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Modulation of D-Serine Levels in Brains of Mice Lacking PICK1

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Abstract

Background: D-serine is an endogenous coagonist of the *N*-methyl-D-aspartate subtype glutamate receptor. Genetic association studies have implicated genes coding for enzymes associated with D-serine metabolism in schizophrenia and bipolar disorder.

Methods: Protein expression of serine racemase (SR) and its binding partner, protein interacting with C-kinase (PICK1), were examined by Western blotting in brains from wildtype and PICK1 knockout mice. Levels of D-serine in wildtype and PICK1 mice were also examined by an established high-pressure liquid chromatography protocol.

Results: Expression of SR and PICK1 proteins was developmentally regulated. Although no change was observed in the level of SR protein, levels of D-serine were selectively decreased in the forebrain of neonatal PICK1 knockout mice, compared with those in wildtype mice.

Conclusions: PICK1 may be involved in the regulation of brain D-serine levels and SR in a spatially and temporally specific manner.

Keywords

Bipolar disorder; D-serine; knockout mice; PICK1; schizophrenia; serine racemase

D-serine is an endogenous coagonist of the *N*-methyl-D-aspartate (NMDA) glutamate receptors (1); it is generated from L-serine by the enzyme serine racemase (SR) and degraded by D-amino acid oxidase (DAO) (2,3). Administration of D-serine together with classic neuroleptics can improve some of the cognitive defects in patients with schizophrenia (SZ) (4,5). Recent genetic association studies have implicated genes coding for enzymes associated with D-serine metabolism in some forms of SZ (6-8). In some studies, these genes, including DAO, are also associated with bipolar disorder, suggesting common mechanisms in SZ and bipolar disorder (8-10). We recently reported that SR binds to protein interacting with C-kinase (PICK1) in glial cells and that the gene for PICK1, located on chromosome 22q13, is associated with disorganized SZ (11). In an independent study, an association of the PICK1 gene with SZ was reported in a Chinese population (12).

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Genetically engineered mice are frequently used to explore the functions of gene products in the brain. Mice with deletion of the PICK1 gene display abnormalities of synaptic plasticity (13,14). In contrast, no genetically engineered mice for DAO are currently available, but spontaneous mutant outbred mice lacking DAO activity display increased levels of D-serine, enhancement of NMDA-receptor-mediated synaptic transmission and marked attenuation of stereotypy, as well as ataxia induced by an antagonist of NMDA receptor, MK-801 (15-17). These mutant mice also show reduced spontaneous locomotor activity and phencyclidine-induced hyperlocomotion (18). No G72 knockout mice are available because there is no mouse ortholog of G72 (19,20). Although the data from mice lacking DAO activity are promising, data from spontaneous mutant mice in an outbred strain are not as readily interpreted as studies with targeted gene deletion.

Accordingly, we explored the well-characterized PICK1 knockout mouse, the genetic background of which has been standardized as C57BL/6, as a possible system of disturbed D-serine metabolism with potential relevance to SZ. As an initial step, we studied expression of PICK1 and SR and examined levels of D-serine in PICK1 knockout mice.

Methods and Materials

Animals

Biochemical experiments were performed on brains from embryonic days 12–19, postnatal days 1–7, or 8-week-old wild-type, PICK1 knockout animals. All animal-use procedures were in accordance with protocols approved by Johns Hopkins University Animal Care and Use Committee.

Assay for D-Serine

We measured D-serine according to a published protocol (21). In brief, brains were homogenized in 20-volume methanol, centrifuged at 4500 g for 10 min, 20 μ L supernatant evaporated at 40°C, followed by addition of 40 μ L .05 mol/L borate (pH 8.0) and 60 μ L 50 mmol/L 4-fluoro-7-nitro-2,1,3-benzoxadiazole, heated at 60°C for 1 min and stopped with 100 μ L 10% CH₃CN and .1% trifluoroacetic-acid, 10 μ L then injected into high-pressure liquid chromatography (HPLC).

Protein Expression Analysis and Western Blotting

Brains were removed and suspended in 20 mmol/L Tris (pH 7.8), 100 mmol/L NaCl, 1% Triton X-100 and protease inhibitors (Roche, Basel, Switzerland), homogenized for 1 min with the Wheaton Overhead Stirrer (Wheaton, Millville, New Jersey), and centrifuged at 16,000 g for 10 min. Protein concentration was determined by Coomassie Plus Reagent (Pierce, Rockford, Illinois). Extracts were resuspended in sample buffer for immuno-blotting, which was performed using antibodies to SR (BD Biosciences, San Jose, California), PICK1 (Chemicon, Temecula, California), and β -Tubulin (Upstate, Lake Placid, New York).

Cell Culture and Transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 2 mmol/L L-glutamine and 10% fetal bovine serum. Transfection of hemagglutinin(HA)-SR and/or myc-PICK1 was carried out by the calcium phosphate method. SR (SR/Y) and PICK1 (K27A/D28A) mutants used previously (11) were also employed, as well as small interfering RNA to PICK1 (Santa Cruz, Santa Cruz, California). L-serine was added to the media immediately posttransfection and D-serine measured 40 hours later.

Statistical Analysis

Data was analyzed by analysis of variance and post hoc comparisons were made with the Scheffé test.

Results

SR and PICK1 Expression in Developing Brain

We studied SR and PICK1 protein expression in wildtype mouse brain at various developmental stages (Figure 1A). Although low levels of PICK1 were observed at embryonic day 14 (E14), reaching nearly adult levels by birth, expression of SR was undetectable until E19, after which it increased steadily until adult levels were reached. The expression pattern of SR is in complete agreement with D-serine levels in the developing brain reported by Hashimoto and colleagues (22), who showed that D-serine is detectable at birth with intermediate levels at week 1 and reaching adult levels by week 3.

SR Expression in Wildtype Versus PICK1 Knockout Brains

SR expression was studied in brains of postnatal day 7 (P7) and 8-week-old adult wildtype (+/+), heterozygote (+/-), and homozygote PICK1 knockout (-/-) mice (Figure 1B). Genetic deletion of PICK1 did not affect the protein levels of SR in P7 or adult mice.

Comparison of D-serine in Wildtype Versus PICK1 Knockout Brains

D-serine was measured by HPLC from wildtype, PICK1 +/-, and -/- mice at P7 and 8 weeks (adult) (Figure 2A). In the hippocampus (HP) and frontal cortex (F-CTX) of P7 PICK1 -/- mice, D-serine levels were reduced by approximately 30% and 40%, respectively, with PICK1 +/- displaying intermediate reductions. In the P7 cerebellum (CB), however, PICK1 -/- does not apparently affect D-serine levels. In adult -/- mice, no overall reduction of D-serine was observed in either the forebrain or cerebellum. Notably, the high levels of D-serine in the forebrain of adult mice in contrast to the residual amounts in the cerebellum are in accordance with previous observations (22).

Effect of SR-PICK1 Interaction on D-serine Production

To address influences of PICK1 on D-serine deriving from its interaction with SR, we transfected SR, PICK1, or both into HEK293 cells and measured D-serine levels in media (Figure 2B). Compared with transfection with SR alone, D-serine was increased in cotransfections with PICK1. An SR mutant with an additional C-terminal tyrosine (SR/Y) or the PICK1 PDZ domain mutant K27A/D28A (11) abolishing the SR-PICK1 interaction prevented this increase indicating that the SR-PICK1 interaction augments D-serine production. Further, small interfering RNA (siRNA) to PICK1 depleted PICK1 and also diminished D-serine levels (Figure 2C).

Discussion

In this study we demonstrate that genetic deletion of PICK1 leads to decreased levels of D-serine in the forebrains of neonatal mice. The decrease in D-serine correlates with the levels of PICK1. The changes are both spatially and temporally specific because D-serine levels were not altered in either the adult mice or the cerebellum of neonatal pups.

In HEK293 cells transfected with wildtype PICK1, SR, or both, we observed an increased ratio of D-serine to SR in cells expressing both SR and PICK1 compared with SR alone. The SR mutant with an additional C-terminal tyrosine or the PICK1 PDZ mutant K27A/D28A, which cannot interact with each other, abolished the increase in D-serine. Reducing PICK1 expression

using siRNA to PICK1 also prevented the increase in D-serine production. These results suggest that PICK1 may be involved in increasing D-serine levels because of its direct interaction with SR in glial cells.

PICK1 is a multifunctional protein with a PDZ domain, which is important for its binding to partner proteins (23,24). In neurons, PICK1 binds to the dopamine and norepinephrine transporters, as well as α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and metabotropic-glutamate receptors (25-29). Furthermore, PICK1 regulates cellular trafficking of the netrin receptor UNC5H1, suggesting a role in neurodevelopment (30). Therefore, this study does not provide conclusive evidence that the impact of PICK1 on D-serine is solely via modulation of SR in vivo. In addition, it is uncertain why there is a reduction in D-serine in neonatal forebrain, but not in adult, PICK1 knockout mice. SR activity in glial cells is also influenced by the glutamate receptor interacting protein (GRIP1) (31). Thus, differential spatiotemporal expression patterns of proteins that modulate SR, such as GRIP1 and PICK1, may account for a more preferential effect of PICK1 on SR/D-serine in the developing forebrain.

Genetically engineered mice that modulate susceptibility genes to SZ and bipolar disorder, as well as their biological cascades, afford promising models for these diseases (32). Synaptic plasticity is diminished in PICK1 knockout mice because of elimination of PICK1-GluR2 interaction (13,14). These mice have normal levels of AMPA receptors but altered GluR2 ultrastructural localization (13). There is no change in NMDA receptor kinetics (CY Kim and RLH, unpublished data), and these mice also show no gross anatomic and behavioral defects associated with learning (13).

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Figure 1. Expression of serine racemase (SR) and PICK1 in mouse brains

(A) PICK1 is expressed from embryonic day 14 (E14) to adulthood. SR is expressed primarily after birth with an increase in its expression during maturation until adulthood. (B) Expression levels of SR are unaltered among wild-type (+/+), heterozygote (+/-), and homozygote (-/-) PICK1 knockout mice at both P7 and 8 weeks (adult). Gene-dose-dependent expression of PICK1 is observed among these mice. These data are representative of three independent experiments.

Figure 2. Levels of D-serine in PICK1 knockout brain and PICK1 overexpressing HEK293 cells
(A) Levels of D-serine in wildtype and PICK1 knockout mice. In the hippocampus (HP) and the frontal cortex (F-CTX) of PICK1 mice at postnatal day 7 (P7), decreased ratios of D-serine to total serine (D/D+L) are observed in a gene dose-dependent manner, whereas in the cerebellum (CB) of P7 mice, PICK1 knockout does not apparently affect D-serine levels. In adult mice, no change in the levels of D-serine is observed in PICK1 knockouts in comparison to wildtype mice. $**p < .01$; $n = 6$ per group). **(B)** Effects of serine racemase (SR)-PICK1 interaction on D-serine production in HEK293 cells. Cotransfection of SR and PICK1 increases D-serine levels in the medium per SR molecules compared with transfection of SR alone. PICK1 PDZ domain mutant K27A/D28A or SR C-terminal mutant SR/Y that abolishes SR-PICK1

interaction had no effect on D-serine production. $**p < .01$, $*p < .05$; $n = 4$ each. (C) Effects of PICK1 knockdown using siRNA to PICK1 on D-serine levels in HEK293 cells. siRNA to PICK1 selectively decreases PICK1 levels without affecting the expression of SR. PICK1 enhances D-serine production, an effect diminished by depleting PICK1 using siRNA to PICK1. $**p < .01$; $n = 4$ per group.