

New insights on the role of free D-aspartate in the mammalian brain

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Abstract Free D-aspartate (D-Asp) occurs in substantial amounts in the brain at the embryonic phase and in the first few postnatal days, and strongly decreases in adulthood. Temporal reduction of D-Asp levels depends on the postnatal onset of D-aspartate oxidase (DDO) activity, the only enzyme able to selectively degrade this D-amino acid. Several results indicate that D-Asp binds and activates *N*-methyl-D-aspartate receptors (NMDARs). Accordingly, recent studies have demonstrated that deregulated, higher levels of D-Asp, in knockout mice for *Ddo* gene and in D-Asp-treated mice, modulate hippocampal NMDAR-dependent long-term potentiation (LTP) and spatial memory. Moreover, similarly to D-serine, administration of D-Asp to old mice is able to rescue the physiological age-related decay of hippocampal LTP. In agreement with a neuromodulatory action of D-Asp on NMDARs, increased levels of this D-amino acid completely suppress long-term depression at corticostriatal synapses and attenuate the prepulse inhibition deficits produced in mice by the

psychotomimetic drugs, amphetamine and MK-801. Based on the evidence which points to the ability of D-Asp to act as an endogenous agonist on NMDARs and considering the abundance of D-Asp during prenatal and early life, future studies will be crucial to address the effect of this molecule in the developmental processes of the brain controlled by the activation of NMDARs.

Keywords D-Amino acids · *N*-methyl D-aspartate receptor · Synaptic plasticity · Learning and memory · Sensorimotor gating

Introduction

Homochirality of amino acids confers proteins their unique three-dimensional shape that allows the discrimination of ligands and substrates, hence permitting a precise execution of biochemical processes. Although L- and D-amino acids display the same chemical and physical properties, only L-enantiomers were selected during evolution as the exclusive building units of proteins. If such a priori selection had not occurred, a random inclusion of D- and L-amino acids into elongating peptide chain would have impeded the proper folding into active proteins. Therefore, the solution to the “dilemma of chirality” by evolution has likely guaranteed the advent and establishment of life. In support of this view, D-amino acids have only been detected in metabolically inert proteins owing to processes of spontaneous racemization occurring with age (Fujii 2002, 2005). The only exception to the rule of stereospecificity of amino acids was believed to be with regard to bacteria, which contain and utilize D-amino acids for the biosynthesis of cell wall peptidoglycans and antibiotics, thus conferring protection against attacks of protease and

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microorganisms (Corrigan 1969). The “unsolved question” of enantiomers’ choice in prokaryotes was explained, at first, as a heritage of the primordial life on Earth. In the light of such belief, the discovery of free D-aspartate (D-Asp) in the brain of cephalopods (D’Aniello and Guiditta 1977, 1978) and, later, in the brain and peripheral organs of rodents and man (Dunlop et al. 1986), sounded like a confutation of the established theory of homochirality in animal tissues. Some years after the first detection of free D-Asp in mammals, another D-amino acid, D-serine (D-Ser), was found in significant amounts in the rat brain (Hashimoto et al. 1992a, b). Over these last few decades, a variety of studies have then established that D-Asp, D-Ser and D-alanine are the only free D-enantiomers occurring in substantial levels in mammalian tissues (Hashimoto and Oka 1997).

Occurrence of D-aspartate in the mammalian brain

D-Ser is found in protoplasmic astrocytes (Schell et al. 1995) and neurons (Miya et al. 2008; Ding et al. 2011), and it is predominantly localized in the forebrain regions where it persists at high levels throughout postnatal life, following the expression pattern of the *N*-methyl-D-aspartate receptors (NMDARs) (Hashimoto et al. 1993a; Schell et al. 1997b). A large bulk of evidence has demonstrated that D-Ser acts as a co-agonist at the strychnine-insensitive glycine site of NMDARs and satisfies the main criteria for a neurotransmitter (Baranano et al. 2001). In contrast to D-Ser, much less is known about the role of D-Asp in mammals. D-Asp is present both in the central nervous system and endocrine glands, appearing with a peculiar temporal occurrence pattern. In endocrine glands, D-Asp content increases during postnatal and adult phase, in concomitance with their functional maturation; hence the appearance of D-Asp has been functionally correlated with the synthesis and/or the release of different hormones in rodents (Furuchi and Homma 2005; D’Aniello 2007).

Transient occurrence of D-Asp is also observed in the brain of mammals, even though in an inverse temporal manner, compared to peripheral organs. Indeed, despite constant levels of L-aspartate (L-Asp), D-Asp is highly concentrated during embryonic stage and in the first few days of life, but strongly decreases onwards (Dunlop et al. 1986; Neidle and Dunlop 1990; Hashimoto et al. 1993b, 1995; Sakai et al. 1998; Lee et al. 1999; Wolosker et al. 2000; Huang et al. 2008). It is surprising that the amount of D-Asp in the human frontal cortex at gestational week 14 even exceeds that of the corresponding L-form (D-Asp: 0.36 $\mu\text{mol/g}$; L-Asp: 0.21 $\mu\text{mol/g}$), to then dramatically diminish starting from postnatal stages (Hashimoto et al. 1993b). This analysis, obtained on human homogenates by

HPLC, has been followed by immunohistochemical studies in the rat brain, addressed to precisely define the localization of D-Asp during ontogenesis. At embryonic day 12 (E12), Sakai et al. (1998) described a faint immunostaining for D-Asp in the ventral area of the caudal forebrain, midbrain and hindbrain. In these regions, D-Asp appears in the cytoplasm of neuroblasts, which have already ceased proliferative activity, but not in mitotic cells. Only in the ventrocaudal forebrain, D-Asp immunoreactivity first appears in cell bodies, when neuroblasts migrate toward the outer layer of neural epithelium, and then shifts to axons once they have extended and the layer has been established. On E14, D-Asp staining becomes more intense and spreads to the basal telencephalon and olfactory bulbs. Between E18 and E20, D-Asp extends to the whole brain even involving the cerebral cortex in the process of stratification. In this area, D-Asp is observed in both cytoplasm and processes of neuroblasts of the intermediate zone, as well as in the marginal zone which mainly consists of axons. At postnatal day 0 (P0), Sakai et al. (1998) found a drastic reduction of D-Asp immunoreactivity throughout the brain. In another work by Wolosker et al. (2000), the occurrence of this D-amino acid was monitored in the rat brain starting from P0 until P28. Differently from Sakai et al. (1998), D-Asp was detected in considerable amount at birth. Intense immunoreactivity was observed in the forebrain, especially in the cerebral cortex, olfactory bulbs, thalamus and hypothalamus, and in part of the midbrain, while it was much less detectable in more posterior brain structures. In P2 brain slices, the staining extends caudally to the hindbrain and cerebellum. At these perinatal stages, D-Asp is concentrated in neuronal sets actively involved in developmental processes, such as the subventricular zone and the cortical plate of the cerebral cortex, pyramidal neurons of the CA1–CA3 area and the dentate gyrus of the hippocampus, as well as the granule cells of the external granular layer of the cerebellum, which have not yet reached their final localization. At P7, D-Asp immunostaining uniformly decreases in the brain, to almost disappear at P28. At this stage, Schell et al. reported that D-Asp immunostaining is visible only in restricted areas of the rat brain, such as the external plexiform layer of the olfactory bulbs, supraoptic and paraventricular nuclei of the hypothalamus, the medial habenula, some scattered brainstem nuclei and a subset of stellate and basket cells of the cerebellum (Schell et al. 1997a). At all phases and in all brain areas, differently from D-Ser, D-Asp is exclusively restricted to neuronal population, localized both in cytoplasm and fiber tracks, without any evident staining in glia (Schell et al. 1997a; Wolosker et al. 2000). It is noteworthy to highlight that in neurosecretory neurons of the rat hypothalamus, D-Asp has been detected in the nucleoli, in close association with heterochromatin (Wang et al. 2002).

This observation prompted authors to hypothesize that this D-amino acid may be involved also in the control of gene expression (Wang et al. 2002).

Metabolism of D-aspartate is regulated by the enzymes aspartate racemase and D-aspartate oxidase

The temporal and regional fluctuations of D-Asp levels in mammalian tissues highlight the existence of a mechanism for the modulation of the endogenous levels of this molecule. In fact, the supposed derivation of D-Asp from exogenous sources, such as the diet and intestinal bacterial flora, or from endogenous metabolically stable proteins, cannot explain the peaks of occurrence of this D-amino acid, especially in the brain where the transport of D-amino acids seems to be limited by the blood–brain barrier (Oldendorf 1973). In this regard, time-dependent accumulation of D-Asp in pheochromocytoma PC12 cells (Long et al. 1998) and conversion of [C^{14}]L-Asp into [C^{14}]D-Asp in primary neuronal cell cultures from rats (Wolosker et al. 2000) suggested that this D-amino acid can be autonomously synthesized in mammalian cells. In line with this evidence, the mammalian aspartate racemase (DR), which converts L-Asp to D-Asp (Kim et al. 2010), has been recently identified and cloned. Like serine racemase (SR), which generates D-Ser from the L-isomer (Wolosker et al. 1999), DR is a pyridoxal 5'-phosphate (PLP)-dependent enzyme and co-localizes with D-Asp in the adult mouse brain (Kim et al. 2010).

While a metabolic mechanism for D-Asp synthesis has been discovered only recently, it has long been known the existence of a catabolic enzyme, D-aspartate oxidase (DDO), able to selectively degrade bicarboxylic D-amino acids, such as D-Asp, D-glutamate and *N*-methyl D-aspartate (NMDA) (Still et al. 1949). DDO is a flavin adenine dinucleotide (FAD)-containing flavoprotein (Van Veldhoven et al. 1991) which oxidizes D-Asp, in presence of H_2O and O_2 , producing α -oxaloacetate, H_2O_2 and NH_4^+ ions (D'Aniello et al. 1993). DDO is inactive toward D-Ser and other D-amino acids that are substrates of the D-amino acid oxidase (DAO) (Martineau et al. 2006), another flavoenzyme belonging to the same family of DDO (Negri et al. 1992). The protein sequence of DDO possesses a functional C-terminal tripeptide for the targeting to peroxisomes (Amery et al. 1998), where this enzyme is supposed to oxidize D-Asp and release its catabolites (Beard 1990). Localization of DDO into peroxisomes, which contain catalase, allows the cell to safely remove H_2O_2 , a toxic product of D-amino acid metabolism (Katane and Homma 2010). DDO is highly expressed in the mammalian adult kidney, liver and brain (Katane and Homma 2010). In the brain, DDO is temporally expressed at postnatal phases, in

concomitance with the decrease in D-Asp levels, since the activity of this enzyme strongly increases from birth until 6 weeks of life (Van Veldhoven et al. 1991). In the adult brain, DDO is widely distributed, it is localized inversely to its substrate, D-Asp (Schell et al. 1997a), and is clearly dominant in neuronal population, even though a weak staining is also detectable in the cytoplasm and at the beginning of extensions of most astrocytes and oligodendrocytes (Zaar et al. 2002), where D-Asp is not detectable (Schell et al. 1997a; Wolosker et al. 2000). In line with a peroxisomal degradation of D-Asp, immunoelectron microscopy revealed that DDO protein is contained in single membrane-bound organelles, while it is absent in axon terminals and synaptic complexes (Zaar et al. 2002). However, in contrast to this last data, another work showed that both enzymatic activity and immunoreactivity of DDO were detectable in postsynaptic membranes isolated from rat brain (D'Aniello et al. 2010).

Pharmacological features of D-aspartate

In the past decade, a considerable number of studies have investigated the molecular binding between the different subclasses of L-glutamate (L-Glu) receptors and various L-Glu analogs with potential ligand affinity, in order to find new pharmacological agents with agonistic or antagonistic activity. Among these compounds, D-Asp emerged as a molecule able to bind NMDA receptors (NMDARs), owing to a relatively high affinity for their Glu binding site (Fagg and Matus 1984; Monahan and Michel 1987; Ogita and Yoneda 1988; Olverman et al. 1988; Ransom and Stec 1988). A comparative binding study in rat brain membranes demonstrated that the potency of D-Asp to displace the binding of the competitive NMDAR antagonist, [H^3]AP5, is the same for NMDA and tenfold lower than Glu (Olverman et al. 1988). In agreement with these in vitro binding assays, voltage-clamp recordings from CA1 pyramidal neurons in mouse hippocampal slices indicate that puff applications of D-Asp are able to induce inward currents, antagonized in a concentration-dependent and reversible manner by competitive and non-competitive blockers of NMDARs, such as D-AP5 and MK-801, respectively (Errico et al. 2008b). Interestingly, residual D-Asp-dependent currents still persist after the simultaneous perfusion of selective antagonists of NR2A, NR2B and NR2C–D subunits of NMDARs or even after the application of high concentrations of D-AP5 or MK-801 (Errico et al. 2008b, 2011a, b), thus suggesting the existence of NMDAR-independent currents triggered by this D-amino acid. In this respect, it has been shown that D-Asp can also activate mGlu5 receptors, coupled to polyphosphoinositide hydrolysis, in neonate rat hippocampal and cortical slices

(Molinaro et al. 2010). These observations expand the range of potential targets for D-Asp action, although the origin of the NMDAR-independent currents stimulated by D-Asp remains to be further clarified.

The ability of D-Asp to activate NMDAR-dependent transmission suggests a role for this D-amino acid in glutamatergic neurotransmission and leads to assume the existence of mechanisms to control its hypothetical release and subsequent removal from synapses. Cellular efflux of D-Asp has been extensively investigated in cell cultures (Homma 2007). In PC12 cell line and subclones, release of endogenous cytosolic D-Asp has been described to occur via a spontaneous and continuous efflux (Adachi et al. 2004; Koyama et al. 2006), or through a volume-sensitive organic anion channel, when PC12 cells are exposed to hypotonic conditions (Koyama et al. 2006; Furuchi et al. 2009). Moreover, in mouse primary cultures of neurons and astrocytes, exogenous radiolabeled D-Asp can be discharged via a heteroexchange mechanism that involves the Glu transporter (Anderson et al. 2001; Bak et al. 2003). The last, yet most suitable, mechanism to explain the neuronal efflux of D-Asp utilizes vesicular Ca^{2+} -mediated exocytotic process (Nakatsuka et al. 2001). The prerequisite of vesicular exocytosis is membrane depolarization, which can be experimentally reproduced by raising the extracellular concentration of K^+ , leading to activation of voltage-gated Ca^{2+} channel and to subsequent increase of intracellular Ca^{2+} . In a flat variant of PC12 cells, it has been observed that D-Asp co-localizes with dopamine in secretory granules and is massively released upon the addition of KCl in a medium supplemented with Ca^{2+} (Nakatsuka et al. 2001). Sensitivity of D-Asp release to different synaptic vesicle toxins further suggests that this D-amino acid may be accumulated in and released from synaptic vesicles (Cousin and Nicholls 1997; Nakatsuka et al. 2001). Besides in vitro experimental conditions, in vivo release of D-Asp has been so far investigated in the adrenal medulla of rats (Wolosker et al. 2000). In this tissue, D-Asp is specifically concentrated in the chromaffin cells, which produce and release epinephrine (Schell et al. 1997a) following activation of nicotinic cholinergic receptors. It has been shown that treatment of rats with nicotine depletes endogenous D-Asp with no significant change in the levels of L-Asp and L-Glu (Wolosker et al. 2000). A study by D'Aniello et al. supports the hypothesis that D-Asp could be physiologically released via Ca^{2+} -dependent exocytotic mechanism since it is shown that endogenous D-Asp is strongly enriched in synaptic vesicles, purified from rat brain. This fraction represents the 8.6 % of total amino acids (D'Aniello et al. 2010). Moreover, when synaptosomes are preloaded with radiolabeled D-Asp and L-Glu, stimulation with K^+ , in the presence of Ca^{2+} , is able to trigger the release of both amino acids (D'Aniello et al. 2010). In line with a vesicular

release of D-Asp, a study performed on whole brain synaptosomes of rats indicated that [^3H]L-Glu, [^3H]L-Asp and [^3H]D-Asp are largely stored in a common vesicular pool, although differences in their apparent affinities and interaction suggest that they may be accumulated by different transporters (Fleck et al. 2001).

The carrier system most likely responsible for the intracellular uptake of neuronal D-Asp is the L-Glu/L-Asp transporter, which utilizes the Na^+/K^+ electrochemical gradient to move excitatory amino acids against their concentration gradient. While, on the one hand, the high-affinity Na^+/K^+ -dependent carrier system recognizes exclusively the L-enantiomer of Glu, on the other, it displays the same affinity for L- and D-Asp and is able to transport both isomers in a stereobind fashion (Palacin et al. 1998). Such peculiar affinity for D-Asp, initially claimed as an “anomaly” of the excitatory amino acids transporters (Gazzola et al. 1981), has been widely exploited in the last few decades for neuroanatomical identification of glutamatergic projection neurons by using labeled D-Asp as a metabolically inert substrate for the high-affinity L-Glu transport system (Davies and Johnston 1976; Streit 1980; Storm-Mathisen and Wold 1981; Wilkin et al. 1982; Taxt and Storm-Mathisen 1984). Thus, in rat hippocampal slices, both [^3H]D-Asp autoradiography (Taxt and Storm-Mathisen 1984) and immunostaining with D-Asp antibody (Gundersen et al. 1993) evidenced that preloaded D-Asp showed a laminar distribution identical to L-Glu, corresponding to the terminal areas of the main excitatory fiber pathways of the hippocampus (Taxt and Storm-Mathisen 1984; Gundersen et al. 1993). In detail, D-Asp immunoreactivity is concentrated in nerve terminals of asymmetrical synapses and, to a lesser extent, in glial cell processes, but absent in postsynaptic spines, dendrites and soma (Gundersen et al. 1993). Uptake of D-Asp is regulated by the functional state of glutamatergic transmission, since synaptic [^3H]D-Asp transport is strongly affected by the lesion of glutamatergic projections (Taxt and Storm-Mathisen 1984; Shifman 1991). Differently from hippocampus, in cerebellar slices [^3H]D-Asp accumulates more in glia than in neuronal endings (Garthwaite and Garthwaite 1985), an evidence that suggests the existence of regional and subtype heterogeneity in the D-Asp transport system (Gundersen et al. 1993). The storage of exogenously supplied D-Asp in glial cells observed in brain slices does not seem to fit with the exclusive neuronal localization of D-Asp (Schell et al. 1997a; Wolosker et al. 2000). However, the weak expression of DDO enzyme in most astrocytes and oligodendrocytes of the human hippocampus (Zaar et al. 2002) allows to hypothesize that also under physiological conditions, extracellular D-Asp may be, at least in part, taken up by glia to be then rapidly metabolized by DDO. Overall, on the basis of the pharmacological

features of D-Asp described above, a stimulatory role for this D-amino acid at glutamatergic synapses could be supposed. In this view, taking into account the hypothesis formulated by Snyder and co-workers (Schell et al. 1997a), D-Asp generated through DR activity may be synaptically released and activate NMDARs, to be then rapidly captured by L-Glu transporter system into neurons and glia where it is finally degraded by DDO enzyme.

Mouse models with deregulated higher levels of D-aspartate

To comprehend the function of the catabolic enzyme DDO and, in turn, the in vivo consequences of deregulated D-Asp levels, in the last few years two knockout mouse models have been independently generated through the targeted deletion of the *Ddo* gene (Errico et al. 2006; Huang et al. 2006). Measurement of endogenous free D-Asp levels by HPLC in the whole brain and in peripheral organs of both knockout lines (*Ddo*^{-/-}) have revealed a strong increase in this D-amino acid, compared to the respective wild-type littermates, while no difference between genotypes was found in the content of bicarboxylic L-amino acids, L-Asp (Errico et al. 2006; Huang et al. 2006) and L-Glu (Huang et al. 2006). A more detailed neurochemical evaluation performed throughout the postnatal life in specific brain regions of *Ddo*^{-/-} mice, like the hippocampus, striatum, cortex, cerebellum and olfactory bulbs, has confirmed that D-Asp increases approximately 10- to 20-fold, in relation to the corresponding wild-type brain areas (Table 1) (Errico et al. 2008a, b, c, 2011a). Therefore, the existence in *Ddo*^{-/-} mice of D-Asp levels largely exceeding those of wild-type animals can be considered as the definitive proof that DDO is the enzyme responsible for the in vivo regulation of endogenous D-Asp levels. Interestingly, as a direct consequence of D-Asp increase, *Ddo*^{-/-} brains also display higher content of endogenous NMDA (Errico et al. 2006, 2011c), the *N*-methyl derivative of D-Asp. Besides HPLC analysis, also immunohistochemical observation has revealed a higher D-Asp labeling in cortical and hippocampal neurons, pinealocytes and, most of all, in the intermediate lobe of the hypophysis of *Ddo*^{-/-} mice (Huang et al. 2006). In this region of the pituitary gland, the dramatic increase in D-Asp levels in knockout mice is reflected by reduced expression of proopiomelanocortin and its derivative α -melanocyte-stimulating hormone (Huang et al. 2006). In turn, the decrease in such hormone levels in *Ddo*^{-/-} mice has been described to induce alterations of melanocortin-dependent behaviors, such as penile erection, self-care activity and appetite with a resulting reduction of successful mating, autogrooming and increase in body weight, respectively (Huang et al. 2006).

Besides *Ddo* gene targeting, an alternative approach has been developed to increase D-Asp levels, based on the oral administration of D-Asp to C57BL/6 mice. In this mouse model, HPLC detection has revealed a significant increase in D-Asp levels in the brain, even though to a lesser extent than in *Ddo*^{-/-} animals. Indeed, depending on the brain region examined and on the schedule of D-Asp administration, the endogenous levels of D-Asp in the hippocampus, cortex, striatum and cerebellum of treated animals increase approximately from two- to five-fold, compared to the same brain areas of untreated mice (Table 2) (Errico et al. 2008a, b, 2011b).

Influence of increased D-aspartate levels on hippocampus-related functions

Overall, both pharmacological and genetic approaches represent experimental tools to unlock the rigorous physiological control exerted by DDO over D-Asp in the adult brain and, in turn, to help in deciphering the in vivo consequences of altered levels of this D-amino acid. Among different brain areas, the hippocampus appears as an elective region in which to study the role of DDO and, in turn, to characterize the effects of deregulated, high levels of D-Asp. Indeed, the adult hippocampus displays very low concentrations of D-Asp paralleled by a strong expression of DDO (Schell et al. 1997a; Zaar et al. 2002), evidence suggesting that the physiological levels of D-Asp must be strictly regulated in this brain region. Moreover, the hippocampus is a region highly enriched with NMDARs, which represent the putative in vivo targets of D-Asp action and, notably, are widely recognized to play a crucial role in learning and memory processes.

Interestingly, in line with the in vitro pharmacological feature of D-Asp to stimulate glutamatergic transmission, electrophysiological data indicate that both adult *Ddo*^{-/-} and D-Asp-treated mice display enhanced NMDAR-dependent long-term potentiation (LTP) in the CA1 area of the hippocampus, compared to their respective controls. Indeed, increased brain levels of D-Asp facilitate the maintenance of hippocampal LTP and prevent synaptic depotentiation (Errico et al. 2008b, 2011a, b). Furthermore, experiments of intermittent oral administration of D-Asp to C57BL/6 animals indicates that the levels of NMDAR-dependent LTP in the hippocampus are strictly regulated by changes in the brain levels of this D-amino acid (Errico et al. 2011b). Even if the variations of hippocampal LTP are tightly regulated by changes in D-Asp brain levels, currently available neurochemical studies on total homogenates cannot clarify to what extent increased concentrations of D-Asp affect the putative extracellular levels of this D-amino acid. In this regard, in vivo microdialysis

Table 1 Free D-aspartate levels in different brain regions of *Ddo*^{-/-} mice

Brain area	Age of mice (month)	D-Asp content (nmol/g tissue)		References
		<i>Ddo</i> ^{+/+} mice	<i>Ddo</i> ^{-/-} mice	
Hippocampus	4–5	119 ± 12	1847 ± 180	Errico et al. (2011b)
	9–10	115 ± 7	1810 ± 129	
	13–14	140 ± 13	2048 ± 276	
Striatum	4	31 ± 6	325 ± 23	Errico et al. (2008a, 2011c)
Cortex		43 ± 6	817 ± 40	
Cerebellum		34 ± 8	447 ± 22	
Olfactory bulbs		33 ± 4	319 ± 34	

Table 2 Free D-aspartate levels in different brain regions of D-aspartate-treated mice

Brain area	20 mM D-asp oral administration delivery time (month)	Age of mice (month)	D-Asp content (nmol/g tissue)		References
			H ₂ O-treated mice	D-Asp-treated mice	
Hippocampus	1	2	22 ± 3	102 ± 11	Errico et al. (2008b)
	3	4.5	41 ± 2	99 ± 5	Errico et al. (2011a)
	12	13.5	71 ± 11	158 ± 29	
Striatum	2	3.5	17 ± 4	91 ± 14	Errico et al. (2008a)
Cortex			58 ± 4	153 ± 11	
Cerebellum			18 ± 1	81 ± 12	

studies would be advisable to elucidate how extracellular concentrations of D-Asp relate to the affinity of this D-amino acid for NMDARs, under both physiological conditions and in mice with enriched content of endogenous D-Asp. Enhancement of LTP induced by high D-Asp levels in adult *Ddo*^{-/-} and D-Asp-treated mice may result from different causes. The ability of NMDAR antagonists to block D-Asp-dependent currents suggests that excess of D-Asp may directly sensitize NMDARs leading to enhancement of LTP. Alternatively, D-Asp may act to increase the sensitivity of NMDARs to endogenous L-Glu released after tetanic stimulation. In any case, it seems plausible to exclude that higher LTP magnitude depends on up-regulation of glutamatergic receptors, since expression levels of hippocampal NMDAR and AMPAR subunits do not change in response to increased levels of D-Asp in both animal models (Errico et al. 2008b, 2011a, b). Interestingly, despite the substantial effect of D-Asp on NMDAR-dependent synaptic plasticity, increased levels of this D-amino acid do not perturb hippocampal presynaptic release probability or the basal properties of synaptic transmission (Errico et al. 2008b, 2011a, b).

Hippocampal LTP has been regarded as a key neural mechanism for the storage of spatial information, since this form of memory is believed to be encoded by modifications of NMDAR-dependent synaptic strength in this brain area

(Morris et al. 2003). Interestingly, in the hidden-platform version of the Morris water maze, *Ddo*^{-/-} mice exhibit an improved spatial memory compared to controls (Errico et al. 2008a, 2011a). Likewise, in the contextual fear conditioning, *Ddo*^{-/-} mice display improved cognitive ability, as evidenced by the longer time spent in freezing, compared to wild-type animals (Errico et al. 2008a, 2011a). On the contrary, D-Asp administration does not produce robust effects in hippocampus-related spatial and emotional memory of D-Asp-treated mice (Errico et al. 2008a, 2011b). Overall, the lack of substantial cognitive effects may depend on the fact that D-Asp-treated mice belong to inbred C57BL/6 strain, a genetic background well characterized to have high cognitive performance (Gerlai 2002; Nguyen and Gerlai 2002) and, therefore, not particularly suitable to show memory improvements associated with pharmacological treatments.

Effect of persistent, higher D-aspartate levels on age-related cognitive processes

The hippocampus of aged mammals undergoes a considerable loss of functional synapses and a strong decrease in NMDAR-dependent responses that, in turn, contribute to deficits of synaptic plasticity, including impairments of

induction and maintenance of LTP. These effects are likely the cause of the cognitive decay experienced by aged mammals (Rosenzweig and Barnes 2003). Therefore, in the light of the ability of D-Asp to increase hippocampal NMDAR-dependent LTP and, in turn, spatial memory of adult $Ddo^{-/-}$ mice, one could question whether the lifelong excess of this D-amino acid may modulate the natural decay of cognitive processes at aging. In response to this question, results indicate that while increased D-Asp content enhances NMDAR-dependent LTP in 4/5-month-old $Ddo^{-/-}$ mice, persistent deregulation of this D-amino acid dramatically accelerates the age-dependent decay of synaptic plasticity in 13-/14-month-old knockout mice (Errico et al. 2011a). Consistently, spatial memory improvement found in 4/5-month-old $Ddo^{-/-}$ mice turns into a drastic worsening of learning and memory abilities in mutants of 13–14 months of age (Fig. 1) (Errico et al. 2011a). Similarly to aged $Ddo^{-/-}$ mice, persistent administration of D-Asp for 12 months to C57BL/6 mice produces a significant but reversible decrease of their NMDAR-dependent synaptic plasticity at CA1 synapses (Errico et al. 2011b). Overall, results obtained in $Ddo^{-/-}$ mice indicate a clear biphasic modulation of persistent higher D-Asp content on hippocampal NMDAR-dependent behavior and synaptic plasticity. In this line, other experiments have revealed a modulation of ERK1/2 activation in the CA1 area of knockout animals that seems to reflect the biphasic changes of LTP and spatial memory (Errico et al. 2011a). This result leads to hypothesize that ERK signalling, known to be implicated in the modulation of LTP and cognition (Davis and Laroche 2006), may have a role in the age-dependent electrophysiological and behavioral phenotypes shown by $Ddo^{-/-}$ animals. Although the molecular mechanisms remain to be clarified, the precocious aging of knockout mice sheds light on a protective contribution of DDO enzyme on the regulation of the physiological decline of hippocampal NMDAR-dependent cognitive processes.

The direct effect of chronic D-Asp exposure on enhancement of hippocampal NMDAR-dependent synaptic plasticity has been further studied in 1-year-old female C57BL/6 mice. Interestingly, in these animals, 1-month treatment with D-Asp produces a consistent improvement of LTP, even exceeding that recorded in naive 2-month-old mice (Errico et al. 2011b). The action of D-Asp administration on hippocampal NMDAR-dependent LTP of aged mice clearly resembles the results obtained with D-Ser, the other endogenous D-amino acid substantially occurring in the brain. Indeed, it has been demonstrated that supplementation of exogenous D-Ser prevents age-related deficits of NMDAR-dependent synaptic responses in elderly rats (Junjaud et al. 2006; Mothet et al. 2006; Potier et al. 2010; Turpin et al. 2011).

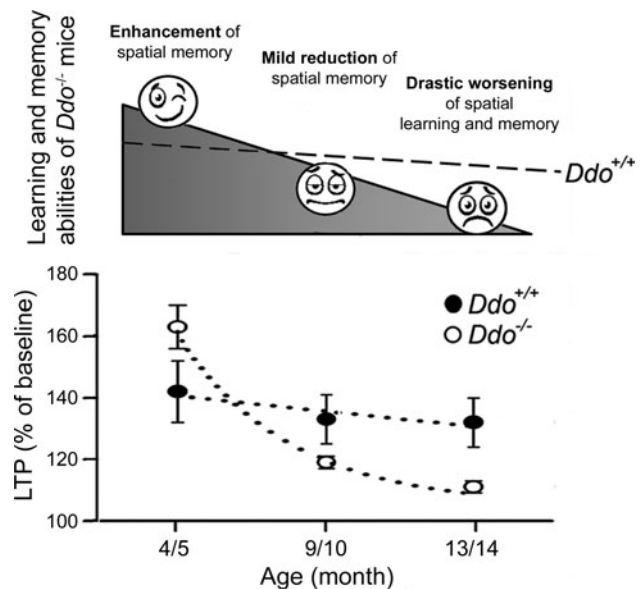


Fig. 1 Schematic representation of the opposite, age-related effects of increased D-aspartate levels on hippocampal LTP and spatial memory of $Ddo^{-/-}$ mice during ontogenesis. LTP values refer to data reported in Errico et al. (2011a)

Influence of increased D-aspartate levels on striatal synaptic plasticity and sensorimotor gating

Evaluation of electrophysiological properties of D-Asp in the striatum indicates that local applications of this D-amino acid induce dose-dependent inward currents in the medium spiny neurons (MSNs) that are antagonized by the NMDAR blockers MK801 and D-AP5. Moreover, similarly to the hippocampus, high concentrations of both antagonists in striatal slices are unable to completely prevent the currents triggered by D-Asp (Errico et al. 2008a), indicating also in this brain region the existence of NMDAR-independent currents activated by D-Asp. The effect of D-Asp on striatal glutamatergic transmission has been also extensively studied in $Ddo^{-/-}$ and D-Asp-treated mice. Electrophysiological data indicate that amplitude and kinetic properties of excitatory postsynaptic currents are unaffected in the striata of both genetic and pharmacological mouse models, while the excess of D-Asp is able to completely abolish corticostriatal long-term depression (LTD) (Errico et al. 2008a) and depotentiation (Errico et al. 2011c). Overall, these alterations are not caused by a differential expression of striatal AMPAR and NMDAR subunits, which remains unchanged in both knockout and D-Asp-treated mice, compared to their respective controls (Errico et al. 2008a, 2011c). Given the ability of D-Asp to activate striatal NMDARs, it is likely that the absence of corticostriatal LTD, in conditions of enhanced levels of D-Asp, may depend on the facilitatory effect of this D-amino acid on NMDAR-dependent transmission. This is

supported by the fact that NMDARs are pivotal in dictating the direction of plasticity events triggered by repetitive stimulation of excitatory corticostriatal synapses *in vitro*. Thus, corticostriatal LTD requires the block of NMDARs, while LTP is favored under conditions that activate this subclass of Glu receptors (Calabresi et al. 1992; Centonze et al. 2007). The effect of increased D-Asp on striatal LTD deserves a special remark if we consider that similar synaptic adaptations occur in the mouse striatum after chronic treatment with the typical antipsychotic haloperidol (Centonze et al. 2004). In support of a potential preclinical value of D-Asp in schizophrenia, *in vivo* experiments of acoustic startle response and prepulse inhibition (PPI), performed in adult *Ddo*^{-/-} and D-Asp-treated C57BL/6 mice, indicate that chronic exposure to higher D-Asp levels significantly attenuates the psychotic-like deficits induced in mice by the treatment with the psychotomimetic drugs amphetamine and MK801 (Errico et al. 2008a). Altogether, these data suggest a potential beneficial effect of D-Asp on sensorimotor filtering abilities of animals and mirror the behavioral results obtained with the other D-amino acid, D-Ser (Tsai et al. 2004; Kanahara et al. 2008).

Future perspectives

Current literature on D-Asp has so far mainly focused on the role of this D-amino acid in the adult brain, where its endogenous content is very low, due to the physiological control exerted by DDO. On the other hand, it is plausible to hypothesize that D-Asp may play neuromodulatory functions at developmental stages, when this D-amino acid prominently occurs in different regions of the brain. Therefore, the possibility to deplete D-Asp stores during brain development, by modifying the physiological expression of the enzymes of D-Asp metabolism, could be a decisive approach to unveil unknown neuronal functions of this molecule. A large bulk of evidence suggests that a transient and limited intervention producing NMDAR hypo-function during development can generate chronic behavioral, neuroanatomic and molecular alterations during adult life. Therefore, in line with the agonistic activity of D-Asp on NMDARs, it could be postulated that early deprivation of D-Asp in the brain may result in a greater vulnerability to alterations related with developmental hypo-functioning of NMDARs and, in turn, influence the physiological maturation of neuronal circuitries and behaviors at adulthood. In particular, many reports point to a reduced functionality of NMDARs during early adulthood for explaining the etiology of neuropsychiatric disorders such as schizophrenia (Sawa and Snyder 2002; Tsai and Coyle 2002). Based on the agonistic activity of D-Asp at NMDARs and in agreement with the ability of higher

D-Asp levels to improve cognition and protect against sensorimotor gating deficits induced by psychotomimetic drugs in preclinical models, we hypothesize that this D-amino acid may have a translational relevance for human neuroscience in connection with neuropsychiatric disorders. With regard to a potential common function of D-Asp and D-Ser in the brain, it is interesting to observe that D-Ser treatment displays beneficial effects on schizophrenia via stimulation of NMDARs (Coyle et al. 2010; Fuchs et al. 2011). Overall, differently to D-Ser, knowledge on D-Asp is still poor, given the recent evolution of the subject matter, and many issues remain to be clarified. For example, we know far too little about the neuronal modalities of D-Asp biosynthesis and release. Likewise, at present our findings about D-Asp effect on hippocampal and striatal NMDAR-dependent functions are not adequately supported by a mechanistic view that explains what happens downstream the D-Asp-dependent activation of NMDARs. In this regard, the parallel stimulation of this subclass of L-Glu receptors by D-Asp and D-Ser upon two different binding sites might suggest a possible interaction between the signalling pathways activated by the two D-amino acids. Moreover, many efforts are still needed to understand the possible abnormalities of D-Asp function in brain disorders associated with NMDARs dysfunction, with a special focus on schizophrenia. In this respect, the possibility to regulate endogenous D-Asp levels, through its exogenous administration or by modulating the activity of its metabolic enzymes, may open attractive avenues for evaluating the effectiveness of new glutamatergic therapeutic approaches. However, future studies on mouse models and humans will help to clarify, respectively, the *in vivo* role of D-Asp in immature mammalian brain and the potential value of this atypical molecule in clinical applications.

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