat SR crystal structures contain an ATP analog. We therefore docked an ATP molecule with a coordinated Mg^{2+} ion into the human SR structure, using the yeast SR structure containing AMP-PCP as a template (Fig. 4 of the Publication IV).

The docking reveals the amino acid residues most probably participating in the ATP binding (Fig. 8). Two ATP molecules are bound per SR dimer with their phosphate groups pointing into the dimerization interface and the pyrimidine moieties facing out to the solution. The presence of H-bond interactions of the γ-phosphate is in accord with the experimental

observations that SR does not hydrolyze the phosphates. The binding pocket of ATP resembles that of other proteins accommodating ATP [178]. Interestingly, the monomeric unit alone does not offer sufficient number of interactions; only the dimeric SR forms enough interactions to accommodate ATP/Mg^{2+} complex.

We observed that C-terminus of the complementary monomeric unit is close to the ATP molecule (within 11 Å). The structures of SR, however, do not contain the last 11 C-terminal residues; they are too flexible to be observed in the diffraction pattern. The C-terminus, thus, may be even closer to interact with the ATP. This may be important in terms of SR regulation *in vivo*, because, as shown before, the last 4 C-terminal residues mediate the interactions with the protein partners PICK1 and GRIP (Chapter 1.2.5).

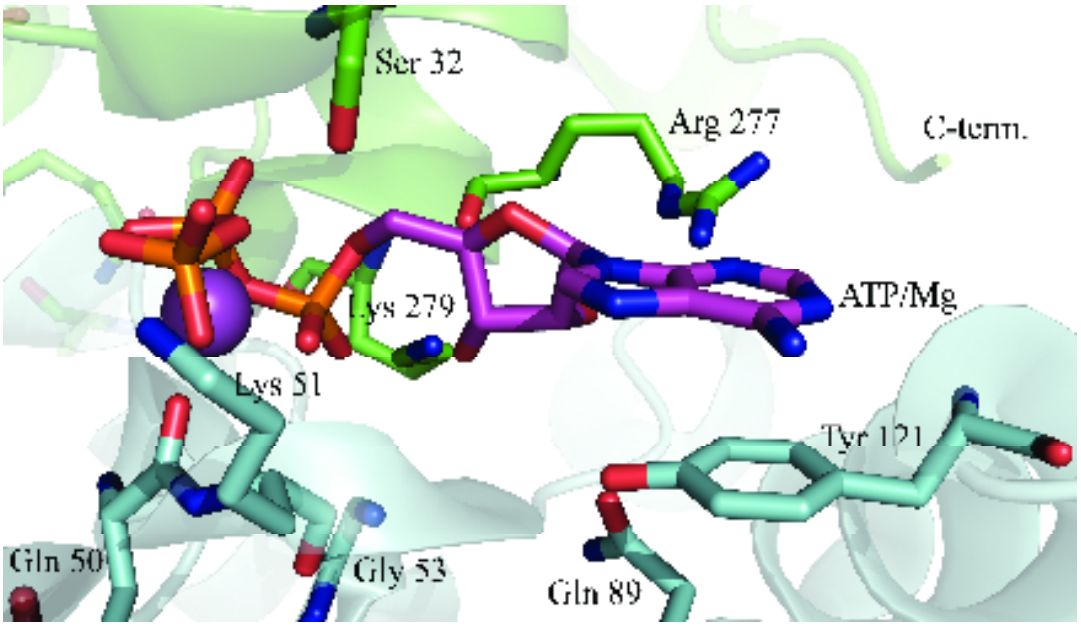


Figure 8 ATP / Mg^{2+} Pocket

ATP/Mg^{2+} complex (magenta) docked into the hSR (PDB code 3L6B) ATP site between the two monomeric units (one in cyan and one in green). Tyr 121, Gln 89, and residues 50-53 belong to one monomeric unit, while Ser 32, Arg 277, and Lys 279 are from the complementary unit. The C-terminus of the complementary unit is marked. In the left upper part of the picture can be noticed some residues of the second ATP-site.

2.6.2 L-EHA in the Active Site

From the docking experiments we can see that the most potent inhibitor, L-EHA, binds with 8 H-bonds. It uses all its functional moieties to anchor in the active site. The molecule of L-EHA fills almost whole active site pocket (Fig. 9).

In the case of analogous inhibitor L-Asp β-hydroxamate, the H-bond to Asn 154 is lost and the H-bonds to Arg 135 and Gly 239 are bifurcated. The orientation of L-THA, SR substrate (Table 2), in the active site is different than that of L-EHA, so that it also lacks the interaction with Asn 154, and it shares an additional H-bond with the PLP phosphate bifurcated between the α-amino and β-hydroxy groups. For comparison, the dicarboxylic inhibitor malonate found in the crystal structure uses the same active site residues, except Asn 154 and Gly 239. From all the docked inhibitors Asn 154 interacts only with L-EHA. Gly 239 forms an H-bond with the α-amino group of the amino acid ligands.

We can speculate that interaction with Asn 154 is responsible for the highest observed affinity of L-EHA. In summary, the docking experiments alone, however, do not explain the different affinity of the compounds.

2.6.3 Serine Racemase Inhibition Summary

Being a PLP-dependent enzyme, serine racemase is a difficult target for inhibition. This fact is usually reflected in low inhibitor specificity. By now, several competitive inhibitors have been identified. Most of the successful inhibitors are small charged molecules, but also several rather bulky tripeptidic competitive inhibitors have been identified. The newly released SR structure indicates that charged molecules with many H-bond donors and acceptors can bind in the active site with high affinity. The highest affinity inhibitors identified to date belong to the family of dicarboxylic acids, with malonic acid and L-EHA being the most potent SR inhibitors published so far. Both have been successfully used in SR research, malonate as an inhibitor assisting human and rat SR crystallization [99] and L-EHA as an inhibitor in a neurophysiologic study of the role of SR [177]. The current known competitive SR inhibitors, along with their respective K_i values, are summarized in Table 6 of the Publication IV.

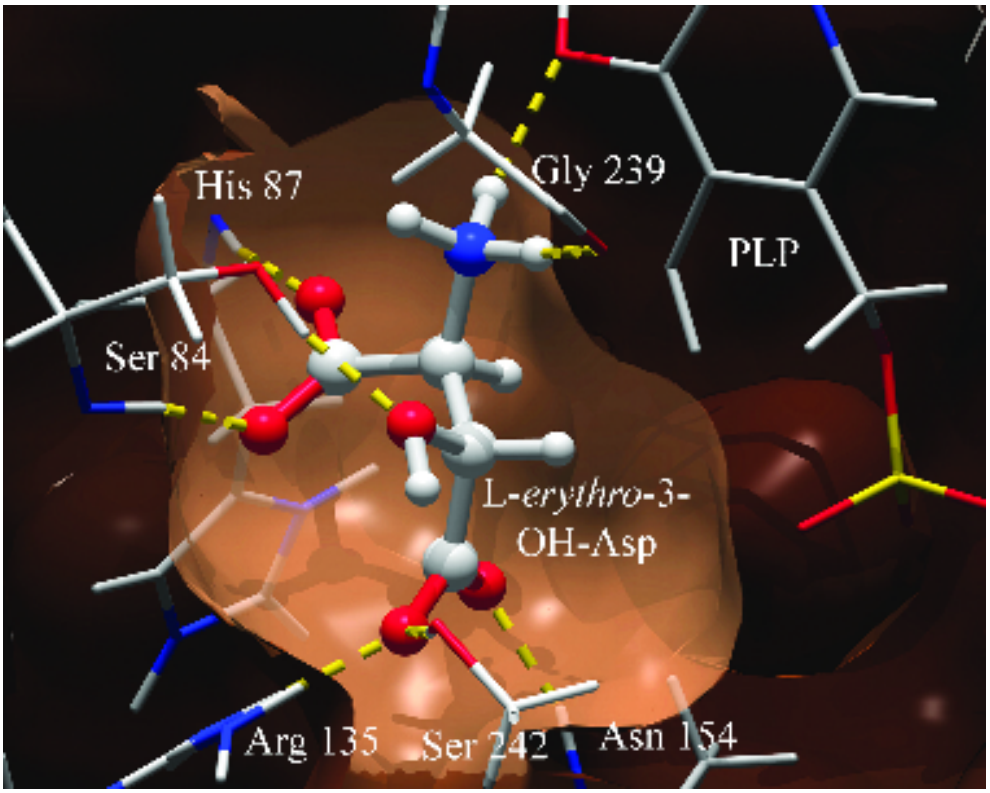


Figure 9 L-EHA in the Active Site

L-EHA docked into the active site of human serine racemase (PDB code 3L6B). The active site pocket is in light brown color. All polar atoms in the inhibitor are involved in hydrogen bonding interactions (yellow dashed lines) with the active site residues and PLP. In addition to the backbone NH groups of Asn154 and His87 and the oxygen of Gly239, the hydrogen bond network involves the side chains of Ser84, Ser242, and Arg135. The picture was taken from the Fig. 5 in the Publication IV.

My contribution to the publication

I participated in the inhibitor screening, structure analysis of the 3D models, and wrote the publication.

Chapter 3 Summary

Serine racemase is a biochemically and pharmaceutically interesting enzyme. It is the first amino acid racemase described in mammals. In human it is abundant throughout the whole body. The role of serine racemase has been largely attributed to D-serine production to co-activate NMDA receptors, the key excitatory glutamate receptors. It has been discussed that serine racemase can participate in DS homeostasis not only by its production but also by its degradation to pyruvate and ammonia. The function of SR in non-neuronal tissues is unknown. The low level of substrate specificity of SR indicates that SR could have other functions too.

The isolation and characterization of mammalian SR opened intensive research of D-serine and its function in NMDA receptor signaling, opening a new space in the field of NMDA recptor physiology and pathology. Neurochemists and physiologists have discovered various DS-mediated effects. DS, together with SR, brings another way to follow in the treatment of the numerous NMDA receptor-connected diseases.

We have been interested in serine racemase and DS from the biochemical point of view. As the first thing we studied recombinant mouse SR in detail (Publication I). We characterized its kinetic parameters, substrate specificity, and inhibition profile using small amino and carboxylic acids. After successful identification of several potent and competitive mSR inhibitors, such as malonic acid and L-EHA, we developed a less time consuming serine separation technique employing capillary electrophoresis to continue with further inhibitor screening (Publication II). The method was used to test inhibition of selected inhibitor hits from a collaborating pharmaceutical company, Guilford Pharmaceuticals, Inc. (Baltimore, USA).

Our second goal was to characterize the 3D structure of the enzyme. Since we were able to prepare a sufficient amount of soluble, active, concentrated and highly pure preparation, we hoped that the crystal structure would be easy to obtain. However, serine racemase failed to crystallize in more than 300 tested conditions. We, therefore, moved a step back to search for better inhibitors. Among the many ligand analogs tested we found small dihydroxamic acids as potent SR inhibitors. Not only did the addition of these compounds not help crystallization, but they turned out to be nonspecific, PLP-interacting compounds. We mapped their mechanism of interaction with PLP and analyzed in detail the specificity of these compounds (Publication III). One of the studied hydroxamic acid analogs, L-aspartic acid β-hydroxamate, behaved as SR-selective competitive inhibitor.

We further enlarged the palette of our serine racemase preparations by purifying a full-length recombinant wild type human ortholog and many rationally as well as randomly prepared mutants [102, 111]. Unfortunately, despite all the efforts, the only crystallization result in our hands was insufficiently diffracting crystals containing inactive protein. Nevertheless, the experimental work revealed new features of mammalian SRs. For example, with the random mutagenesis strategy, we identified residues Cys 217 and Lys 222, adjacent to the cation binding site, as crucial for SR activity [111].

Through screening of various compounds for their SR inhibition, we identified dicarboxylic and tricarboxylic acid inhibitors, revealed moieties that are important for the inhibitor to bind, and summarized our results and results by others in an invited review for *Current Drug Targets* (Publication IV).

An important milestone in the serine racemase field was recently achieved by the company Evotec (Oxon, UK). They succeeded in serine racemase crystallization and solved the human and rat SR 3D structures. Since we have long awaited the release of a mammalian serine racemase structure, we are now prepared to use the structure as part of a rational search for SR inhibitors. We already employed the hSR structure (PDB code 3L6B) in docking experiments to map the APT binding site and the interactions of the most potent inhibitor L-EHA within the active site (Publication IV).

References

[1] Pasteur L. Researches on the molecular asymmetry of natural organic products. Edinburgh: The Alembic Club ; Chicago : University of Chicago Press; 1906.
[2] Sanger F, Thompson EO. The amino-acid sequence in the glycyl chain of insulin. Biochem J. 1952 Sep;52(1):iii.
[3] Sanger F. The arrangement of amino acids in proteins. Adv Protein Chem. 1952;7:1-67.
[4] Edman P, Hammarsten E, et al. Partition chromatographic separation of adenine and guanine. J Biol Chem. 1949 Mar;178(1):395-8.
[5] Plankensteiner K, Reiner H, Rode BM. From earth's primitive atmosphere to chiral peptides--the origin of precursors for life. Chem Biodivers. 2004 Sep;1(9):1308-15.
[6] McBride JM, Tully JC. Physical chemistry: did life grind to a start? Nature. 2008 Mar 13;452(7184):161-2.
[7] Noorduin WL, Izumi T, Millemaggi A, Leeman M, Meekes H, Van Enckevort WJ, Kellogg RM, Kaptein B, Vlieg E, Blackmond DG. Emergence of a single solid chiral state from a nearly racemic amino acid derivative. J Am Chem Soc. 2008 Jan 30;130(4):1158-9.
[8] Heck SD, Faraci WS, Kelbaugh PR, Saccomano NA, Thadeio PF, Volkmann RA. Posttranslational amino acid epimerization: enzyme-catalyzed isomerization of amino acid residues in peptide chains. Proc Natl Acad Sci U S A. 1996 Apr 30;93(9):4036-9.
[9] Kreil G. D-amino acids in animal peptides. Annu Rev Biochem. 1997;66:337-45.
[10] Fujii N. D-amino acid in elderly tissues. Biol Pharm Bull. 2005 Sep;28(9):1585-9.
[11] Waite ER, Collins MJ, Ritz-Timme S, Schutz HW, Cattaneo C, Borrman HI. A review of the methodological aspects of aspartic acid racemization analysis for use in forensic science. Forensic Sci Int. 1999 Jul 26;103(2):113-24.
[12] Krebs HA. Metabolism of amino-acids: Deamination of amino-acids. Biochem J. 1935 Jul;29(7):1620-44.
[13] 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 Nov 26;141(1):27-32.
[14] Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T, Takahashi K. The presence of free D-serine in rat brain. FEBS Lett. 1992 Jan 13;296(1):33-6.
[15] Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T, Takashima S, Doi N, Mizutani Y, Yamazaki T, et al. Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. J Neurochem. 1993 Jul;61(1):348-51.
[16] Hamase K, Homma H, Takigawa Y, Fukushima T, Santa T, Imai K. Regional distribution and postnatal changes of D-amino acids in rat brain. Biochim Biophys Acta. 1997 Mar 15;1334(2-3):214-22.
[17] 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 Jul;85(3):168-80.
[18] Wroblewski JT, Fadda E, Mazzetta J, Lazarewicz JW, Costa E. 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. 1989 May;28(5):447-52.
[19] Siegel G, editor. Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th ed. Philadelphia: Lippincot Williams and Wilkins; 1999.
[20] 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 Jul;65(1):454-8.
[21] 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 Mar;31(5):808-16.
[22] Mothet JP, Parent AT, Wolosker H, Brady RO, Jr., Linden DJ, Ferris CD, Rogawski MA, Snyder SH. 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 Apr 25;97(9):4926-31.
[23] Miller RF. D-Serine as a glial modulator of nerve cells. Glia. 2004 Aug 15;47(3):275-83.
[24] Betz H, Kuhse J, Fischer M, Schmieden V, Laube B, Kuryatov A, Langosch D, Meyer G, Bormann J, Rundstrom N, et al. Structure, diversity and synaptic localization of inhibitory glycine receptors. J Physiol Paris. 1994;88(4):243-8.
[25] Tsuzuki H, Tanaka S, Maekawa M, Hori Y. Behavioral Characteristics and the Expression of Nmda Receptor Subunit in the Serine Racemase Knockout Mice. Journal of Physiological Sciences. 2009;59:517-.

[26] Oliet S. Glial cells and excitatory neurotransmission. International Congress on Amino Acids, Peptides and Proteins. Vienna, Austria2009.
[27] Baranano DE, Ferris CD, Snyder SH. Atypical neural messengers. Trends Neurosci. 2001 Feb;24(2):99-106.
[28] Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C, Poo M, Duan S. Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. Proc Natl Acad Sci U S A. 2003 Dec 9;100(25):15194-9.
[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] Danysz W, Parsons CG. Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol Rev. 1998 Dec;50(4):597-664.
[31] Gardoni F, Di Luca M. New targets for pharmacological intervention in the glutamatergic synapse. Eur J Pharmacol. 2006 Sep 1;545(1):2-10.
[32] Parsons C. http://www.chrisparsons.de/Chris/nmda.htm.
[33] Gao X, Xu X, Pang J, Zhang C, Ding JM, Peng X, Liu Y, Cao JM. NMDA receptor activation induces mitochondrial dysfunction, oxidative stress and apoptosis in cultured neonatal rat cardiomyocytes. Physiol Res. 2007;56(5):559-69.
[34] Ma MC, Huang HS, Chen YS, Lee SH. Mechanosensitive N-methyl-D-aspartate receptors contribute to sensory activation in the rat renal pelvis. Hypertension. 2008 Nov;52(5):938-44.
[35] Omori T, Mihara H, Kurihara T, Esaki N. Occurrence of phosphatidyl-D-serine in the rat cerebrum. Biochem Biophys Res Commun. 2009 May 1;382(2):415-8.
[36] Inoue R, Hashimoto K, Haraj T, Mori H. NMDA-and beta-Amyloid(1-42) -Induced Neurutoxicity Is Attenuated in Serine Racemase Knock-Out Mice (December, pg 14486, 2008). Journal of Neuroscience. 2009 Jan 21;29(3):-.
[37] Basu AC, Tsai GE, Ma CL, Ehmsen JT, Mustafa AK, Han L, Jiang ZI, Benneyworth MA, Froimowitz MP, Lange N, Snyder SH, Bergeron R, Coyle JT. Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. Mol Psychiatry. 2009 Jul;14(7):719-27.
[38] Mustafa AK, Ahmad AS, Zeynalov E, Gazi SK, Sikka G, Ehmsen JT, Barrow RK, Coyle JT, Snyder SH, Dore S. Serine racemase deletion protects against cerebral ischemia and excitotoxicity. J Neurosci. 2010 Jan 27;30(4):1413-6.
[39] Miya K, Inoue R, Takata Y, Abe M, Natsume R, Sakimura K, Hongou K, Miyawaki T, Mori H. Serine racemase is predominantly localized in neurons in mouse brain. J Comp Neurol. 2008 Oct 20;510(6):641-54.
[40] Bauer D, Hamacher K, Broer S, Pauleit D, Palm C, Zilles K, Coenen HH, Langen KJ. Preferred stereoselective brain uptake of d-serine--a modulator of glutamatergic neurotransmission. Nucl Med Biol. 2005 Nov;32(8):793-7.
[41] Goodnough DB, Lutz MP, Wood PL. Separation and quantification of D- and L-phosphoserine in rat brain using N alpha-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) by high-performance liquid chromatography with ultraviolet detection. J Chromatogr B Biomed Appl. 1995 Oct 20;672(2):290-4.
[42] 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 Sep;69(3):1286-90.
[43] Dunlop DS, Neidle A. The origin and turnover of D-serine in brain. Biochem Biophys Res Commun. 1997 Jun 9;235(1):26-30.
[44] Iwama H, Takahashi K, Kure S, Hayashi F, Narisawa K, Tada K, Mizoguchi M, Takashima S, Tomita U, Nishikawa T. Depletion of cerebral D-serine in non-ketotic hyperglycinemia: possible involvement of glycine cleavage system in control of endogenous D-serine. Biochem Biophys Res Commun. 1997 Feb 24;231(3):793-6.
[45] Wolosker H, Sheth KN, Takahashi M, Mothet JP, Brady RO, Jr., Ferris CD, Snyder SH. Purification of serine racemase: biosynthesis of the neuromodulator D-serine. Proc Natl Acad Sci U S A. 1999 Jan 19;96(2):721-5.
[46] Halassa MM, Fellin T, Haydon PG. The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol Med. 2007 Feb;13(2):54-63.
[47] Allen NJ, Barres BA. Neuroscience: Glia - more than just brain glue. Nature. 2009 Feb 5;457(7230):675-7.
[48] Darra E, Ebner FH, Shoji K, Suzuki H, Mariotto S. Dual cross-talk between nitric oxide and D-serine in astrocytes and neurons in the brain. Cent Nerv Syst Agents Med Chem. 2009 Dec;9(4):289-94.
[49] Halassa MM, Fellin T, Haydon PG. Tripartite synapses: roles for astrocytic purines in the control of synaptic physiology and behavior. Neuropharmacology. 2009 Sep;57(4):343-6.
[50] Faissner A, Pyka M, Geissler M, Sobik T, Frischknecht R, Gundelfinger ED, Seidenbecher C. Contributions of astrocytes to synapse formation and maturation - Potential functions of the perisynaptic extracellular matrix. Brain Res Rev. 2010 May;63(1-2):26-38.
[51] Dodla MC, Mumaw J, Stice SL. Role of Astrocytes, Soluble Factors, Cells Adhesion Molecules and Neurotrophins in Functional Synapse Formation: Implications for Human Embryonic Stem Cell Derived Neurons. Curr Stem Cell Res Ther. 2010 Mar 8.

[52] Mothet JP, Pollegioni L, Ouanounou G, Martineau M, Fossier P, Baux G. Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter D-serine. Proc Natl Acad Sci U S A. 2005 Apr 12;102(15):5606-11.
[53] Martineau M, Galli T, Baux G, Mothet JP. Confocal imaging and tracking of the exocytotic routes for D-serine-mediated gliotransmission. Glia. 2008 Sep;56(12):1271-84.
[54] Yamamoto T, Nishizaki I, Nukada T, Kamegaya E, Furuya S, Hirabayashi Y, Ikeda K, Hata H, Kobayashi H, Sora I, Yamamoto H. Functional identification of ASCT1 neutral amino acid transporter as the predominant system for the uptake of L-serine in rat neurons in primary culture. Neuroscience Research. 2004 May;49(1):101-11.
[55] Ribeiro CS, Reis M, Panizzutti R, de Miranda J, Wolosker H. Glial transport of the neuromodulator D-serine. Brain Res. 2002 Mar 8;929(2):202-9.
[56] Shao Z, Kamboj A, Anderson CM. Functional and immunocytochemical characterization of D-serine transporters in cortical neuron and astrocyte cultures. J Neurosci Res. 2009 Aug 15;87(11):2520-30.
[57] Sikka P, Walker R, Cockayne R, Wood MJ, Harrison PJ, Burnet PW. D-Serine metabolism in C6 glioma cells: Involvement of alanine-serine-cysteine transporter (ASCT2) and serine racemase (SRR) but not D-amino acid oxidase (DAO). J Neurosci Res. 2010 Jun;88(8):1829-40.
[58] Molla G, Sacchi S, Bernasconi M, Pilone MS, Fukui K, Polegioni L. Characterization of human D-amino acid oxidase. FEBS Lett. 2006 Apr 17;580(9):2358-64.
[59] Wang LZ, Zhu XZ. Spatiotemporal relationships among D-serine, serine racemase, and D-amino acid oxidase during mouse postnatal development. Acta Pharmacol Sin. 2003 Oct;24(10):965-74.
[60] 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 FP, Maurice K, Essioux L, Millasseau P, Grel P, Debailleul V, Simon AM, Caterina D, Dufaure I, Malekzadeh K, Belova M, Luan JJ, Bouillot M, Sambucy JL, 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 PC, Straub RE, Weinberger DR, Cohen N, Cohen D. 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 Oct 15;99(21):13675-80.
[61] Madeira C, Freitas ME, Vargas-Lopes C, Wolosker H, Panizzutti R. Increased brain D-amino acid oxidase (DAAO) activity in schizophrenia. Schizophr Res. 2008 Apr;101(1-3):76-83.
[62] Habl G, Zink M, Petroianu G, Bauer M, Schneider-Axmann T, von Wilmsdorff M, Falkai P, Henn FA, Schmitt A. Increased D: -amino acid oxidase expression in the bilateral hippocampal CA4 of schizophrenic patients: a post-mortem study. J Neural Transm. 2009 Oct 13.
[63] Verrall L, Walker M, Rawlings N, Benzel I, Kew JN, Harrison PJ, Burnet PW. d-Amino acid oxidase and serine racemase in human brain: normal distribution and altered expression in schizophrenia. Eur J Neurosci. 2007 Sep;26(6):1657-69.
[64] 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. Identification of multiple serine racemase (SRR) mRNA isoforms and genetic analyses of SRR and DAO in schizophrenia and D-serine levels. Biol Psychiatry. 2005 Jun 15;57(12):1493-503.
[65] Habl G, Zink M, Petroianu G, Bauer M, Schneider-Axmann T, von Wilmsdorff M, Falkai P, Henn FA, Schmitt A. Increased D-amino acid oxidase expression in the bilateral hippocampal CA4 of schizophrenic patients: a post-mortem study. J Neural Transm. 2009 Dec;116(12):1657-65.
[66] Almond SL, Fradley RL, Armstrong EJ, Heavens RB, Rutter AR, Newman RJ, Chiu CS, Konno R, Hutson PH, Brandon NJ. Behavioral and biochemical characterization of a mutant mouse strain lacking D-amino acid oxidase activity and its implications for schizophrenia. Mol Cell Neurosci. 2006 Aug;32(4):324-34.
[67] Labrie V, Wang W, Barger SW, Baker GB, Roder JC. Genetic loss of D-amino acid oxidase activity reverses schizophrenia-like phenotypes in mice. Genes Brain Behav. 2009 Aug 5.
[68] Strisovsky K, Jiraskova J, Barinka C, Majer P, Rojas C, Slusher BS, Konvalinka J. Mouse brain serine racemase catalyzes specific elimination of L-serine to pyruvate. FEBS Lett. 2003 Jan 30;535(1-3):44-8.
[69] Foltyn VN, Bendikov I, De Miranda J, Panizzutti R, Dumin E, Shleper M, Li P, Toney MD, Kartvelishvily E, Wolosker H. Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity. J Biol Chem. 2005 Jan 21;280(3):1754-63.
[70] Baumgart F, Rodriguez-Crespo I. D-amino acids in the brain: the biochemistry of brain serine racemase. FEBS J. 2008 Jul;275(14):3538-45.

[71] Zhao S, Yuan H, Xiao D. Detection of d-Serine in rat brain by capillary electrophoresis with laser induced fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2005 Aug 5;822(1-2):334-8.
[72] Koval D, Jiraskova J, Strisovsky K, Konvalinka J, Kasicka V. Capillary electrophoresis method for determination of D-serine and its application for monitoring of serine racemase activity. Electrophoresis. 2006 Jul;27(13):2558-66.
[73] Bruckner H, Westhauser T, Godel H. Liquid-Chromatographic Determination of D-Amino-Acids and L-Amino-Acids by Derivatization with O-Phthaldialdehyde and N-Isobutyryl-L-Cysteine - Applications with Reference to the Analysis of Peptidic Antibiotics, Toxins, Drugs and Pharmaceutically Used Amino-Acids. Journal of Chromatography A. 1995 Sep 8;711(1):201-15.
[74] Goodnough DB, Lutz MP, Wood PL. Rapid and Sensitive Procedure for the Separation and Quantitation of D-Serine and L-Serine in Rat-Brain Using Gas-Chromatography Mass-Spectrometry. Journal of Chromatography B-Biomedical Applications. 1995 May 19;667(2):223-32.
[75] Patzold R, Schieber A, Bruckner H. Gas-chromatographic quantification of free D-amino acids in higher vertebrates. Biomedical Chromatography. 2005 Jul;19(6):466-73.
[76] Bhushan R, Bruckner H. Marfey's reagent for chiral amino acid analysis: A review. Amino Acids. 2004 Dec;27(3-4):231-47.
[77] Fuchs SA, de Sain-van der Velden MGMD, de Barse MMJ, Roeleveld MW, 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. Clinical Chemistry. 2008 Sep;54(9):1443-50.
[78] Grant SL, Shulman Y, Tibbo P, Hampson DR, Baker GB. Determination of d-serine and related neuroactive amino acids in human plasma by high-performance liquid chromatography with fluorimetric detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2006 Dec 5;844(2):278-82.
[79] Schomburg I, Chang A, Schomburg D. BRENDA, enzyme data and metabolic information. Nucleic Acids Res. 2002 Jan 1;30(1):47-9.
[80] http://www.ncbi.nlm.nih.gov/protein.
[81] Shikata Y, Watanabe T, Teramoto T, Inoue A, Kawakami Y, Nishizawa Y, Katayama K, Kuwada M. Isolation and Characterization of a Peptide Isomerase from Funnel-Web Spider Venom. Journal of Biological Chemistry. 1995 Jul 14;270(28):16719-23.
[82] Arias CA, Weisner J, Blackburn JM, Reynolds PE. Serine and alanine racemase activities of VanT: a protein necessary for vancomycin resistance in Enterococcus gallinarum BM4174. Microbiology. 2000 Jul;146 (Pt 7):1727-34.
[83] Ohnishi M, Saito M, Wakabayashi S, Ishizuka M, Nishimura K, Nagata Y, Kasai S. Purification and characterization of serine racemase from a hyperthermophilic archaeon, Pyrobaculum islandicum. J Bacteriol. 2008 Feb;190(4):1359-65.
[84] Goto M, Yamauchi T, Kamiya N, Miyahara I, Yoshimura T, Mihara H, Kurihara T, Hirotsu K, Esaki N. Crystal structure of a homolog of mammalian serine racemase from Schizosaccharomyces pombe. J Biol Chem. 2009 Sep 18;284(38):25944-52.
[85] Wada M, Nakamori S, Takagi H. Serine racemase homologue of Saccharomyces cerevisiae has L-threo-3-hydroxyaspartate dehydratase activity. FEMS Microbiol Lett. 2003 Aug 29;225(2):189-93.
[86] Gogami Y, Ito K, Kamitani Y, Matsushima Y, Oikawa T. Occurrence of D-serine in rice and characterization of rice serine racemase. Phytochemistry. 2009 Feb;70(3):380-7.
[87] Fujitani Y, Horiuchi T, Ito K, Sugimoto M. Serine racemases from barley, Hordeum vulgare L., and other plant species represent a distinct eukaryotic group: gene cloning and recombinant protein characterization. Phytochemistry. 2007 Jun;68(11):1530-6.
[88] Fujitani Y, Nakajima N, Ishihara K, Oikawa T, Ito K, Sugimoto M. Molecular and biochemical characterization of a serine racemase from Arabidopsis thaliana. Phytochemistry. 2006 Apr;67(7):668-74.
[89] Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR, Miller RF. 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.
[90] Xia M, Liu Y, Figueroa DJ, Chiu CS, Wei N, Lawlor AM, Lu P, Sur C, Koblan KS, Connolly TM. Characterization and localization of a human serine racemase. Brain Res Mol Brain Res. 2004 Jun 18;125(1-2):96-104.
[91] Mehta PK, Christen P. The molecular evolution of pyridoxal-5'-phosphate-dependent enzymes. Adv Enzymol Relat Areas Mol Biol. 2000;74:129-84.
[92] Eliot AC, Kirsch JF. Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. Annu Rev Biochem. 2004;73:383-415.

[93] Dunathan HC. Conformation and reaction specificity in pyridoxal phosphate enzymes. Proc Natl Acad Sci U S A. 1966 Apr;55(4):712-6.
[94] Toney MD. Reaction specificity in pyridoxal phosphate enzymes. Arch Biochem Biophys. 2005 Jan 1;433(1):279-87.
[95] Bunik VI, Schloss JV, Pinto JT, Gibson GE, Cooper AJ. Enzyme-catalyzed side reactions with molecular oxygen may contribute to cell signaling and neurodegenerative diseases. Neurochem Res. 2007 Apr-May;32(4-5):871-91.
[96] Battaglioli G, Liu H, Martin DL. Kinetic differences between the isoforms of glutamate decarboxylase: implications for the regulation of GABA synthesis. J Neurochem. 2003 Aug;86(4):879-87.
[97] Copley SD. Enzymes with extra talents: moonlighting functions and catalytic promiscuity. Curr Opin Chem Biol. 2003 Apr;7(2):265-72.
[98] 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 Oct 4;44(39):13091-100.
[99] Smith MA, Mack V, Ebneth A, Moraes I, Felicetti B, Wood M, Schonfeld D, Mather O, Cesura A, Barker J. The structure of mamalian serine racemase: Evidence for conformational changes upon inhibitor binding. J Biol Chem. 2010 Jan 27.
[100] Murakami T, Maeda T, Yokota A, Wada M. Gene cloning and expression of pyridoxal 5'-phosphate-dependent L-threo-3-hydroxyaspartate dehydratase from Pseudomonas sp. T62, and characterization of the recombinant enzyme. J Biochem. 2009 May;145(5):661-8.
[101] Yamauchi T, Goto M, Wu HY, Uo T, Yoshimura T, Mihara H, Kurihara T, Miyahara I, Hirotsu K, Esaki N. Serine racemase with catalytically active lysinoalanyl residue. J Biochem. 2009 Apr;145(4):421-4.
[102] 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 Jan;63(1):62-7.
[103] Dunlop DS, Neidle A. Regulation of serine racemase activity by amino acids. Brain Res Mol Brain Res. 2005 Feb 18;133(2):208-14.
[104] Wu S, Barger SW, Sims TJ. Schwann cell and epineural fibroblast expression of serine racemase. Brain Res. 2004 Sep 10;1020(1-2):161-6.
[105] Zanatta A, Schuck PF, Viegas CM, Knebel LA, Busanello EN, Moura AP, Wajner M. In vitro evidence that D-serine disturbs the citric acid cycle through inhibition of citrate synthase activity in rat cerebral cortex. Brain Res. 2009 Nov 17;1298:186-93.
[106] Jirásková J, Ettrich R, Vorlová B, Hoffman HE, Lepšík M, Jansa P, Konvalinka J. Inhibition of Human Serine Racemase, an Emerging Target for Medicinal Chemistry. 2010.
[107] Panizzutti R, De Miranda J, Ribeiro CS, Engelender S, Wolosker H. 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. 2001 Apr 24;98(9):5294-9.
[108] 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 Nov 9;96(23):13409-14.
[109] Zhao H, Hamase K, Morikawa A, Qiu Z, Zaitsu K. Determination of d- and l-enantiomers of threonine and allo-threonine in mammals using two-step high-performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2004 Oct 25;810(2):245-50.
[110] Cook SP, Galve-Roperh I, Martinez del Pozo A, Rodriguez-Crespo I. Direct calcium binding results in activation of brain serine racemase. J Biol Chem. 2002 Aug 2;277(31):27782-92.
[111] Hoffman H, Jiraskova J, Zvelebil M, Konvalinka J. Random mutagenesis of human serine racemase reveals residues important for the enzymatic activity. Collect Czech Chem Commun. 2010 Jan;75(1):59-79.
[112] Brocard JB, Rajdev S, Reynolds IJ. Glutamate-induced increases in intracellular free Mg2+ in cultured cortical neurons. Neuron. 1993 Oct;11(4):751-7.
[113] 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 Oct 29;99(22):14542-7.
[114] Neidle A, Dunlop DS. Allosteric regulation of mouse brain serine racemase. Neurochem Res. 2002 Dec;27(12):1719-24.
[115] Mustafa AK, Kumar M, Selvakumar B, Ho GP, Ehmsen JT, Barrow RK, Amzel LM, Snyder SH. Nitric oxide S-nitrosylates serine racemase, mediating feedback inhibition of D-serine formation. Proc Natl Acad Sci U S A. 2007 Feb 20;104(8):2950-5.

[116] Lopez-Mirabal HR, Winther JR. Redox characteristics of the eukaryotic cytosol. Biochim Biophys Acta. 2008 Apr;1783(4):629-40.
[117] Hoffman HE. Characterization of recombinant human serine racemase. Prague: Charles University; 2010.
[118] Dumin E, Bendikov I, Foltyn VN, Misumi Y, Ikehara Y, Kartvelishvily E, Wolosker H. Modulation of D-serine levels via ubiquitin-dependent proteasomal degradation of serine racemase. J Biol Chem. 2006 Jul 21;281(29):20291-302.
[119] Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol. 1999 Dec 17;294(5):1351-62.
[120] Balan L, Foltyn VN, Zehl M, Dumin E, Dikopoltsev E, Knoh D, Ohno Y, Kihara A, Jensen ON, Radzishevsky IS, Wolosker H. 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.
[121] Kim PM, Aizawa H, Kim PS, Huang AS, Wickramasinghe SR, Kashani AH, Barrow RK, Huganir RL, Ghosh A, Snyder SH. Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. Proc Natl Acad Sci U S A. 2005 Feb 8;102(6):2105-10.
[122] Baumgart F, Mancheno JM, Rodriguez-Crespo I. Insights into the activation of brain serine racemase by the multi-PDZ domain glutamate receptor interacting protein, divalent cations and ATP. FEBS J. 2007 Sep;274(17):4561-71.
[123] Fujii K, Maeda K, Hikida T, Mustafa AK, Balkissoon R, Xia J, Yamada T, Ozeki Y, Kawahara R, Okawa M, Huganir RL, Ujike H, Snyder SH, Sawa A. Serine racemase binds to PICK1: potential relevance to schizophrenia. Mol Psychiatry. 2006 Feb;11(2):150-7.
[124] DeLano WL. *The PyMol Molecular Graphics System.*: DeLano Scientific, San Carlos (CA)
2002.
[125] Meldrum BS. Excitatory amino acid receptors and disease. Curr Opin Neurol Neurosurg. 1992 Aug;5(4):508-13.
[126] Holtzheimer PE, Nemeroff CB. Novel targets for antidepressant therapies. Curr Psychiatry Rep. 2008 Dec;10(6):465-73.
[127] Thomas RJ. Excitatory amino acids in health and disease. J Am Geriatr Soc. 1995 Nov;43(11):1279-89.
[128] Kalia LV, Kalia SK, Salter MW. NMDA receptors in clinical neurology: excitatory times ahead. Lancet Neurol. 2008 Aug;7(8):742-55.
[129] Davidson ME, Kerepesi LA, Soto A, Chan VT. D-Serine exposure resulted in gene expression changes implicated in neurodegenerative disorders and neuronal dysfunction in male Fischer 344 rats. Arch Toxicol. 2009 Aug;83(8):747-62.
[130] Ro JY, Nies M, Zhang Y. The role of peripheral N-methyl-D-aspartate receptors in muscle hyperalgesia. Neuroreport. 2005 Apr 4;16(5):485-9.
[131] Kinkelin I, Brocker EB, Koltzenburg M, Carlton SM. Localization of ionotropic glutamate receptors in peripheral axons of human skin. Neurosci Lett. 2000 Apr 7;283(2):149-52.
[132] Chen Z, Huang W, Srinivas SR, Jones CR, Ganapathy V, Prasad PD. Serine racemase and D-serine transport in human placenta and evidence for a transplacental gradient for D-serine in humans. J Soc Gynecol Investig. 2004 Jul;11(5):294-303.
[133] 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 Jun;112(6):805-11.
[134] 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 Mar 1;17(5):1604-15.
[135] 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 Aug;98(4):1159-66.
[136] Turpin FR, Potier B, Dulong JR, Sinet PM, Alliot J, Oliet SH, Dutar P, Epelbaum J, Mothet JP, Billard JM. Reduced serine racemase expression contributes to age-related deficits in hippocampal cognitive function. Neurobiol Aging. 2009 Oct 1.
[137] Takayasu N, Yoshikawa M, Watanabe M, Tsukamoto H, Suzuki T, Kobayashi H, Noda S. The serine racemase mRNA is expressed in both neurons and glial cells of the rat retina. Arch Histol Cytol. 2008 Sep;71(2):123-9.
[138] Dememes D, Mothet JP, Nicolas MT. Cellular distribution of D-serine, serine racemase and D-amino acid oxidase in the rat vestibular sensory epithelia. Neuroscience. 2006 Feb;137(3):991-7.
[139] Maekawa M, Okamura T, Kasai N, Hori Y, Summer KH, Konno R. D-amino-acid oxidase is involved in D-serine-induced nephrotoxicity. Chem Res Toxicol. 2005 Nov;18(11):1678-82.

[140] Kantrowitz JT, Javitt DC. N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: The final common pathway on the road to schizophrenia? Brain Res Bull. 2010 Apr 24.
[141] Bendikov I, Nadri C, Amar S, Panizzutti R, De Miranda J, Wolosker H, Agam G. A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. Schizophr Res. 2007 Feb;90(1-3):41-51.
[142] 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 Jun;29(5):767-9.
[143] Tsai G, Yang P, Chung LC, Lange N, Coyle JT. D-serine added to antipsychotics for the treatment of schizophrenia. Biol Psychiatry. 1998 Dec 1;44(11):1081-9.
[144] Tsai GE, Yang P, Chung LC, Tsai IC, Tsai CW, Coyle JT. D-serine added to clozapine for the treatment of schizophrenia. Am J Psychiatry. 1999 Nov;156(11):1822-5.
[145] Lane HY, Lin CH, Huang YJ, Liao CH, Chang YC, Tsai GE. A randomized, double-blind, placebo-controlled comparison study of sarcosine (N-methylglycine) and d-serine add-on treatment for schizophrenia. Int J Neuropsychopharmacol. 2009 Nov 4:1-10.
[146] Coyle JT, Tsai G. The NMDA receptor glycine modulatory site: a therapeutic target for improving cognition and reducing negative symptoms in schizophrenia. Psychopharmacology (Berl). 2004 Jun;174(1):32-8.
[147] Labrie V, Fukumura R, Rastogi A, Fick LJ, Wang W, Boutros PC, Kennedy JL, Semeralul MO, Lee FH, Baker GB, Belsham DD, Barger SW, Gondo Y, Wong AH, Roder JC. Serine racemase is associated with schizophrenia susceptibility in humans and in a mouse model. Hum Mol Genet. 2009 Sep 1;18(17):3227-43.
[148] 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. A genetic variant of the serine racemase gene is associated with schizophrenia. Biol Psychiatry. 2007 May 15;61(10):1200-3.
[149] Strohmaier J, Georgi A, Schirmbeck F, Schmael C, Jamra RA, Schumacher J, Becker T, Hofels S, Klopp N, Illig T, Propping P, Cichon S, Nothen MM, Rietschel M, Schulze TG. No association between the serine racemase gene (SRR) and schizophrenia in a German case-control sample. Psychiatr Genet. 2007 Apr;17(2):125.
[150] Hikida T, Mustafa AK, Maeda K, Fujii K, Barrow RK, Saleh M, Huganir RL, Snyder SH, Hashimoto K, Sawa A. Modulation of D-serine levels in brains of mice lacking PICK1. Biol Psychiatry. 2008 May 15;63(10):997-1000.
[151] Rothstein JD. Excitotoxicity and neurodegeneration in amyotrophic lateral sclerosis. Clin Neurosci. 1995;3(6):348-59.
[152] Eisen A, Weber M. Treatment of amyotrophic lateral sclerosis. Drugs Aging. 1999 Mar;14(3):173-96.
[153] Yamin G. NMDA receptor-dependent signaling pathways that underlie amyloid beta-protein disruption of LTP in the hippocampus. J Neurosci Res. 2009 Jun;87(8):1729-36.
[154] Hashimoto K, Fukushima T, Shimizu E, Okada S, Komatsu N, Okamura N, Koike K, Koizumi H, Kumakiri C, Imai K, Iyo M. Possible role of D-serine in the pathophysiology of Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry. 2004 Mar;28(2):385-8.
[155] Sasabe J, Chiba T, Yamada M, Okamoto K, Nishimoto I, Matsuoka M, Aiso S. D-serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. EMBO J. 2007 Sep 19;26(18):4149-59.
[156] Wu SZ, Bodles AM, Porter MM, Griffin WS, Basile AS, Barger SW. Induction of serine racemase expression and D-serine release from microglia by amyloid beta-peptide. J Neuroinflammation. 2004 Apr 20;1(1):2.
[157] Inoue R, Hashimoto K, Harai T, Mori H. NMDA- and beta-Amyloid(1-42)-Induced Neurotoxicity Is Attenuated in Serine Racemase Knock-Out Mice. Journal of Neuroscience. 2008 Dec 31;28(53):14486-91.
[158] Loscher W. Pharmacology of glutamate receptor antagonists in the kindling model of epilepsy. Prog Neurobiol. 1998 Apr;54(6):721-41.
[159] Kohl BK, Dannhardt G. The NMDA receptor complex: a promising target for novel antiepileptic strategies. Curr Med Chem. 2001 Sep;8(11):1275-89.
[160] White HS, Harmsworth WL, Sofia RD, Wolf HH. Felbamate modulates the strychnine-insensitive glycine receptor. Epilepsy Res. 1995 Jan;20(1):41-8.
[161] De Sarro G, Trimarchi GR, Sinopoli S, Masuda Y, De Sarro A. 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. 1993 Mar;24(2):439-47.
[162] Liu YH, Wang L, Wei LC, Huang YG, Chen LW. Up-regulation of D-serine might induce GABAergic neuronal degeneration in the cerebral cortex and hippocampus in the mouse pilocarpine model of epilepsy. Neurochem Res. 2009 Jul;34(7):1209-18.
[163] Xie X, Dumas T, Tang L, Brennan T, Reeder T, Thomas W, Klein RD, Flores J, O'Hara BF, Heller HC, Franken P. Lack of the alanine-serine-cysteine transporter 1 causes tremors, seizures, and early postnatal death in mice. Brain Res. 2005 Aug 9;1052(2):212-21.

[164] Pittenger C, Sanacora G, Krystal JH. The NMDA receptor as a therapeutic target in major depressive disorder. CNS Neurol Disord Drug Targets. 2007 Apr;6(2):101-15.
[165] Wood PL. The NMDA receptor complex: A long and winding road to therapeutics. Idrugs. 2005 Mar;8(3):229-35.
[166] Wang C, Sadovova N, Hotchkiss C, Fu X, Scallet AC, Patterson TA, Hanig J, Paule MG, Slikker W, Jr. Blockade of N-methyl-D-aspartate receptors by ketamine produces loss of postnatal day 3 monkey frontal cortical neurons in culture. Toxicol Sci. 2006 May;91(1):192-201.
[167] Li Q, Clark S, Lewis DV, Wilson WA. NMDA receptor antagonists disinhibit rat posterior cingulate and retrosplenial cortices: a potential mechanism of neurotoxicity. J Neurosci. 2002 Apr 15;22(8):3070-80.
[168] Muir KW, Lees KR. Clinical experience with excitatory amino acid antagonist drugs. Stroke. 1995 Mar;26(3):503-13.
[169] Ikonomidou C, Stefovska V, Turski L. Neuronal death enhanced by N-methyl-D-aspartate antagonists. Proc Natl Acad Sci U S A. 2000 Nov 7;97(23):12885-90.
[170] Olney JW, Labruyere J, Wang G, Wozniak DF, Price MT, Sesma MA. NMDA antagonist neurotoxicity: mechanism and prevention. Science. 1991 Dec 6;254(5037):1515-8.
[171] Parsons CG, Stoffler A, Danysz W. Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system--too little activation is bad, too much is even worse. Neuropharmacology. 2007 Nov;53(6):699-723.
[172] Yang CR, Svensson KA. Allosteric modulation of NMDA receptor via elevation of brain glycine and D-serine: the therapeutic potentials for schizophrenia. Pharmacol Ther. 2008 Dec;120(3):317-32.
[173] Matsui T, Nagafuji T, Kumanishi T, Asano T. Role of nitric oxide in pathogenesis underlying ischemic cerebral damage. Cell Mol Neurobiol. 1999 Feb;19(1):177-89.
[174] Dixon SM, Li P, Liu R, Wolosker H, Lam KS, Kurth MJ, Toney MD. Slow-binding human serine racemase inhibitors from high-throughput screening of combinatorial libraries. J Med Chem. 2006 Apr 20;49(8):2388-97.
[175] Hoffman HE, Jiraskova J, Cigler P, Sanda M, Schraml J, Konvalinka J. Hydroxamic acids as a novel family of serine racemase inhibitors: mechanistic analysis reveals different modes of interaction with the pyridoxal-5'-phosphate cofactor. J Med Chem. 2009 Oct 8;52(19):6032-41.
[176] Mustafa AK, van Rossum DB, Patterson RL, Maag D, Ehmsen JT, Gazi SK, Chakraborty A, Barrow RK, Amzel LM, Snyder SH. Glutamatergic regulation of serine racemase via reversal of PIP2 inhibition. Proc Natl Acad Sci U S A. 2009 Feb 24;106(8):2921-6.
[177] Henneberger C, Papouin T, Oliet SH, Rusakov DA. Long-term potentiation depends on release of D-serine from astrocytes. Nature. 2010 Jan 14;463(7278):232-6.
[178] Kubala M, Teisinger J, Ettrich R, Hofbauerova K, Kopecky V, Jr., Baumruk V, Krumscheid R, Plasek J, Schoner W, Amler E. Eight amino acids form the ATP recognition site of Na(+)/K(+)-ATPase. Biochemistry. 2003 Jun 3;42(21):6446-52.

Declaration

Prohlašuji, že jsem tuto práci, ani její podstatnou část, nepředložila k získání stejného ani jiného akademického titulu.

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Jana Jirásková

V _____ Dne _____

My Contribution to the Publications Included in the Thesis

I

I participated in the inhibitor screening, established the oxo-acid detection method,. and analyzed the oxo-acid reactions. I performed some mechanism of inhibition experiments with L-EHA.

II

I prepared the activity reactions, performed the HPLC measurements and the application part, and contributed to the text.

III

I participated in the initial inhibitor screening and mass spectrometry study, performed the analysis of mechanism of inhibition, and contributed to the text.

IV

I participated in the inhibitor screening, structure analysis of the 3D models, and wrote the publication.

Jana Jirásková

I hereby confirm

Jan Konvalinka
Supervisor