D-Serine Influences Synaptogenesis in a P19 Cell Model

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Abstract Recently, D-serine has been identified as an important NMDA-receptor co-agonist, which might play a role in central nervous system development. We investigated this by studying rat P19 cells, an established model for neuronal and glial differentiation. Our results show that (1) the D-serine synthesizing enzyme serine racemase was expressed upon differentiation, (2) extracellular D-serine concentrations increased upon differentiation, which was inhibited by serine racemase antagonism, and (3) inhibition of D-serine synthesis or prevention of D-serine binding to the NMDA-receptor increased synaptophysin expression and intercellular connections, supporting a role for NMDA-receptor activation by D-serine, synthesized by serine racemase, in shaping synaptogenesis and neuronal circuitry during central nervous system development. In conjunction with recent evidence from literature, we therefore suggest that D-serine deficiency might be responsible for the severe neurological phenotype seen in patients with serine deficiency disorders. In addition, this may provide a pathophysiological mechanism for a role of D-serine deficiency in psychiatric disorders.

Introduction

Central nervous system (CNS) development is a complex process, in which N-Methyl D-Aspartate receptors (NMDARs) play an essential role; NMDAR activation is involved in neuronal migration, proliferation, maturation and survival, dendritic outgrowth, synaptic formation, brain plasticity, and the onset of long-term potentiation (Fuchs et al. 2008a). For activation, NMDARs require simultaneous binding by glutamate to their NR2 subunit and glycine or D-serine to their NR1 subunit. D-serine appears to be the main co-agonist in most areas of human CNS (Mothet et al. 2000), where it can be synthesized from L-serine by serine racemase (SR, EC 5.1.1.18) and metabolized by D-amino acid oxydase (DAO, EC 1.4.3.3).

As an endogenous NMDAR agonist, D-serine is likely to be involved in CNS development. This is supported by specifically elevated D-serine concentrations in human and rodent CNS during the intense period of embryonic and early postnatal CNS development, which coincides with a transient expression and increased activity of NMDARs (Fuchs et al. 2006; Fuchs et al. 2008a). The severe CNS abnormalities upon failure to achieve these high D-serine concentrations, as seen in patients (Fuchs et al. 2006; Jaeken et al. 1996) and mutant mice (Yoshida et al. 2004) with 3-phosphoglycerate dehydrogenase deficiency (3-PGDH, OMIM 601815), a rare inherited disorder in L-serine and hence D-serine synthesis, underscore the putative role of D-serine in CNS development. Degradation of D-serine by DAO and selective inhibition of SR in 8-day-old mouse cerebellar slices significantly reduced granule cell migration, whereas D-serine activated this process (Kim et al. 2005). However, no evidence of disrupted neuronal migration was observed in mutant mice with a targeted disruption in exon 1 of SR, thereby lacking the ability to

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produce D-serine endogenously (Basu et al. 2009). These mice displayed altered glutamatergic neurotransmission and attenuated synaptic plasticity and subtle behavioral and memory abnormalities.

To gain insight into the role of D-serine in CNS development and in serine deficiency disorders, we studied rat P19 cells, an established model for neuronal and glial differentiation (Bain et al. 1994). Neurons developing from these cells strongly resemble normal mammalian embryonic neurons, with functional glutamatergic receptors (NR1, NR2A/B, AMPA/kainate receptors, and non-NMDARs (GluR1-4)). In this model, we demonstrate that D-serine was actively synthesized by SR upon differentiation and appeared to shape synaptogenesis, potentially by preventing widespread untargeted synaptogenesis. As an NMDAR co-agonist, our results contribute to the expanding evidence indicating a role for NMDAR activation in synaptic shaping and wiring of neuronal circuitry (Rabacchi et al. 1992; Yang et al. 2003; Adesnik et al. 2008), and provide new evidence for a role of D-serine and SR in this process.

Materials and Methods

Cell Differentiation

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

D-Serine Analysis

D-serine was quantified according to our stable isotope dilution LC-MS method described previously (Fuchs et al. 2008b).

Western Blots

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

Results

Undifferentiated P19 Cells

Undifferentiated P19 cells did not express the neuronal marker neuron-specific β-III-tubulin, the glial marker GFAP nor SR (Fig. 1a) and did not excrete D-serine in supernatant (Fig. 1b). In fact, D-serine concentrations in external medium were somewhat lower than in fresh medium supplemented with fetal calf serum (2.88 µM vs. 3.13 µM).

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

Fig. 2 Differentiation in the presence and absence of D-serine-induced NMDAr activation. The top panels show undifferentiated and differentiated cells (at day 15) under normal conditions and with exogenous supplementation of D-serine. In order to simulate

conditions without NMDAr activation by D-serine, LSOS, an SR antagonist (middle panel) and DCKA, an antagonist of the NR1 subunit of the NMDAr (lower panel) were employed. By addition of D-serine, we attempted to rescue these conditions

Differentiated P19 Cells

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

Is D-Serine Synthesis by SR Cause or Consequence of Differentiation?

To distinguish between these options, we attempted to create conditions without D-serine induced NMDAr activation by inhibiting D-serine synthesis (LSOS) and D-serine binding to NMDAr (DCKA). Figure 2 demonstrates that differentiation was not prevented by LSOS or DCKA. Interestingly, dendritic outgrowths appeared less concentrated and cells less interconnected upon exogenous

D-serine addition, while dendritic outgrowths appeared more concentrated and cells more interconnected upon treatment with LSOS and DCKA (Fig. 2, the two lower left panels). D-serine appeared to overcome the latter in the sample treated with LSOS, but not in the sample treated with DCKA. Similarly, western blots of these samples (Fig. 3) showed decreased synaptophysin expression upon exogenous D-serine addition and increased expression of synaptophysin upon treatment with LSOS and DCKA, when compared with differentiated P19 cells with normal NMDAr activation. D-serine rescue normalized synaptophysin expression in LSOS-treated cells, but not in DCKA-treated cells.

Discussion

In this study, we induced and visualized P19 cell differentiation into neurons and glia and evidenced for the first time D-serine synthesis by SR upon differentiation. Similar to GFAP, SR expression was not evidently increased by ARA-C, concurring with the predominant glial localization of D-serine and SR (Schell et al. 1995). Inhibition of D-serine synthesis or D-serine binding to NMDAr increased synaptophysin expression and the

Fig. 3 Expression of synaptophysin in the presence or absence of D-serine-induced NMDAr activation. This western blot shows the expression of synaptophysin, neuron-specific β -III-tubulin and loading control actin in undifferentiated P19 cells (–RA, lane 1), differentiated cells (C, lane 2), differentiated cells with additional

exogenous D-serine to 10 μ M (C+DS, lane 3), differentiated cells in the presence of the SR antagonist LSOS (LSOS, lane 4), the rescue of the former with D-serine to 10 μ M (LSOS+DS), differentiated cells in the presence of the NR1 antagonist DCKA (DCKA, lane 5) and the rescue of the latter with D-serine to 50 μ M (DCKA+DS)

former was overcome by D-serine supplementation. This might signify that suppression of NMDAr activation by decreased D-serine synthesis or NR1 antagonism leads to increased synaptic formation. This concurs with the finding that NMDAr activation is critical in the regression of functional synapses in the developing rat cerebellum (Rabacchi et al. 1992). Similarly, partial deletion of NR1 subunits of NMDAr in organotypic hippocampal cells profoundly increased numbers of functional synapses between neurons and strength of unitary connections in vitro and in vivo (Adesnik et al. 2008). Conversely, reintroduction of NMDAr in NR1-deficient neurons reduced the number of functional inputs. Based on these findings, the authors proposed a new model for maturation of excitatory synapses in which ongoing activation of NMDAr prevents premature synaptic maturation by ensuring that only punctuated bursts of activity lead to induction of functional synapses for the activity-dependent wiring of neuronal circuitry (Adesnik et al. 2008). Our results imply that endogenous D-serine might be a crucial factor in activating NMDAr, thereby preventing premature synaptic maturation. Further support for our finding of D-serine in a regulatory role in tissue development comes from chondrogenesis, where SR negatively regulated maturation in chondrocytes (Takarada et al. 2008). D-Serine suppressed several chondrocytic maturation markers in rat chondrocytes and delayed chondral mineralization in mouse metatarsals (Takarada et al. 2009). D-Serine, synthesized by SR, may thus negatively regulate chondrocyte differentiation, similar to the negative regulatory activity during neuronal differentiation in our results.

Evaluation of CNS development in SR knockout mice is interesting in this respect. These mice display attenuated synaptic plasticity, a spatial memory deficit, and subtle behavioral abnormalities, including mild hyperactivity and

increased anxiety (Basu et al. 2009). According to our results, this might be attributable to differences in synaptic shaping induced by decreased D-serine concentrations. In fact, NMDAr antagonists influenced rat hippocampal mossy fiber synaptogenesis and inhibited spatial learning (Ramirez-Amaya et al. 2001). Similarly, NMDAr seem to be involved in synaptic plasticity in the amygdala, which appears to play a role in anxiety (Pape and Pare 2010). Since no structural CNS abnormalities were described in the SR knockout mice, a vast migration defect induced by D-serine depletion seems less likely, but compensations in the constitutive knockout and residual D-serine concentrations (10% of the wild type (Basu et al. 2009)) may occlude the effects of D-serine depletion. Conversely, 3-PGDH knockout mice display a lethal phenotype and evident structural CNS abnormalities, with hypoplasia of the telencephalon, diencephalon, and mesencephalon and in particular the olfactory bulbs, ganglionic eminence and cerebellum appearing as indistinct features (Yoshida et al. 2004). Patients with 3-PGDH deficiency – who might represent the milder nonlethal end of the disease spectrum with some residual enzyme activity – do not exhibit vast structural CNS abnormalities, but show evidence of hypomyelination and white matter attenuation (de Koning et al. 2000) and a severe neurological phenotype, evidenced by microcephaly, profound mental retardation and intractable seizures (Jaeken et al. 1996; De Koning and Klomp 2004). While normally D-serine concentrations peak during the neonatal period of intense CNS development, these patients with defective L-serine and hence D-serine biosynthesis had virtually no residual D-serine concentrations in their cerebrospinal fluid after birth (Fuchs et al. 2006), thus differing from SR knockout mice in this respect. Postnatal treatment with L-serine and/or glycine results in a major reduction in seizure frequency, but no evident effect on head circumference or psychomotor retardation (de Koning

et al. 2002). Interestingly, upon restoration of D-serine concentrations by prenatal maternal L-serine treatment of a patient with 3-PGDH deficiency, the complete neurological phenotype was reversed (De Koning et al. 2004). This led us to hypothesize that D-serine deficiency during the critical period of prenatal and early postnatal CNS development is responsible for the severe neurological phenotype in 3-PGDH deficiency. Our present results support this hypothesis and present altered synaptic shaping of the brain as the putative mechanism. In addition, D-serine has been implicated in lipid oxidative damage and decreased antioxidant defenses in the striatum (Leipnitz et al. 2010) and cerebral cortex (da Silva et al. 2009) of young rats and in impairment of the citric acid cycle, thereby compromising energy production in the cerebral cortex of young rats (Zanatta et al. 2009). Hypothetically, these mechanisms might underlie the inhibition of synaptogenesis by D-serine seen in our study or contribute additionally to the neurological damage in patients affected by disorders in D-serine metabolism.

Schizophrenia has been strongly associated with both altered synaptic shaping (Eastwood 2004) and decreased D-serine concentrations, alterations in D-serine synthesizing and metabolizing enzymes and genes encoding for these enzymes (Detera-Wadleigh and McMahon 2006). Our results putatively link altered synaptic shaping and decreased D-serine concentrations in schizophrenia. Similarly, in bipolar disorder, an increase in synaptophysin and synaptosomal-associated protein-25 was observed in post-mortem brains, when compared to control brains (Scarr et al. 2006), which, considering our results, might be due to decreased D-serine concentrations, caused by altered D-serine metabolism, because genes coding for enzymes associated with D-serine metabolism have been implicated in bipolar disorder (Detera-Wadleigh and McMahon 2006). Likewise, D-serine showed some anxiolytic properties in patients with posttraumatic stress disorder (Heresco-Levy et al. 2009), suggesting an absolute or relative D-serine deficiency, potentially leading to altered synaptic plasticity, which has been associated with anxiety (Pape and Pare 2010).

In conclusion, our P19 cell studies showed that (1) SR was expressed upon differentiation, (2) extracellular D-serine concentrations increased upon differentiation, which was inhibited by SR antagonism, (3) inhibition of D-serine synthesis or prevention of D-serine binding to NMDARs appeared to lead to altered synaptogenesis, supporting a role for NMDAR activation by D-serine, synthesized by SR, in shaping synaptogenesis and neuronal circuitry. In conjunction with recent evidence from literature, we therefore suggest that D-serine deficiency might be responsible for the severe neurological phenotype seen in patients with serine deficiency disorders. In addition, this may

provide a pathophysiological mechanism for a role of D-serine deficiency in psychiatric disorders.

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Concise 1 Sentence Take-Home Message

D-serine, synthesized by serine racemase might play an important role in shaping synaptogenesis and neuronal circuitry during central nervous system development.

Details of the Contributions of Individual Authors

- Sabine A. Fuchs: planned the study, designed and performed the experiments (with the exception of the LC-MS analyses), interpreted results, and wrote the manuscript
- Martin W. Roeleveld: performed all LC-MS analyses, revised and agreed with the manuscript
- Leo W.J. Klomp: participated in study conception and design, data interpretation and manuscript revision
- Ruud Berger: supervised study conception and design, data interpretation and manuscript revision.
- Tom J. de Koning: participated in study conception and design, data interpretation and manuscript revision

Guarantor for the Article

Tom J. de Koning

A Competing Interest Statement

None of the authors has any conflict of interest to declare, thus none of the authors has

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Details of Ethics Approval, Patient Consent Statement or Approval from the Institutional Committee for Care and Use of Laboratory Animals

No ethics approval, patient consent statement or approval from the Institutional Committee for Care and Use of Laboratory Animals was required for these research studies

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