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New insights on the influence of free D-aspartate metabolism in the mammalian brain during prenatal and postnatal life

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ABSTRACT

Free D-aspartate is abundant in the mammalian embryonic brain. However, following the postnatal onset of the catabolic D-aspartate oxidase (DDO) activity, cerebral D-aspartate levels drastically decrease, remaining constantly low throughout life. D-Aspartate stimulates both glutamatergic NMDA receptors (NMDARs) and metabotropic Glu5 receptors. In rodents, short-term D-aspartate exposure increases spine density and synaptic plasticity, and improves cognition. Conversely, persistently high D-Asp levels produce NMDAR-dependent neurotoxic effects, leading to precocious neuroinflammation and cell death. These pieces of evidence highlight the dichotomous impact of D-aspartate signaling on NMDAR-dependent processes and, in turn, unveil a neuroprotective role for DDO in preventing the detrimental effects of excessive D-aspartate stimulation during aging. Here, we will focus on the *in vivo* influence of altered D-aspartate metabolism on the modulation of glutamatergic functions and its involvement in translational studies. Finally, preliminary data on the role of embryonic D-aspartate in the mouse brain will also be reviewed.

1. Introduction

Several free D-amino acids are present in the Central Nervous System (CNS) and peripheral organs of mammals [1–4]. The long-established existence of flavoenzymes responsible for their degradation [5] supported the idea that these atypical amino acids might have a biological meaning in mammals.

Among the free D-amino acids detected in mammals, D-serine (D-Ser) and D-aspartate (D-Asp) are the most abundant in the CNS [6]. Functionally, D-Ser acts as a physiological co-agonist of NMDA receptors (NMDARs) at central excitatory synapses. Changes in cerebral D-Ser concentrations affect the activation of the glutamatergic system by modulating NMDAR signaling and, ultimately, influencing fundamental neuronal processes and behaviours [7–9]. Therefore, abnormal brain and cerebrospinal fluid (CSF) levels of this D-amino acid are associated to different neurological and psychiatric pathologies, including post-traumatic stress disorders, traumatic brain injury, amyotrophic lateral sclerosis, Alzheimer's disease (AD) and schizophrenia [9–15].

In contrast to the well-recognized role of D-Ser on NMDAR-related transmission, the biological relevance of the endogenous free D-Asp in the mammalian CNS is much less detailed. In this review article, we will focus on the emerging neuroprotective role of the enzyme involved in D-Asp degradation, D-aspartate oxidase (DDO), along with the epigenetic events controlling the time-dependent expression of *Ddo* gene in the mammalian brain. Moreover, we will describe the findings concerning D-Asp metabolic alterations in neurological and psychiatric disorders, and the potential therapeutic effects of D-Asp administration in translational studies. Finally, we will show the earliest findings on the possible role of embryonic cerebral D-Asp metabolism in modulating brain morphology and functioning.

2. The time-dependent occurrence of D-aspartate in the mammalian brain is regulated by the enzyme D-aspartate oxidase

D-Asp displays a distinctive temporal pattern of occurrence since it is abundant at prenatal stages and drastically decreases after birth [16–23]. Although this trend is common to both human and rodent brains, HPLC analyses suggest that the relative abundance of D-Asp,

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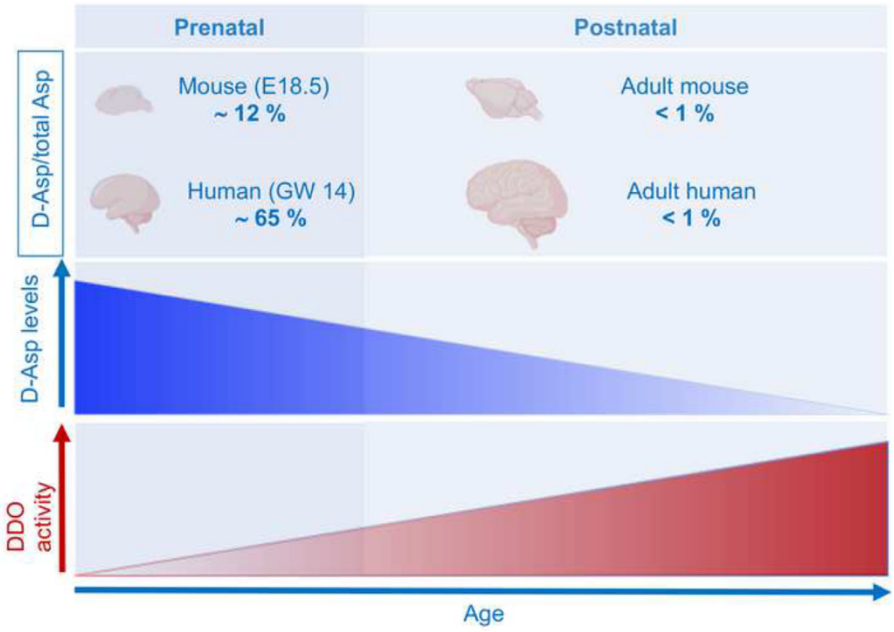

Fig. 1. Time-dependent relationship between cerebral D-aspartate content and enzymatic DDO activity. Cerebral D-Asp levels in the mammalian brain are the highest during the prenatal phase. However, the prenatal levels of D-Asp, with respect to the total Asp content (D-Asp/total Asp ratio), substantially change between species. Indeed, the relative abundance of D-Asp in the embryonic human brain is considerably higher than in the embryonic mouse brain (approximately 65% *vs* 12%, upper panel). During postnatal life, D-Asp levels progressively decrease (middle panel). In this phase, the relative abundance of D-Asp is very low in both mouse and human brain (upper panel). D-Asp reduction is a consequence of the increased enzymatic DDO activity (lower panel). Abbreviations: E18.5, embryonic day 18.5; GW14, gestational week 14.

expressed as the ratio between the D-enantiomer and the total content of the amino acid (D + L), might significantly differ between these two species during prenatal life (Fig. 1). Indeed, while in the human prefrontal cortex (PFC) D-Asp levels reach as high as the 65% of total Asp content (in the first trimester of pregnancy) [17], in the whole mouse brain, D-Asp levels never exceed the 12% of total Asp (at embryonic day 18.5) [23]. However, it is important to remark that the difference in prenatal D-Asp levels between humans and mice may, in part, be due to the difference in the tissues analyzed (PFC and whole brain from human and mouse, respectively). Therefore, further studies in homologous brain regions of humans and mice are needed to confirm or clarify the extent of the variations in relative D-Asp abundance existing between embryonic human and mouse brain.

The emergence of D-Asp in the developing brain follows specific spatial patterns of distribution. Indeed, immunohistochemical experiments showed that D-Asp emerges in the rat brain at embryonic day 12 in the ventrocaudal regions of the forebrain, in the midbrain and hindbrain while it spreads to the whole brain and cortical areas towards the end of gestation [21]. Interestingly, during the embryonic phase, D-Asp is initially localized in the cytoplasm of migrating neuroblasts and then shifts from the cell body to axons once neuroblasts reach their final regional position. The changes in intracellular D-Asp localization observed in the rat prenatal brain may unveil multiple roles for this D-amino acid in neural cells during development, thus further increasing the interest for studying such a molecule.

As for other amino acids, convergent biosynthetic and degradative pathways regulate D-Asp metabolism in living organisms. Previous observations supported the existence of a specific mammalian aspartate racemase able to convert L-Asp in D-Asp in the brain [24]. However, further studies using homologous recombination strategies failed to confirm such evidence [25,26]. At present, although the precise mechanisms of D-Asp biosynthesis remain unclear, recent findings have suggested that the enzyme Serine racemase (SR), responsible for D-serine biosynthesis [27], may have a role also in D-Asp generation within specific brain regions [28,29] (Fig. 2).

With regard to the catabolic pathway, it is well-known that D-aspartate oxidase (DDO or DASPO EC 1.4.3.1), a flavin adenine dinucleotide-containing enzyme, degrades D-Asp in the peroxisomes producing α-oxaloacetate, H_2O_2 and NH_4^+ ions [30–33] (Fig. 2). DDO is inactive towards basic and neutral D-amino acids, including D-Ser [30,32], which are degraded by a flavoenzyme homologous to DDO, D-amino acid oxidase (DAAO, EC 1.4.3.3) [34,35].

3. Regulation of *D-aspartate oxidase* gene expression in the mouse and human brain

Consistent with the robust time-dependent decrease of cerebral D-Asp levels, enzymatic DDO activity sharply increases in the brain from birth to adulthood [36] (Fig. 1). Molecular studies have revealed that the postnatal expression of DDO protein is most likely regulated at the transcriptional level since *Ddo* mRNA content dramatically rise in the whole mouse brain from embryonic to adult stage [20,23,37]. Based on this evidence, analysis of mRNA amount and methylation studies have been carried out to investigate the regulatory mechanisms at the basis of the cerebral *Ddo* gene expression during lifespan. In this regard, a work performed in the whole mouse brain at different prenatal and postnatal stages revealed that increased *Ddo* transcription is tightly associated with progressive temporal demethylation within the *Ddo* putative promoter region (at 8 CpG residues surrounding the transcription start site, from −363 to +113 bp) [20]. In agreement with a regulatory role of methylation on gene expression, experiments of azacytidine-mediated DNA demethylation showed that this drug was able to activate *Ddo* gene in primary neurons deriving from the embryonic mouse cortex [20]. Following these early studies, other investigations have evaluated in more detail the involvement of methylation on *Ddo* gene expression, as well as on the expression of the genes involved in D-serine metabolism, such as *Daao*, *Sr* and *G72* [38], both in the mouse and human brain [39–42].

The state of DNA methylation within *Ddo* gene promoter region has been deeply explored among different cell types and brain areas at various developmental stages taking advantage of a novel ultradeep methylation analysis type [40]. In particular, the analysis of specific combinations of methylated CpGs, defined as “epialleles”, indicated the presence of high rate of cell to cell heterogeneity of *Ddo* methylation profiles. Neurons, oligodendrocytes, astrocytes, and microglial cells display cell-type-specific methylation features at *Ddo* promoter, indicating that epialleles distribution could mark the cell-type identity. Moreover, undifferentiated embryonic stem cells (ESCs) at *Ddo* locus exhibited an apparently disorganized distribution of epialleles combinations, with a slight prevalence of monomethylated molecules that evolve in a specific and highly reproducible rearrangement of the epiallele frequency distribution profile upon neural differentiation

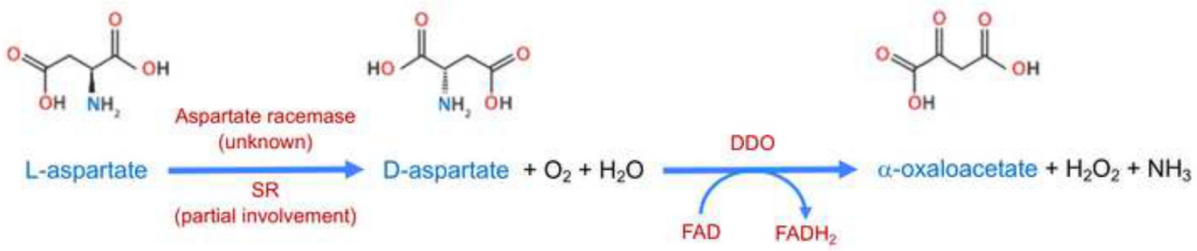

Fig. 2. Figure 1 Schematic pathway of the biochemical metabolism of D-aspartate in the mammalian brain. The mechanisms regulating D-Asp biosynthesis are still unknown. Serine racemase (SR) could be partially involved in D-Asp production. The existence of a selective enzymatic activity converting L-Asp into D-Asp has been so far only hypothesized. Conversely, the enzyme responsible for D-Asp degradation, D-aspartate oxidase (DDO), has long been known. DDO catalyzes the deaminative oxidation of D-Asp in the presence of O_2 and H_2O, producing the ketoacid α-oxaloacetate, hydrogen peroxide (H_2O_2), and ammonia (NH_3). In this reaction, the coenzyme flavin adenine dinucleotide (FAD) serves as a hydrogen acceptor.

[40]. Therefore, these heterogeneous patterns appeared to be non-stochastic among different cells, developmental stages and brain areas, thus suggesting the existence of predetermined methylation trajectories. Taken together, these methylation studies highlighted the importance of postnatal cycling epigenetic events in the proper control of *Ddo* transcription.

Based on these observations, the relationship between the status of *DDO* promoter methylation and mRNA expression in the human brain, under both physiological and psychiatric conditions, was also investigated [42]. In particular, a comprehensive *DDO* methylation analysis in three different *post-mortem* brain areas (dorsolateral prefrontal cortex, hippocampus and cerebellum) of patients with schizophrenia and non-psychiatric controls suggested the existence of peculiar epigenetic brain region signatures for this gene. In line with this, despite the lack of main alterations between diagnosis groups, *DDO* promoter region exhibited specific methyl-CpG distribution that allowed to distinguish among the different brain regions analyzed [42], thus revealing the involvement of epigenetic mechanisms on the control of D-Asp metabolism in the human brain.

Less characterized is the post-transcriptional regulation of *DDO* gene, *i.e.* at splicing, transcript stability and protein synthesis levels. Indeed, like promoter methylation, these molecular mechanisms represent potential regulatory processes for downstream DDO protein synthesis and activity. Among gene expression regulation, microRNAs represent crucial players due to their ability to regulate transcript levels in time- and cell-specific manner. Remarkably microRNAs play a significant role for nervous system development and physiology since they are involved in the spatiotemporal regulation of neuronal gene expression and ultimately neural differentiation, development and activity of neuronal networks [43]. Interestingly, an *in silico* preliminary analysis of the 3'UTR sequence of human *DDO* and other genes involved in D-amino acid metabolism, as *SR* and *DAAO*, disclosed the occurrence of several potential binding sites for microRNAs that are expressed in the CNS, and bind to sequences whose conservation is, in some cases, restricted to mammals (http://www.targetscan.org). Specifically, human *DDO* 3'UTR contains several sites that are evolutionary poorly conserved sites for miRNA families conserved among vertebrates and mammals (TargetScan), with few microRNAs experimentally validated (miRTarBase). From a comparative analysis of predicted binding sites among human, rat and mouse *DDO* 3'UTRs, very few sequences resulted as shared sites for microRNAs mediated regulation. Lack of evolutionary conservation of 3'UTR sequences may account for species-specific mechanisms of finely tuned expression regulation. On the other hand, the occurrence of binding sites for miRNAs in both human and rodents could represent a valuable aspect to study mechanisms of gene expression regulation in those animal models that could be then translated to human studies.

4. Changes in cerebral D-aspartate levels impact on NMDAR-dependent functions in adulthood

Several biochemical and electrophysiological studies have demonstrated that D-Asp binds to and stimulates the ionotropic NMDAR subclass of L-glutamate receptors [44–48]. Previous pharmacological evidence has shown that D-Asp is also able to activate metabotropic Glu5 receptors (mGluR5). Indeed, the stimulation of polyphosphoinositide hydrolysis mediated by mGluR5 is enhanced in rat hippocampal or cortical slices by D-Asp application, or inhibited by the addition of MPEP, a selective mGluR5 blocker [49]. Moreover, microdialysis experiments carried out in the PFC of freely moving mice revealed that D-Asp is present at nanomolar concentrations in the extracellular space and its release occurs *via* Ca^{2+}-dependent mechanisms [20,50]. In support of these *in vivo* results, previous *in vitro* findings showed that D-Asp can be stored in secretory granules [51], released through vesicular Ca^{2+}-mediated exocytosis [22,51–53] and taken up at both nerve terminals and glia *via* Na^+/K^+ electrochemical L-Glu/L-Asp transporter system [54–56]. Besides a direct effect of D-Asp on the activation of postsynaptic NMDAR and mGluR5, recent research showed that exogenous administration of this D-amino acid evokes a considerable extracellular release of L-glutamate in the PFC of freely moving mice through the stimulation of presynaptic NMDA, AMPA and mGlu5 receptors [50].

The influence of D-Asp signaling on NMDAR-mediated transmission has been demonstrated in different rodent models characterized by increased endogenous D-Asp levels, obtained either by genetic *Ddo* ablation (*Ddo*$^{-/-}$) or chronic oral D-Asp administration. Investigations in these animal models during young adulthood revealed that the upregulation of cerebral D-Asp levels is always associated with increased NMDAR-dependent functions, including the enhancement of hippocampal long-term potentiation (LTP) [44–46,57]. The considerable NMDAR-dependent synaptic strengthening promoted by abnormally high D-Asp levels most likely explains the changes in dendritic spine morphology and cognitive skills reported in mice. Indeed, both *Ddo* gene ablation and chronic D-Asp treatment are associated with increased dendritic length and spine density in pyramidal neurons of the hippocampus and PFC [57], and with improved NMDAR-dependent spatial memory [44,45,47,58] (Fig. 3). The enhancing effect of D-Asp on the genesis of dendritic spines has been recently confirmed also *in vitro* through the application of this D-amino acid on rat hippocampal slices [59]. Taken together, these findings suggest that deregulation of D-Asp levels during adulthood has a structural and functional influence on the activation of glutamatergic cortico-hippocampal circuits involved in cognition (Fig. 3). In this line of reasoning, analysis of basal cerebral blood volume (CBV) by functional magnetic resonance imaging (fMRI) has revealed that both oral and intragastric D-Asp administration promote basal metabolic activity in fronto-hippocampal areas of adult mice [57] and rats [59], respectively.

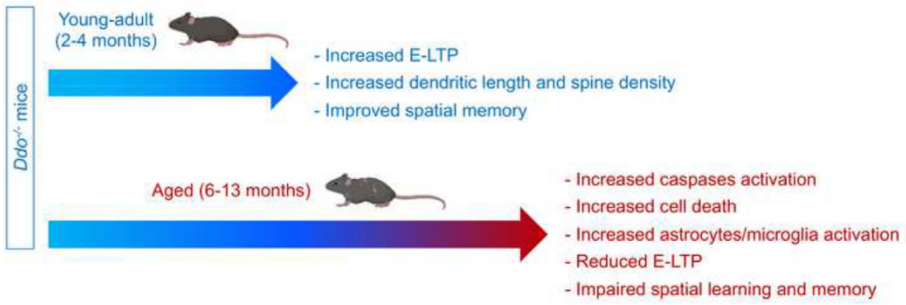

Fig. 3. Lack of DDO unmasks the dichotomous effect of cerebral D-aspartate on NMDA receptor-dependent functions. The study of genetic mouse models lacking DDO (*Ddo*$^{-/-}$ mice) has shown that constitutive upregulation of D-Asp content produces beneficial or harmful effects in a time-dependent manner. Increased content of D-Asp improves morphological, functional and cognitive processes dependent on NMDAR stimulation in young-adult *Ddo*$^{-/-}$ mice (2–4 months of age). Conversely, persistent increase of cerebral D-Asp content over longer periods of time (from six months of life onwards) has detrimental consequences in *Ddo*$^{-/-}$ mice, resulting in precocious brain aging. These findings highlight the importance of DDO as a crucial enzyme counteracting the potential deleterious consequences of excessive NMDAR stimulation through the regulation of cerebral D-Asp levels.

5. Prenatal D-aspartate content influences brain morphology and memory in adult mice

It is well established that glutamatergic neurotransmission is involved in several neurodevelopmental processes (including neurogenesis, proliferation, migration, differentiation and apoptosis/survival) through the early activation of NMDARs and mGluR5 [60–63]. Based on the ability of D-Asp to stimulate such L-glutamate receptor subclasses, a very recent work has described for the first time the influence of embryonic D-Asp depletion on adult brain morphology and behaviours, employing a novel genetic mouse model characterized by non-physiological, prenatal onset of *Ddo* gene expression [23]. To generate these animals, an inducible *Ddo* cassette was targeted into the genomic locus of *Rosa26*. The subsequent Cre-mediated removal of the stop cassette (triple poly-A), included in the recombinant DNA sequence, allowed to obtain mice with constitutive expression of *Ddo* driven by *Rosa26* promoter. *Ddo* knockin mice showed anticipated cerebral expression of *Ddo*, paralleled by a concomitant increase in enzymatic DDO activity that, in turn, caused the removal of cerebral D-Asp since the earliest phases of brain development. In line with a selective role of DDO on D-Asp catabolism, neurochemical results obtained by HPLC showed that *Ddo* overexpression in mice does not affect the prenatal and postnatal metabolism of other amino acids directly or indirectly involved in NMDAR activity, such as L-glutamate, L-Asp, D-Ser and L-Ser. Moreover, the selective depletion of embryonic D-Asp is unable to alter the expression levels of essential components of glutamatergic synapses in the adult brain, such as NMDAR and AMPAR subunits, mGluR5 and the scaffolding post-synaptic proteins, PSD-95 and Homer1b/c. Interestingly, *in vivo* results also demonstrated that *Ddo* knockin mice are viable, fertile and show improved spatial memory abilities, as revealed in the hidden-platform version of the Morris water maze and novel object recognition task. Such a behavioural phenotype in D-Asp-deficient mice seems paradoxical for two main reasons: 1) physiological NMDAR activation plays a central role in memory formation [64,65]; 2) cognitive ameliorations were previously found also in young-adult mice with increased cerebral D-Asp levels [44,45,47]. Therefore, it has been hypothesized that memory improvement in *Ddo* knockin mice could be independent from a direct effect of D-Asp depletion. Instead, it might be the consequence of unknown secondary/aspecific functions of DDO overexpression. In this regard, enzymatic activity assay revealed that *Ddo* knockin brains show excessive cerebral DDO activity (up to one hundred times higher than wild-type controls in developmental phases). Finally, no main neuroanatomical changes in the overall adult brain architecture, neuronal and myelin distribution were found in *Ddo* knockin mice. Nevertheless, in support of a role for D-Asp in brain development, a selective increase in the number of parvalbumin-positive interneurons was observed in the PFC of adult mice with early D-Asp depletion. Overall, the absence of more substantial morphological alterations in the mouse brain devoid of D-Asp could be explained by the physiological low relative abundance of this D-amino acid in the embryonic mouse brain (less than the 12% of total Asp content) (Fig. 1). Conversely, based on the high relative abundance of D-Asp in the human PFC (around the 65% of total Asp) (Fig. 1), it could be hypothesized that D-Asp signaling has a more profound neurodevelopmental influence in human rather than mouse brain.

5.1. Control of D-aspartate levels by D-aspartate oxidase has neuroprotective effects in the mouse brain during aging

It is well known that a physiological stimulation of NMDARs is crucial for promoting synaptic plasticity, brain connectivity and cognition [64,65]. Conversely, “too little” or “too much” activation of NMDARs can lead to neuronal death, being harmful to brain functioning. In particular, chronic elevation of extracellular L-glutamate levels can exceed the survival-promoting effect of synaptic NMDARs and activate extrasynaptic NMDARs, coupled to pro-death signaling, thus triggering cell death and related brain damages [66,67].

Based on the pharmacological ability of D-Asp to stimulate post-synaptic NMDAR activity, it has been demonstrated that persistent elevation of D-Asp content in *Ddo*$^{-/-}$ mice at elderly stages results in the precocious appearance of brain deterioration hallmarks in the hippocampus and PFC, including synaptic plasticity and memory deficits, caspases activation and cell death [20,45,68] (Fig. 3). These phenotypes were accompanied by early emergence of dystrophic microglia and reactive astrocytes [20,68] (Fig. 3), well-recognized cellular neuroinflammation markers involved in the progression of neurodegenerative disorders, including AD, multiple sclerosis (MS), amyotrophic lateral sclerosis and Parkinson's disease (PD) [69–71]. Overall, these observations disclose a physiological neuroprotective role for the catabolic enzyme DDO, which acts by hindering the detrimental cerebral effects that would be produced by uncontrolled, persistent elevation of D-Asp levels.

In support of a physiological role for DDO against precocious cell death caused by persistent D-Asp stimulation, a direct link between abnormally high D-Asp levels, NMDAR signaling, and neuronal demise has been recently established using *in vitro* primary cortical neurons [72]. In these cells, D-Asp is able to cause neuronal death in a time- and dose-dependent manner and this effect was blocked by MK801, a non-competitive NMDAR antagonist. In line with previous pharmacological studies [44], D-Asp-induced cell death was also dramatically attenuated by Ifenprodil, which blocks GluN2B-containing NMDARs, indicating that this GluN2 subunit plays a significant role in D-Asp-mediated toxicity. Similarly, MTEP, a selective inhibitor of mGluR5, was able to rescue a fraction of cortical neurons from D-Asp-mediated toxicity [72]. This is consistent with results showing that D-Asp-induced effects on neuronal physiology also involve this metabotropic receptor [49].

As reported in aged *Ddo*$^{-/-}$ animals [20,68], exposure to high D-Asp concentration induced severe toxicity in primary cortical neurons by activating apoptosis-promoting capase-3 and necrosis-associated release of lactate dehydrogenase (LDH) [72]. Furthermore, administration of D-Asp to primary cortical neurons induces the modulation of JNK phosphorylation through NMDAR stimulation in a time- and dose-

dependent manner. This effect confirms the influence of D-Asp exposure on neurotoxicity events modulated by NMDAR-dependent signaling, and it is in line with the crucial role of JNK activation in NMDAR-related oxidative stress and cell death [73,74]. Notably, altered JNK phosphorylation is associated with extensive Tau hypophosphorylation at PHF1 and AT8 sites in the PFC and hippocampus of aged *Ddo*$^{-/-}$ mice. Similarly, experiments in primary cortical neurons also revealed that D-Asp exposure induces MK801-responsive changes in Tau phosphorylation at the same sites analyzed in the cortex of *Ddo*$^{-/-}$ mice [72]. Overall, these observations suggest the existence of a functional link between increased D-Asp signaling, enhanced NMDAR activation and downstream changes in JNK and Tau phosphorylation state.

The results so far described in mouse models indicate that persistent D-Asp elevation due to the lack of DDO catabolism induces a dramatic NMDAR-dependent cell death and, in turn, exacerbates precocious synaptic plasticity deterioration and cognitive decline occurring during aging. However, what happens in wild-type aged animals if the cerebral D-Asp upregulation lasts only for a shorter time? This question has been addressed in a study in which D-Asp was administered to aged (12-month-old) C57BL/6 J mice for one month [44]. The results showed that short-term treatment with D-Asp was able to reverse the age-dependent LTP decay occurring in untreated elderly mice. Altogether, the results described in this section suggest that the upregulation of D-Asp levels may reveal to be either detrimental or beneficial for NMDAR-related aging processes depending on the time of D-Asp deregulation (Fig. 3). Consistent with this evidence, the enzymatic DDO activity plays a physiological role in limiting D-Asp content under a certain "danger threshold", thus preventing the potentially toxic effects of protracted D-Asp stimulation upon NMDAR-mediated signaling.

6. Altered D-aspartate metabolism in neurological and psychiatric disorders

Given the relevance of NMDAR and mGluR5 dysfunction in neurological and psychiatric pathologies [75–80], different works have evaluated whether metabolic D-Asp alterations could be involved in some of these disorders and in related animal models.

Dysfunctional L-glutamate-mediated transmission in basal ganglia circuit has long been related to PD and motor disturbance associated to L-3,4-dihydroxyphenylalanine (L-DOPA) treatment [81,82]. In this regard, a recent study suggested that such dysfunction also involves the metabolism of D-Asp [83]. Indeed, increased D-Asp levels have been found in the putamen of a primate PD model treated with the dopaminergic neurotoxin MPTP. Interestingly, such deregulation was absent in PD monkeys treated with L-DOPA. Besides D-Asp-related alterations, it should be pointed out that PD monkeys also showed a selective D-Ser reduction in the *substantia nigra*, compared to the unlesioned controls [83]. Since a previous work reported a beneficial effect of D-Ser add-on treatment on PD symptoms [84], the observation carried out in PD monkeys might suggest that the recovery of D-Ser levels in midbrain residual neurons is a therapeutic mechanism that contributes to restoring dysfunctional NMDAR-dependent transmission in patients. Interestingly, D-Ser but not D-Asp concentration was also found to be reduced in the cerebrospinal fluid of L-DOPA-free PD patients [83]. Overall, the changes in D-Asp and D-Ser levels found in the putamen and *substantia nigra* of PD monkeys let hypothesize the existence of potential adaptive metabolic mechanisms limiting NMDAR-dependent midbrain neurotoxicity in PD.

Different pre-clinical and clinical works in the last years have also evaluated the potential involvement of D-Asp metabolism in a psychiatric disorder like schizophrenia. This interest comes from two primary pieces of evidence: 1) schizophrenia is characterized by a documented glutamatergic hypofunctionality, which severely affects both NMDAR- and mGluR5-mediated signaling [75,76,78,79]; 2) the risk factors associated to schizophrenia are likely to arise during developmental periods [85–88] when cerebral D-Asp reaches the highest levels [16,17,19–21,23]. Two different works have so far analyzed the content of D-Asp in schizophrenia brains using two different cohorts of *post-mortem* samples [89,90]. Both studies revealed a substantial D-Asp reduction in prefrontal areas of schizophrenia patients (about 30–40% decrease, compared with non-psychiatric subjects), associated with increases in either *DDO* gene expression [89] or enzymatic DDO activity [90]. The potential influence of D-Asp metabolism in PFC circuits involved in schizophrenia has been indirectly supported also in a study performed on healthy individuals, which investigated the association of single nucleotide polymorphisms (SNPs) in *DDO* gene with functional prefrontal phenotypes relevant to schizophrenia [57]. The *in silico* section of this study (performed on 268 brain samples from the *post-mortem* collection bank, Braincloud (http://braincloud.jhmi.edu) [91]) showed the existence of an intronic *DDO* variant (rs3757351) in which the C allele predicts reduced *DDO* mRNA expression, compared to the T allele. The subsequent *in vivo* analyses performed in healthy individuals revealed that this SNP is also associated to a functional increase in prefrontal gray matter volume and greater prefrontal activity during working memory tasks, respectively measured by voxel-based morphometry (n = 152) and blood oxygen level-dependent (BOLD) fMRI (n = 143), compared to subjects with the T allele. Mouse models with increased D-Asp levels have served to shed further light on the impact of D-Asp on phenotypes relevant to psychiatric disorders. In this regard, it has been demonstrated that both adult *Ddo*$^{-/-}$ and D-Asp-treated mice (one- or two-month chronic oral administration) display reduced sensorimotor gating deficits, motor hyperactivity and ataxic responses after acute treatment with phencyclidine (PCP) [92,93] or other psychotomimetic drugs, like amphetamine and MK801 [47]. In addition to behavioural resiliency, PCP-treated *Ddo*$^{-/-}$ mice also show reduced functional activation of cortico-limbo-thalamic circuits, compared to PCP-treated wild-type mice, as measured by fMRI [93]. Moreover, in contrast to brain dysconnectivity found in animal models and patients with schizophrenia [94,95], it has been demonstrated that higher D-Asp content produces greater connectivity in cortico-hippocampal networks of both *Ddo*-deficient mice [93] and rats with intragastric administration of this D-amino acid [96]. In line with potential protective effect of D-Asp, another study in preclinical models revealed that the administration of the second-generation antipsychotic drug, olanzapine, stimulates the cortical release of both D-Asp and L-glutamate. These data were supported by *in vitro* enzymatic assays showing that olanzapine inhibits the activity of both human and murine recombinant DDO enzymes [50]. Based on the ability of D-Asp to evoke L-glutamate release [50], these findings let hypothesize that the mechanism of action of olanzapine may involve the enhancement of L-glutamate-mediated transmission through the regulation of D-Asp catabolism.

7. Applications of D-aspartate supplementation in translational studies

The association of several neurological and neuropsychiatric disorders with synaptic defects depending on glutamatergic system dysfunctions [75–80] has led to assess whether supplementation with D-Asp may prove to show therapeutic benefits.

The influence of D-Asp signaling on glutamatergic synaptic plasticity in animal models has been exploited in a recent clinical trial carried out in patients with MS [97]. This study was based on the principle that improving the efficiency of synaptic transmission may affect clinical recovery in progressive forms of MS, characterized by reduced synaptic plasticity reserve. Interestingly, the authors found that four-week oral D-Asp intake is sufficient to enhance transcranial magnetic stimulation-induced LTP and intracortical facilitation in progressive MS patients, suggesting an improvement in synaptic plasticity reserve and trans-synaptic gluatamatergic transmission [97]. The potential influence of D-Asp in the pathophysiological mechanisms of MS has been also addressed in preclinical models. Indeed, based on the involvement of glutamatergic system on the maturation of oligodendrocyte precursor

cells and myelin regeneration, it has been recently demonstrated that D-Asp administration stimulates oligodendrocytes differentiation in primary cell cultures and attenuates demyelination in a cuprizone mouse model of MS [98]. Other beneficial effects of oral D-Asp treatment have been found in a mouse model of experimental autoimmune encephalomyelitis (EAE). In these mice, exogenous D-Asp administration is associated with an overall reduction of EAE-related indexes, including clinical disease severity score, histopathological brain inflammation, serum glutathione reductase activity and interleukin 6 levels [99]. Taken together, these studies might pave the way for future researches addressing the clinical effects of D-Asp on the onset and progression of MS-related disabilities.

Inappropriate L-glutamate-mediated signaling has been linked to chronic pain conditions [100,101]. A recent study investigated the effects of one-month D-Asp supplementation to a mouse model of neuropathic pain induced by the spared nerve injury of the sciatic nerve. The results showed that exogenous D-Asp is able to reduce chronic neuropathic pain and related motor coordination, sensorial and cognitive symptoms, likely through the restoration of glutamatergic synaptic transmission within the PFC and hippocampus [102].

Given the neurotoxic effects of persistent D-Asp exposure in mouse models, possible disadvantages of D-Asp supplementation should be taken into account to develop appropriate administration schedules, based on short-term/intermittent treatments. However, studies performed so far in humans have shown that D-Asp administration has no toxicological consequences and does not affect parameters related to the skeletal muscle mass or hormonal biomarkers associated with hypothalamic-pituitary-gonadal axis [103–107]. As an alternative to oral D-Asp treatment, new therapeutic strategies focused on compounds with inhibitory activity against DDO are also being evaluated to improve cerebral D-Asp availability and reduce potential side-effects associated with excessive D-Asp stimulation [108].

8. Conclusions and future perspectives

While the significance of embryonic cerebral D-Asp has been only recently addressed, the functional relevance of the physiological D-Asp reduction at adulthood has been the subject of several studies. Based on the limited current knowledge, it will be important to further investigate the role of embryonic D-Asp in animal models. Moreover, as discussed in the previous sections of this review, it will be mandatory to understand the importance of precocious D-Asp in human brain development. Indeed, the high relative abundance of D-Asp in the human embryonic brain suggests that this D-amino acid might have a role in early cerebral processes involved in neurodevelopmental syndromes, like schizophrenia and autism spectrum disorders. In this regard, future studies might unveil whether eventual *DDO* gene aberrations associated with increased *DDO* gene expression and, hence, with cerebral down-regulation of D-Asp levels, are linked to the emergence of neurodevelopmental disorders.

In line with the excitotoxic effect produced by abnormally high NMDAR stimulation, we have herein described that persistent exposure to elevated D-Asp levels are able to trigger L-glutamate-mediated signaling pathways implicated in neurodegeneration in cell systems and mice, thus producing deleterious effects on neuronal survival, synaptic plasticity and, finally, on cognitive skills during aging. These findings highlight the importance of DDO catabolism in preventing the deleterious consequences of long-lasting glutamatergic stimulation through the control of post-natal D-Asp levels. Like in animal models, synaptic plasticity and cognitive abilities show also in humans a progressive temporal decline. Therefore, extending the research from preclinical models to clinical practice will help to unveil whether DDO-dependent deregulations of D-Asp levels are involved in physiological forms of brain aging, cognitive frailty conditions and/or neurodegenerative disorders. Besides D-Ser and D-Asp, another D-amino acid, D-glutamate, is gaining attention for its potential involvement in cognitive impairment of patients with AD and mild cognitive decline [14,15]. In this regard, the assessment of D-Asp interactions with D-serine and D-glutamate in neurodegeneration could be an interesting research issue to be developed in future studies.

Based on the crucial temporal relationship between catabolic DDO activity and endogenous D-Asp content, a recent line of research has focused on the comprehension of the mechanisms regulating *Ddo* gene expression. At present, promoter-mediated methylation has emerged as an epigenetic mechanism used by neural cells to control the time-dependent *Ddo* gene expression and to confer, in turn, neuroprotection against the potential NMDAR-dependent damages caused by excessive D-Asp stimulation. In this context, the dissection of the mechanisms involved in transcriptional and post-transcriptional *DDO* expression could shed light on metabolic D-Asp variations that have been so far associated with neuropsychiatric disorders, including schizophrenia, or potentially linked to aging processes and neurodegenerative disorders.

In conclusion, despite the recent advances, there is still a long way to go for fully comprehending the role of D-Asp and its catabolic enzyme, DDO, in the mammalian brain. The deepening of basic research and clinical application issues will help to further comprehend the unresolved questions related to D-Asp metabolism and the potentialities of D-Asp supplementation in the attempt to identify new therapeutic targets for L-glutamate-mediated brain dysfunctions.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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