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Decreased levels of free D-aspartic acid in the forebrain of serine racemase (*Srr*) knock-out mice

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ABSTRACT

D-Serine, an endogenous co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor is synthesized from L-serine by serine racemase (SRR). A previous study of *Srr* knockout (*Srr*-KO) mice showed that levels of D-serine in forebrain regions, such as frontal cortex, hippocampus, and striatum, but not cerebellum, of mutant mice are significantly lower than those of wild-type (WT) mice, suggesting that SRR is responsible for D-serine production in the forebrain. In this study, we attempted to determine whether SRR affects the level of other amino acids in brain tissue. We found that tissue levels of D-aspartic acid in the forebrains (frontal cortex, hippocampus and striatum) of *Srr*-KO mice were significantly lower than in WT mice, whereas levels of D-aspartic acid in the cerebellum were not altered. Levels of D-alanine, L-alanine, L-aspartic acid, taurine, asparagine, arginine, threonine, γ-amino butyric acid (GABA) and methionine, remained the same in frontal cortex, hippocampus, striatum and cerebellum of WT and mutant mice. Furthermore, no differences in D-aspartate oxidase (DDO) activity were detected in the forebrains of WT and *Srr*-KO mice. These results suggest that SRR and/or D-serine may be involved in the production of D-aspartic acid in mouse forebrains, although further detailed studies will be necessary to confirm this finding.

1. Introduction

It was a long held belief that only the L-isomers of amino acids existed in mammals. However, with recent advances in analytical methods, free D-amino acids, including D-aspartic acid and D-serine, have been found in the tissues of mammals, including humans (Dunlop et al., 1986; Hashimoto et al., 1992; Nagata, 1992; Nagata et al., 1992a,b; Brückner and Hausch, 1993). Subsequent studies using two-dimensional high performance liquid chromatography (HPLC), detected very low levels of free D-amino acids in a variety of mammalian tissues (Hamase et al., 2001, 2005; Morikawa et al., 2001; Miyoshi et al., 2009, 2012; Yamanaka et al., 2012). It is therefore not unreasonable to conclude that D-amino acids may play a role in physiological and biological functions in mammals. Of the free D-amino acids, the roles of D-aspartic acid, D-serine and D-alanine have been well investigated in animal brains (Hashimoto and Oka, 1997; Hamase, 2007; Yamanaka et al., 2012).

D-Serine, an endogenous co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor, plays an important role in excitatory neurotransmission, via the NMDA receptor (Hashimoto et al., 1993; Schell et al., 1997). D-Serine is synthesized from L-serine by the pyridoxal-5′ phosphate-dependent enzyme, serine racemase (SRR) (Wolosker et al., 1999a, b), and metabolized by D-amino acid oxidase (DAAO) (Wolosker and Mori, 2012). Studies using *Srr* knock-out (*Srr*-KO) mice have shown that SRR is predominantly localized to forebrain neurons (Miya et al., 2008) and that levels of D-serine in the forebrain of these animals are 80–90% lower than in wild-type (WT) mice (Inoue et al., 2008; Basu et al., 2009; Horio et al., 2011), implying that D-serine production in the forebrain is largely dependent on SRR activity. In contrast, levels of L-serine, glycine, glutamine and glutamate, which are also related to NMDA receptor neurotransmission, were similar between brain tissue from *Srr*-KO and WT mice (Horio et al., 2011).

D-Aspartic acid was the first D-amino acid found in mammalian brains (Dunlop et al., 1986) and it is observed in many neuroendocrine and endocrine organs (Hashimoto et al., 1995). It is directly involved in the secretion of hormones, such as melatonin and testosterone (D'Aniello et al., 1996; Takigawa et al., 1998; Huang et al., 2006). D-Aspartic acid is synthesized by the pyridoxal-5′ phosphate-dependent enzyme, aspartate racemase in adult mouse brain (Kim et al., 2010), and degraded by D-aspartate oxidase (DDO) (Van Veldhoven et al., 1991; Huang et al., 2006). Due to its structural similarity to NMDA, D-aspartic acid binds to the NMDA receptor and potentiates NMDA receptor-mediated neurotransmission (Fagg and Matus, 1984; D'Aniello et al., 2011; Yamanaka et al., 2012). Like D- serine, D-alanine is also an

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endogenous co-agonist of the NMDA receptor although its synthetic pathway is unknown (Kleckner and Dingledine, 1988; McBrain et al., 1989). Interestingly, increased levels of D-alanine in brain tissue and plasma were observed in patients with Alzheimer's (Fisher et al., 1991) and renal diseases (Nagata et al., 1992a).

In this study, we measured levels of D-aspartic acid and D-alanine, as well as the amino acids, L-alanine, L-aspartate, taurine, asparagine, arginine, threonine, γ-amino butyric acid (GABA) and methionine, from frontal cortex, hippocampus, striatum and cerebellum of wild-type (WT) and *Srr*-KO mice. Additionally, we measured the activity of DDO in the forebrain of WT and *Srr*-KO mice.

2. Materials and methods

2.1. Animals

The *Srr*-KO mice were generated from C57BL/6- derived embryonic stem cells transfected with a gene-targeting vector containing C57BL/6 mouse genomic DNA, and the colony expanded by crossing with C57BL/6 mice (Miya et al., 2008). The generation and genotyping of *Srr*-KO mice and wild-type (WT) control mice with a pure C57BL/6 genetic background has been reported previously (Miya et al., 2008). WT and *Srr*-KO male mice aged 2–3 months were used for analyses. The mice were housed in clear polycarbonate cages (22.5 × 33.8 × 14.0 cm) in groups of 5 or 6 per cage under a controlled 12/12-h light–dark cycle (lights on from 7:00a.m. to 7:00p.m.), with a room temperature of 23 ± 1 °C and humidity of 55 ± 5%. The mice were given free access to water and food pellets. Experimental procedures were approved by the Animal Care and Use Committee of Chiba University.

2.2. Sample preparation

Twenty-four hours after fasting, mice were sacrificed by decapitation. Then, brain regions, the frontal cortex, hippocampus, striatum, and cerebellum were dissected on ice. All samples were stored at −80 °C before analysis.

2.3. HPLC system for the determination of D- and L-aspartic acid, and D- and L-alanine

Tissues were homogenized in 1.5 mL of methanol (HPLC grade) on ice. The homogenates were centrifuged at 3000*g* for 6 min at 4 °C, and 20 μL of supernatant was evaporated to dryness at 40 °C. To the residue, 20 μL of H_2O (HPLC grade), 20 μL of 0.1 M borate buffer (pH 8.0) and 60 μL of 50 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) in CH_3CN (HPLC grade) were added. The reaction mixture was then heated to 60 °C for 2 min, and immediately supplemented with 100 μL of H_2O/acteonitrile (92/8) containing 0.05% trifluoroacetic acid to stop the reaction. These procedures were fully automated using a 3023 auto sampler.

Measurement of D- and L-aspartic acid, and D- and L-alanine was performed using the previous reports with a slight modification (Morikawa et al., 2001; Miyoshi et al., 2009, 2012). The HPLC system (NANOSPACE SI-2 series, Shiseido Ltd, Tokyo, Japan) consisted of a type 3202 degasser, 3101 and 3201 pumps, a 3023 auto sampler, 3004 and 3014 column oven, two 3213 fluorescence detectors, a 3011 column-switching high pressure valve and dual-loop valve. A data processing programs, EzChrom Elite Client, was used to monitor the detectors response and a column-switching valve and a dual-loop valve were controlled by a KSAA valve controlling system (Shiseido Ltd, Tokyo, Japan).

The analytical column for the reversed-phase separation was a Nucleonavi (250 mm × 1.0 mm i.d., Shiseido Ltd., Tokyo, Japan) maintained at 40 °C. Mobile phase 1a consisted of H_2O/acetonitrile (92/8) containing 0.05% TFA, and phases 1b, H_2O/acetonitrile (10/90) containing 0.1% TFA and acetonitrile, respectively. The gradient elution of the mobile phase was kept at a constant flow rate of 50 μL/min. The time program for gradient elution was as follows: 0–40.0 min 1a: 1b = 100: 0, 40.0–40.1 min liner gradient from 0% 1b to 100% 1b,40.1–50.0 min 1a: 1b = 0: 100, 50.0–50.1 min liner gradient from 0% 1a to 100% 1a, and 50.1–120 min 1a: 1b = 100: 0. The chiral column (Column 2) used for the separation and quantification of D- and L-aspartic acid, and D- and L-alanine with NBD-F comprised two KSAA-OA2500 columns (S) (250 mm × 2.0 mm i.d., Shiseido Ltd., Tokyo, Japan), which were connected in tandem. The mobile phase was 15 mM citric acid in methanol. The flow rate was isocratically pumped at 200 μL/min. The column temperature was maintained at 25 °C for all columns. Fluorescence detection was performed at 530 nm with an excitation wavelength at 470 nm.

2.4. HPLC system for the determination of various amino acids

Measurements of taurine, asparagine, arginine, threonine, γ-amino butyric acid (GABA) and methionine were carried out using a HPLC system with fluorescence detection, as previously reported (Aoyama et al., 2004). Tissues were homogenized in 1.5 mL of methanol (HPLC grade) on ice. The homogenates were centrifuged at 3000*g* for 6 min at 4 °C, and 20 μL of supernatant was evaporated to dryness at 40 °C. To the residue, 25 μL of H_2O (HPLC grade), 20 μL of internal standard solution (375 nM ε-amino-*n*-caproic acid in water), 25 μL of 0.2 M borate buffer (pH 9.5) and 30 μL of 10 mM NBD-F (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) in CH_3CN (HPLC grade) were added. The reaction mixture was put at room temperature for 40 min, and immediately supplemented with 50 μL of 1 M tartrate buffer (pH 2.0) to stop the reaction. These procedures were fully automated using a SIL-20A auto sampler.

The HPLC system (SCL-10A vp series, Shimadzu Ltd., Tokyo, Japan) consisted of a type DGU-20A_5 degasser, a LC-20A pumps, a SIL-20AC auto sampler, a CTO-20A column oven, a RF-10A_{XL} fluorescence detectors, for the determination of taurine, asparagine, arginine, threonine, GABA and methionine, a cadenza CD-C18 ODS column (250 mm × 4.6 mm i.d., Imtakt, Ltd., Kyoto, Japan) was used. The gradient elution of the mobile phase was kept at a constant flow rate of 0.8 mL/min. Mobile phase A consisted of H_2O/acetonitrile/2-propanol (90/10/0.8) containing 0.08% TFA, and phases B, C and D, of H_2O/acetonitrile/2-propanol (90/10/5) containing 0.08% TFA, H_2O/acetonitrile (90/10) and H_2O/acetonitrile (10/90) containing 0.08% TFA acetonitrile, respectively.

The time program for gradient elution was programmed as follows: 0–25 min A: B: C: *D* = 100: 0: 0: 0, 25–32 min liner gradient from 0% B to 100% B, 32–35 min A: B: C: *D* = 0: 100: 0: 0, 35–39 min liner gradient from 100% B to 97% B, 39–45 min A: B: C: *D* = 0: 97: 0: 3, 45–45.1 min liner gradient from 97% B to 0% B, 45.1–60 min A: B: C: *D* = 0: 0: 90: 10, 60–60.1 min liner gradient from 90% C to 65% C, 60.1–63 min A: B: C: *D* = 0: 0: 65: 35, 63–63.1 min liner gradient from 0% B to 15% B, 63.1–68 min A: B: C: *D* = 0: 15: 55: 30, 68–70 min liner gradient from 55% C to 50% C, 70–82 min A: B: C: *D* = 0: 15: 50: 35, 82–85 min liner gradient from 15% B to 0% B, 85.1–100 min A: B: C: *D* = 0: 0: 40: 60, 100–100.1 min liner gradient from 0% A to 100% A and 100–120 min, A: B: C: *D* = 100: 0: 0: 0. Injection volume was 20 μL. All column temperatures were maintained at 35 °C. Fluorescence detection was performed at 530 nm with an excitation wavelength at 470 nm.

2.5. Measurement of DDO activity

The activity of DDO in the forebrain and kidney (a high DDO activity tissue) was measured as previously reported (Yamada et al., 1988; D'Aniello et al., 1993). Briefly, tissue was homogenized