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Persistent increase of D-aspartate in D-aspartate oxidase mutant mice induces a precocious hippocampal age-dependent synaptic plasticity and spatial memory decay

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Abstract

The atypical amino acid D-aspartate (D-Asp) occurs at considerable amounts in the developing brain of mammals. However, during postnatal life, D-Asp levels diminish following the expression of D-aspartate oxidase (DDO) enzyme. The strict control of DDO over its substrate D-Asp is particularly evident in the hippocampus, a brain region crucially involved in memory, and highly vulnerable to age-related deterioration processes. Herein, we explored the influence of deregulated higher D-Asp brain content on hippocampus-related functions during aging of mice lacking DDO ($Ddo^{-/-}$). Strikingly, we demonstrated that the enhancement of hippocampal synaptic plasticity and cognition in 4/5-month-old $Ddo^{-/-}$ mice is followed by an accelerated decay of basal glutamatergic transmission, NMDAR-dependent LTP and hippocampus-related reference memory at 13/14 months of age. Therefore, the precocious deterioration of hippocampal functions observed in mutants highlights for the first time a role for DDO enzyme in controlling the rate of brain aging process in mammals.

Keywords: D-Aspartate; NMDA receptors; Synaptic plasticity; Reference memory; Hippocampus; Brain aging

1. Introduction

Today a clear role for the atypical amino acid D-aspartate (D-Asp) is mainly limited to endocrine physiology (D'Aniello, 2007; Huang et al., 2006), while its nervous system function remains to be clarified. D-Asp is highly expressed in the whole brain during embryonic and perinatal periods, and strongly decreases during adulthood (Hashimoto et al., 1993; Sakai et al., 1998; Wolosker et al., 2000). The drastic postnatal decline of D-Asp levels in the mammalian brain has been correlated with the onset of D-aspartate oxidase (DDO) activity (Van Veldhoven et al., 1991), the only enzyme that selectively metabolizes this D-amino acid (D'Aniello et al., 1993) and directly regulates its endogenous levels (Errico et al., 2006; Huang et al., 2006). To understand the biological significance of D-Asp in mammals, different mouse models with deregulated high levels of this D-amino acid have been recently generated, either by ablating *Ddo* gene ($Ddo^{-/-}$ mice) or through chronic oral administration of D-Asp (Errico et al., 2008a,b, 2006; Huang et al., 2006). Overall, such experimental manipulations produced a significant increase of D-Asp in adult brain regions, such as hippocampus, cortex

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and striatum, which physiologically display very low levels of this molecule because of strong local DDO activity (Schell et al., 1997). In accordance with early *in vitro* binding studies (Fagg and Matus, 1984; Olverman et al., 1988), higher brain levels of D-Asp in these mice have highlighted a modulatory role for this D-amino acid at N-methyl D-aspartate receptors (NMDARs) (Errico et al., 2008a,b). Accordingly, augmented D-Asp content is able to completely suppress Long-Term Depression (LTD) in the striatum, and to increase NMDAR-dependent hippocampal Long-Term Potentiation (LTP) and spatial memory (Errico et al., 2008a,b).

The hippocampus, a structure involved in learning and memory processes, is a brain region known to be vulnerable to age-related loss of functional synapses and NMDAR-mediated responses, including LTP (Rosenzweig and Barnes, 2003). Therefore, based on the ability of D-Asp to considerably enhance hippocampal synaptic plasticity in adult mice, in this work we evaluated how this in-embryo-enriched molecule and, indirectly, its regulating enzyme DDO influence the decay of the above mentioned functions during aging. Remarkably, our findings indicate that increased hippocampal levels of D-Asp in $Ddo^{-/-}$ mice induce striking biphasic age-related changes on the state of ERK1/2 phosphorylation, coupled with potentiated LTP and spatial memory abilities during adulthood and accelerated deterioration of hippocampus-dependent processes at the beginning of senescence phase.

Overall, these findings suggest that free D-Asp acts as an endogenous NMDAR agonist in the mammalian brain and, in turn, unveil a biological role for the enzyme DDO in preventing, by strictly controlling its substrate, an overusage of NMDARs thought to trigger an early deterioration of hippocampus-dependent functions.

2. Materials and methods

2.1. Animals

Knockout mice for the *Ddo* gene were generated as previously described (Errico et al., 2006). Male wild type ($Ddo^{+/+}$) and knockout ($Ddo^{-/-}$) mice of approximately 4–5, 9–10 and 13–14 months of age were used in this study and derived from mating of heterozygous ($Ddo^{+/-}$) mice, back-crossed to F5 generation to C57BL/6J strain (Banbury Conference on genetic background in mice, 1997). Animals were genotyped by polymerase chain reaction according to Errico et al. (2006). Mice were housed in groups ($n = 4$–5) in standard cages (29 cm × 17.5 cm × 12.5 cm) at constant temperature (22° ± 1 °C) and maintained on a 12/12 h light/dark cycle, with food and water *ad libitum*. Experiments were conducted in conformity with protocols approved by the veterinary department of the Italian Ministry of Health and in accordance with the ethical and safety rules and guidelines for the use of animals in biomedical research provided by the relevant Italian laws and European Union's directives (no. 86/609/EC). All efforts were made to minimize the animal's suffering. The experimental schedule was designed so that animals involved in behavioral tasks were then channelled into subsequent neurochemical and electrophysiological studies while biochemical and immunohistochemical experiments were executed on naïve animals. For each behavioral task performed, we used an independent group of mice in order to avoid the chance that behavioral responses were altered by prior test history (McIlwain et al., 2001).

2.2. Drugs

D-(−)-2-Amino-5-phosphonoeptanoic acid (AP5), D-aspartate, 5S,10R-(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5,10-imine maleate (MK801), cadmium chloride, L-β-threo-benzyl-aspartate (DL-TBOA), nifedipine and tetrodotoxin (TTX) were purchased from Sigma–Aldrich (Milan, Italy); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (RS)-a-methyl-4-carboxyphenylglycine (MCPG) from Tocris Cookson Ltd. (Bristol, UK).

2.3. HPLC analysis

Mice were killed and the hippocampus dissected and stored at −80 °C. The determination of D-Asp content was performed by HPLC technique, based on the diastereomeric separation of D-Asp from the L-form and other L-amino acids, as previously described (D'Aniello et al., 2000). Data was analyzed using two-way analysis of variance (ANOVA), followed by the appropriate *post hoc* comparison.

2.4. Western blotting

$Ddo^{+/+}$ and $Ddo^{-/-}$ mice were killed and their heads immediately immersed in liquid nitrogen for 5–6 s, as previously described (Svenningsson et al., 2000). The brains were then removed and the hippocampi dissected out within 20 s on an ice-cold surface, sonicated in 200 μl of 1% SDS and boiled for 10 min. The effectiveness of this extraction procedure in preventing protein phosphorylation and dephosphorylation, hence ensuring that the level of phosphoproteins measured *ex vivo* reflects the *in vivo* situation, has been tested previously (Errico et al., 2008c; Santini et al., 2009; Svenningsson et al., 2000). Aliquots (2 μl) of the homogenate were used for the protein determination using Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Equal amounts of total proteins (40 μg) for each sample were loaded onto 10% polyacrylamide gels. Proteins were separated by SDS-PAGE and transferred overnight to membranes (PVDF) (Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were immunoblotted overnight using selective antibodies against NR1 (1:1000; Sigma, St. Louis, MO), NR2A (1:1000; Sigma), NR2B (1:1000; Upstate, Lake Placid, NY), GluR1 (1:5000; Chemicon, Temecula, CA), GluR2/3 (1:1000; Upstate), phospho-Thr286-